DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR FIELD DETECTION AND DISCRIMINATION OF *FUSARIUM CIRCINATUM* FROM *FUSARIUM OXYSPORUM* AND *DIPLODIA PINEA* IN PINE SEEDLINGS

By

Phumzile Mkhize

University of KwaZulu-Natal, BSc, Honours.

Submitted in partial fulfillment of the requirements for the degree of

Master of Science (Plant Pathology)

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Science

College of Agriculture, Science and Engineering

University of KwaZulu-Natal

Pietermaritzburg

Republic of South Africa

May 2013

DISSERTATION SUMMARY

Fusarium circinatum is a fungal pathogen that has had a serious impact on pine production throughout the world. It attacks most *Pinus* species including *Pinus elliottii, Pinus patula* and *Pinus radiata*. Infections in South Africa (SA) are largely on seedlings, and result in fatal seedling wilt. Accurate and quick detection systems suitable for field use are needed to monitor the spread of the disease and optimize fungicide applications. Detection of *F. circinatum* is currently based on visual observations of typical symptoms. However, symptoms are not unique to the pathogen and can be caused by other biotic and abiotic stress factors. Nucleic acid-based identification techniques using PCR are available for different fungal species. These are sensitive and accurate, but they are expensive and require skilled biotechnologists to conduct the assays.

In this study an enzyme-linked immunosorbent assay (ELISA) was developed to identify *F. circinatum* in infected seedlings. This optimized ELISA is able to discriminate between *F. circinatum* and two other fungi that frequently affect pine. This method has advantages over other assays because of its ease of operation and sample preparation, sensitivity and the ability to run multiple tests simultaneously. Mycelium-soluble antigens from *Diplodia pinea* (=*Sphaeropsis sapinea*), *F. circinatum* and *F. oxysporum* were prepared in nutrient broth. Analysis of these antigens on SDS-PAGE indicated the presence of common antigens between the different fungal pathogens. Some antigens were expressed more by some isolates than by others. Separate groups of chickens were immunised with mycelium-soluble antigens from *D. pinea*, *F. circinatum* and *F. oxysporum* and exo-antigen from *F. circinatum* prepared in nutrient broth. A 34 kDa protein purified from SDS-PAGE specific for *D. pinea* was also used for immunisation. Five sets of antibodies were obtained including anti-*D. pinea*, anti-*F. circinatum*, anti-*F. oxysporum*, anti-*F. circinatum*exo and anti-*D. pinea* 34 kDa antibodies, respectively. Reactivity of these antibodies was evaluated against antigens prepared in nutrient broth using western blotting and ELISA.

Western blot analysis indicated that immuno-dominant antigens for *F. circinatum* were larger than 34 kDa and their reactivity was not the same between different isolates. Each of the

antibodies prepared using mycelium-soluble antigens showed increased reactivity when detecting its own specific pathogen, but cross-reactivity was observed. Anti-*D.pinea*antibodies showed minimal cross-reactivity with antigens from *F. circinatum* and *F. oxysporum*. Anti-*F. circinatum* antibodies cross-reacted with antigens from *F. oxysporum* but showed little cross-reactivity with *D. pinea* antigens. Anti-*F. oxysporum* antibodies showed more cross-reactivity towards antigens from *F. circinatum* than those from *D. pinea*. No reactivity was observed when anti-*F. circinatum*-exo antigen and anti-*D. pinea* 34 kDa antibodies were used in immuno-blotting analysis.

Evaluation of antibody reactivity using indirect ELISA showed patterns similar to those observed on western blotting, where anti-*D. pinea,* anti-*F. circinatum* and anti-*F. oxysporum* antibodies showed the same cross-reactivity relationships. Anti-*F. circinatum* and anti-*F. oxysporum*antibodies showed a significant difference when reacting with antigens isolated from other pathogens including *D. pinea*, *F. circinatum*, *F. oxysporum*, *F. solani, F. graminearum* and *F. culmorum* (P = 0.001). No significant difference was observed when the antigens were detected with anti-*D. pinea* antibodies. Reactivity of anti-*F. circinatum*-exo and anti-*D. pinea*34 kDa antibodies was mostly similar to that of non-immune antibodies and showed no significant difference between detection of different antigens.

Pine seedlings were artificially infected with the three fungal pathogens using a spore concentration of 1 – 1 x 10^6conidiaml^{-1}.Infection was monitored using scanning electron microscopy. Results showed increased levels of mycelium growth on the stem and roots of the *F. circinatum* and *F. oxysporum* infected seedlings and on the leaves and stem in the case of *D. pinea* infected seedlings. These plant parts were used in ELISA tests for the detection of antigens. Isolation of antigens from the plant materials involved crushing plant parts in buffer and centrifugation of the suspension. The supernatant obtained was directly used in the assay. ELISA tests prepared in this study were sensitive enough to detect infection caused by 1 conidium ml^{-1}at two weeks post inoculation. A positive reaction for detection of *F. circinatum* and *F. oxysporum* was indicated by an ELISA reading above an optical density at 405 nm

The plant material used in ELISA tests were further analysed using PCR. Results indicated that there was no cross-infection between seedlings and served as a confirmation of the

disease-causing pathogen. This indicated that cross-reactivity observed was due to other factors such as common epitopes on the major antigens. Use of an ELISA dip-stick or ELISA using these antibodies should provide an easy, fast field test to identify infections of pine, discriminating between *F. circinatum*, *F. oxysporum* and *D. pinea*.

PREFACE

The experimental work presented in this dissertation was carried out in the School of Agricultural, Earth and Environmental Science, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Mark D. Laing and Professor T.H.T. Coetzer

These studies represent my original work and have not been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

……

P. Mkhize (Candidate)

……

Professor M. D. Laing (Supervisor)

……..

Professor T.H.T. Coetzer (Co-supervisor)

DECLARATION

I, Phumzile Mkhize declare that: The work presented in this thesis is my original research and it has not been submitted for any degree or examination. Acknowledgment has been specified where another person' work, data, table and pictures are used. Research from other persons' has been re-written and reference.

Signed: …….

ACKNOWLEDGEMENTS

First and foremost, I wish to express my greatest appreciation and sincere gratitude to my supervisor and co-supervisor for their support, patience and guidance throughout this project.

Professor M.D. Laing, for his supervision throughout this study, sharing his knowledge, expertise in Plant Pathology, his contribution to laboratory work and editing of different chapters in this dissertation.

Professor T.H.T. Coetzer, for sharing her expertise in the field of Biochemistry, for giving direction, encouragement and constructive suggestion in the laboratory work and editing different chapters in this dissertation. Her assistance is greatly valued.

SAPPI for providing pine seedlings used in this study

Professor T. Coutinho, for providing *Fusarium* and *D. pinea* isolates

Fusarium Working Team, for their assistance and sharing of expertise in the disease and field work

Mr R. Burgdorf, for his contribution to the project and technical assistance

Post graduate students from Plant Pathology and Biochemistry, for their assistance and contribution to the success of this project

Plant Pathology and Biochemistry staff, for their technical assistance

Very special thanks go to The Mkhize Family, for all their support and sacrifices that have enabled me to pursue my studies.

DEDICATION

To the Mkhize Family, especially my mother for the support, understanding and spiritual encouragement during my studies

TABLE OF CONTENTS

DISSERTATION INTRODUCTION

Fusarium circinatum is one of the most important pathogens of pines. It is a causal agent of two distinct pine diseases: pine pitch canker on mature pine trees and pine seedling wilt. It has had a serious impact on pine production in South Africa and other countries throughout the world, leading to serious losses in the pine industry (Wingfield *et al.*, 2008). The pathogen has been reported in California, Japan, Mexico and South Africa (Muramoto and Dwinell, 1990; Viljoen *et al.*, 1994; Storer *et al.*, 1998; Britz *et al.*, 2001). In South African nurseries *F. circinatum* epidemics have been reported mostly on *Pinus patula* Schldl. *et* Cham seedlings. On mature pine trees few reports of pitch canker have been made on *P. radiate* D. Don, *P. greggii* Engelm. ex Parl. and *P. elliottii* Engelm. (Viljoen *et al.*, 1994; Mitchell *et al.*, 2011; Mitchell *et al.*, 2012). The main focus of this study was on seedling wilt which is the prevalent disease in South Africa. *Fusarium circinatum* has a huge impact on ecological, economic and timber production via impaired tree growth and reduced yields of timber (Conradie *et al.*, 1990; Wingfield *et al.*, 2001). These losses have been documented in 16 different sites where 42% of the dying plants were infected with *F. circinatum* (Crous, 2005). In the case of *P. patula*, low seedling survival rates result in losses of over R11 million lost per year in South Africa. Infection of *P. radiata* and *P. patula* in new plantations in South Africa has resulted in a loss of close to R12 million per year over the period 2005 to 2010(Mitchell *et al.*, 2009; Mitchell *et al.*, 2011). It is estimated that by 2020 close to 5 billion Rand will be lost by the forestry industry due to *F. circinatum*.

Symptoms observed in nurseries include root and collar rot, tip wilting followed by discolouration beneath the growing tip, which then progresses to other parts of the seedling, resulting in death. On mature trees, pitch canker is marked by branch die-back, development of resinous cankers on the stem and resin-soaked wood (Nelson, 1981; Storer *et al.*, 1998). More rapid death is associated with the infection of seedlings than established trees (Barnard and Blakeslee, 1980). In most countries *F. circinatum* is known to cause damage on established trees, whereas in South Africa *F. circinatum* emerged as a nursery pathogen posing a threat to new plantations especially to *P. patula*. In nurseries *F. circinatum* spreads from contaminated planting containers, irrigation water and planting media (Coutinho *et al.*,

2007; Wingfield *et al.*, 2008). Infection on mature trees has been associated with wounding that becomes the site for *F. circinatum* to gain entry. These wounds are commonly created by beetles i.e. *Ips conophthorus* Hopkins, *Ernobius* Fall and *Pissodes nemorensis* Germar the latter is a common beetle of timber found in South Africa. The absence of the other two beetles (*Ips conophthorus* and *Ernobius*) species in the country is believed to be the reason for pitch canker to be absent from South Africa for some time (Storer *et al.*, 1998; Wingfield *et al.*, 2002a; Coutinho *et al.*, 2007).

Early detection of the causal pathogen plays a major role in ensuring proper control and limiting the spread of the disease. Methods used for fungal detection such as those that are nucleic-acid based need to be effective enough for detection before symptom development. Fungal plant pathogens have been identified using conventional methods such as the investigation of visible symptoms, culturing on selective media and microscopic analysis. These methods may require the use of a skilled plant pathologist for accurate diagnosis. They are time consuming since waiting for symptom development or culturing may take weeks. This makes such methods impractical where quick detection is required. The major drawbacks of these detection systems are the inaccuracy and lack of sensitivity. The use of nucleic acid based detection methods using PCR, and protein based immunochemical techniques are more sensitive, quick and specific. The use of PCR methods have been well documented for detection of fungal pathogens (Grimm and Geisen, 1998; O'Donnell *et al.*, 1998; Fraaije *et al.*, 1999; Weiland and Sundsbak, 2000; Schweigkofler *et al.*, 2004; Luchi *et al.*, 2007).

Immunochemical detection systems mostly entail the use of enzyme-linked immunosorbent assay (ELISA). In this assay, specific antibodies are developed against proteins for detection. ELISAs are much easier and quicker to use for detection and quantification of the pathogen in both the laboratory and field (Lievens and Thomma, 2005; Lievens *et al.*, 2008). ELISAs are ideal methods to use in complex mixtures such as plant extracts and soil mixtures because there are no purification steps required prior to testing. This is because specific antibodies preferentially bind to one protein of interest in the mixture, and unbound material is washed off during subsequent washing steps carried out during the assay. This method has an advantage over PCR and culturing methods because it allows for detection of multiple samples in a single test simultaneously.

There are commercially available kits for field detection of fungal pathogens. Dip-stick assays have been successfully used in studies that tested at the *Fusarium* mycotoxins such as T-2 toxin (de Saeger and van Peteghem, 1996). Tube-like immuno-assays for the detection of *Botrytis, Aspergillus* and *Penicillium* on grape fruits have been developed. This assay was reported to be user friendly and can be used on site at wineries. The principle of this assay is that of a plate-trapped antigen (PTA) ELISA: antigens are directly coated onto the wells of micro-titre plates and incubated with a specific primary antibody and an enzyme-linked secondary antibody. For the detection of the immunochemical reaction a chromogenic substrate solution is added (Dewey and Meyer, 2004). Also, PTA-ELISA for the detection of *Phytophthora, Pythium* and *Rhizoctonia* in the field have been developed,in which the detector enzyme was linked to the primary antibody, making this assay even quicker as there was no need for incubation with the detector-linked secondary antibody (Ali-Shtayeh *et al.*, 1991). In this test, the presence of the pathogen can be detected within ten min, marked by a colour change that can be easily interpreted by a nurseryman. ELISA tests have been reported for quantification of *F. poae* (Peck) Wollenw, *F. graminearum* (Schw.) Petch, *F. oxysporum f.sp. albedinis, F. culmorum* (W.G.) Smith, *Thielaviopsis basicola* (Berk. & Br.) and *F. avenaceum* (Fr.) Sacc. (Kitagawa *et al.*, 1989; Beyer *et al.*, 1993; de Saeger and van Peteghem, 1996; Gan *et al.*, 1997).

Despite the reports of different ELISAs that are available for the detection of different fungal pathogens, there is still a need to develop an ELISA system for the detection of *F. circinatum* in the field. This research was motivated by the need of most pine nurseries in South Africa to have a detection system that can be used in the nursery and in the field for quick, accurate and simple detection of *F. circinatum*. This is very important for farmers because it will help them ensure that the pathogen is correctly identified and correct control measures are implemented. Most importantly, the use of an accurate detection system will ensure that infected seedlings do not go out for planting.

The aim of this research was to develop an ELISA that can be used in the field for the detection and discrimination of *F. circinatum* from *D. pinea* and *F. oxysporum* in infected pine seedlings. This is achievable by ensuring that antigens especially those that are unique and different between the three fungal pathogens are properly analysed using protein separating

techniques and used to develop more specific antibodies. Other fungal pathogens closely related to *F. circinatum* and *F. oxysporum* were also analysed to optimize the specificity and reactivity of the ELISA developed. This is covered in details in Chapter 2 of this thesis where the research focuses on the development and characterisation of chicken antibodies raised against *F. circinatum*, *F. oxysporum* and *D. pinea* using ELISA and western blotting. Also, to achieve the goals of this research tests were carried out in the field on infected seedlings. Different fungal spore concentrations were used to inoculate pine seedlings and different antigen isolation methods were evaluated during the ELISA tests in the field. This ensured that the ELISA developed was able to detect the presence of the fungus at different fungal spore concentration in the infected pine seedlings and that the antigen was easily isolated for the testing using the ELISA. The field tests were properly covered in Chapter 3 of this thesis where the research focuses on the discriminatory detection of *F. circinatum*, from *F. oxysporum* and *D. pinea* in infected pine seedlings using ELISA.

Some of the limitations that were expected included cross-reactivity from closely related fungal pathogens especially those form the gunus, *Fusarium*. Determining specific antigens for each of the fungal pathogen was expected to be a problem because fungal pathogens have very low protein concentration and these became degraded during culture storage (Gan *et al.*, 1997). Also, ELISA test that have been developed in the past have used soluble and homogeneous suspensions of antigens, while in this study antigens will be suspended with debris from the pine seedlings (Arie *et al.*, 1991; Arie *et al.*, 1995).

This assay should be user-friendly and results easily interpreted by nursery staff with little biotechnology background. Field stations can be provided with an ELISA plate coated with the anti-fungus antibody and the necessary enzyme-labeled secondary antibody as well as substrate. A dipstick-test based on this sandwich ELISA using these antibodies should provide an easy, fast field test to identify infections of pine, discriminating between *F. circinatum*, *F. oxysporum* and *D. pinea*.

CHAPTER 1

Literature Review

1.1 Introduction

The forestry industry plays a major role in the economy of South Africa, through the production of structural timber and fiber for cellulosic pulp. However, this industry is negatively affected by man-made fires as well as pests and diseases. These have a considerable impact on the ecological, economic and social dynamics of forestry (Conradie *et al.*, 1990; Wingfield *et al.*, 2001). *Fusarium circinatum* Nireberg and O'Donnell [=*F. subglutinans* (Wollenw. and Reinking) Nelson *et al. f.sp. pini* Corell *et al*] is amongst the most important fungal pathogens of *Pinus* species (Dwinell, 1978; Carey and Kelley, 1994; Clark and Gordon, 1998; Wingfield *et al.*, 2002a). It is the causal agent of two distinct diseases of pines, namely, pine pitch canker and seedlings wilt. Both these diseases are a big threat to countries where extensive planting of susceptible pine trees is carried out, because it affects timber and pulp production and quality and causes extensive tree mortality (Storer *et al.*, 2002). Pine pitch canker has been reported in Japan where it was associated with bleeding resinous cankers observed on *P. lunchuensis* Mayr (Muramoto and Dwinell, 1990). Pitch canker fungus has also been recorded in Chile, northern Spain and California (Viljoen *et al.*, 1994; Wingfield *et al.*, 2002a; Landeras *et al.*, 2005; Carlucci *et al.*, 2007). Seedling wilt is a common disease in South Africa. The first report of infections by this fungus in the southern hemisphere was in the Mpumalanga Province on *Pinus patula* Schldl. *et* Cham where it caused seedling wilt (Dwinell, 1978; Viljoen *et al.*, 1994). Subsequent to the first report of the pathogen it spread throughout the nurseries in the country resulting in severe damages (Coutinho *et al.*, 2007). There have not been any serious outbreaks of pine pitch canker in South Africa. Symptoms resembling those of pitch canker were observed on five and nine year old *P. radiata var. Binata* in the Western Cape Province (Coutinho *et al.*, 2007). This study will focus on seedling wilt which is an important disease in South Africa. In seedling nurseries, infections cause seedling damping-off, stem lesions, reddish-brown lesions on the

roots and terminal wilt that lead to seedling death (Correll *et al.*, 1991; Carey and Kelley, 1994).

There is no absolute means of controlling seedling wilt in the infected trees but for planting, more resistant species, such as Monterey pine (*P. radiata*) for control of *F. circinatum* (Bonello *et al.*, 2001; Vivas *et al.*, 2012). The management of *F. circinatum* in seedling nurseries requires that effective hygiene measures are maintained. These include treatment of the seeds by either soaking them in ethanol or hydrogen peroxide suspended in hot water (Wingfield *et al.*, 2008). The steam and copper treatments can be used to sterilize polysterene planting trays. Nursery staff need to ensure that irrigation water is free of *F. circinatum* by treating it with a sterilant such as hydrogen peroxide (Dwinell and Fraedrich, 1998; Storer *et al.*, 1998). Fungicides may be applied in an attempt to control *F. circinatum* but they have been deemed as ineffective (Runion *et al.*, 1993; Storer *et al.*, 1998).There is also an option of ensuring that the fungus is not introduced into areas that are currently free from infection by thorough screening of seeds. Proper control of insect vectors that carry the fungus may reduce new infections (Sakamoto and Gordon, 2006). Early and accurate detection of infection and the causal agent plays a major role in preventing the spread of the disease and major losses in this agricultural sector. Correct identification of *F. circinatum* as the cause of seedling wilt may be difficult because similar symptoms are caused by other fungal pathogens, water and chemical stress (Ward *et al.*, 2004). The development of detection systems for plant pathogens, especially antibody-based and nucleic-acid based, are reviewed, with a focus on fungal pathogens.

1.2 The pathogen

Fusarium species are economically important plant pathogens. There are 17 known species of *Fusarium* that target a number of cultivated plants, trees and cereals, causing a decrease in their quality and yield (Barrows-Broaddus and Dwinell, 1985b; Storer *et al.*, 1998). These pathogens include *F. graminearum Schw*, *F. solani (Mart.) Sacc*, *F. culmorum* (Wm.G. Sm.) *Sacc.*, *F. roseum Link.*, *F. oxysporum f.sp. albedinis* and *F. circinatum*. The main focus of this study is on *F. circinatum* and *F. oxysporum* strains that affect various *Pinus* species (Nelson, 1981; Wingfield *et al.*, 2008).

Fusarium circinatum was first discovered in 1946 causing severe damage in southern USA (Hepting and Roth, 1953). The first symptoms observed on pine trees were cankers flowing from bark and soaking the wood. Subsequent studies associated *F. circinatum* with the development of root diseases, root rot, seed decay and stem cankers (Landeras *et al.*, 2005; Carlucci *et al.*, 2007; Wingfield *et al.*, 2008; Mitchell *et al.*, 2011). Supplementary studies on the disease showed that the infectious agent produced microconidia, few macroconidia and no chlamydoconidia (Smith and Snyder, 1975).

Fusarium oxysporum Schlecht is sub-divided into races referred to as *formae speciales*. These races are separated based on pathogenicity and host specificity (Nelson, 1981). Close to 70 *F. oxysporum formae speciales* have been described (Nelson, 1981). For some of these *formae speciales* the primary host has not been identified as yet and these are referred to as non-pathogenic strains, some of which are beneficial. The pathogenic strains of *F. oxysporum* can attack a diverse group of plants, including crops such as tomato, cabbage, banana, flax, sweet potato and watermelon. Some trees such as oil date, palm date and some pine species have been reported to be susceptible to this pathogen (Bloomberg, 1981; Nelson, 1981; Toussoun, 1981; Arie *et al.*, 1998).

Most strains of *F. oxysporum* can only infect one or a few plant species. Under unfavourable conditions *F. oxysporum* survives as chlamydoconidia in the soil or in decaying host tissue. When favourable conditions prevail, conidia are stimulated to germinate (Smith and Snyder, 1975; Nelson, 1981; Toussoun, 1981). Upon germination, hyphae form that may penetrate the plant through wounds but for some*F.formae speciales*, wounding is not required (Pascholati *et al.*, 2002). In crops such as tobacco, infection is largely dependent on the presence of a wound, while in cabbages,*F. oxysporum f. sp. conglutinans* [(Wr) Snyd. & Hans)] Race 1 is able to penetrate intercellularly in the apical meristematic regions of uninjured roots (Nelson, 1981). In British Columbian nurseries, *F. oxysporum* was reported to cause over 90% of damping-off, root-rot and wilting on the lower shoots on pine seedlings (Bloomberg, 1971).

Diplodia pinea (Fr) Dyko & B. Sutton, also known as *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton and first described as *Sphaeria pinea* Desm., is an opportunistic pathogen of conifers (de Wet *et al.*, 2000; Juhasova *et al.*, 2006). *D. pinea* has a worldwide distribution, where it is generally associated with shoot death of pines (Stanosz *et al.*, 1996). This pathogen has been

reported in many countries but has been most notorious in South Africa (Wingfield and Knox-Davies, 1980; Swart and Wingfield, 1991; Smith *et al.*, 1996). This is due to the common occurrence of heavy rainfall, droughts and hailstorms in South Africa that enhances the susceptibility of *P. radiata* and *P. patula* Schldl. *et* Cham trees to infection (Brookhouser and Peterson, 1971; Wingfield and Knox-Davies, 1980).

There are three different morphological forms of *D. pinea* that have been identified; these are referred to as morphotypes. These morphotypes can be separated by their molecular characteristics and pathogenicity (de Wet *et al.*, 2000; de Wet *et al.*, 2003). A study carried out by Brookhouser and Peterson (1971) evaluated the site and mode of penetration for *D. pinea*. They found that *D. pinea* can penetrate needles through the stomata of uninjured Australian pine [*Pinus nigra* (Visiani) Franco], Scots pine (*P. sylvestris* L. var. *hamata* Steven), Pondorosa pine (*P. ponderosa* Douglas ex C. Lawson) and *P. radiata*D. Don (Chou, 1978). In South Africa *D. pinea* causes dieback of *P. patula* and *P. radiata* (Wingfield, 1980; de Wet *et al.*, 2000). Infection occurs when the plant is under stress conditions, such as harsh weather conditions and shortage of nutrients and water (Paoletti *et al.*, 2001; Stanosz *et al.*, 2001).

Diplodia pinea has been isolated from healthy branches, twigs and wood from mature trees, indicating that it remains asymptomatic in most trees worldwide (Stanosz *et al.*, 1996). The source of inoculum in the USA was found to be infected pine trees used as windbreaks, and the presence of leftover pruned branches (Stanosz *et al.*, 2007). In South Africa seeds are thought to have been the initial source of inoculum, followed by dispersal of conidia by wind and splashed water (Burgess and Wingfield, 2002).

A wide range of *Pinus* species throughout the world are susceptible to infection by *D. pinea* (Waterman, 1943; Brookhouser and Peterson, 1971; Stanosz *et al.*, 2007). Like *F. circinatum*, *D. pinea* targets all plant developmental stages from seedlings in nurseries to plantations and natural stands (Waterman, 1943; Luchi *et al.*, 2007; Stanosz *et al.*, 2007). *Pinus radiata* and *P. patula* are the main hosts of *D. pinea*. Upon infection of young seedlings, a high mortality rate is observed. Infection of mature trees results in stem malformation and reduction in usable length of the bole (Zwolinski *et al.*, 1990). Multiple infections result in death of the mature tree.

1.3 Symptoms

The primary symptom observed in the pine seedling nursery upon infection with *F. circinatum* is the wilting of the seedling tip. At early stages of infection death of root tips occurs, roots are then unable to take up water and supply the rest of the seedling. Seedling wilt is also marked by wilting and fading of colour in the needles that are closer to the tip of young branches (Gordon *et al.*, 1998; Mitchell *et al.*, 2011). Following the colour change of the needles, dieback of terminal and lateral branches within the crown becomes severe, especially after repeated infections (Figure 1.1, A) (Gordon *et al.*, 2001). Tips turn purple and collar rot is observed. Development of hyphae on dead seedling stems occurs at late stages on infection (Figure 1.1, B).

Pine pitch canker is marked by bleeding, resin-soaked wood and formation of resinous canker on the trunk and branches (Figure 1.1, C) (Storer *et al.*, 1998; Coutinho *et al.*, 2007). Each canker greatly affects the wood beneath it, which appears deeply pitch-soaked with resin-soaked lesions on the lower stem while the bark is retained (Figure 1.1, C) (Barnard and Blakeslee, 1980; Dwinell *et al.*, 1985). The damaged shoots are unable to supply water to the expanding buds above them and therefore the shoots die. On the older tissue, shoot growth may be fully expanded before being killed by the infection. A good indicator for this disease is that dead shoots remain in the crown for several years. The needles on dead shoots turn gray and wilt on the tree tip is also observed (Figure 1.1, D).

The development of symptoms may vary depending on the *Pinus species* affected. The development of cankers on the trunks, branches and exposed roots is a common phenomenon in *P. radiata*, *P. palustris* Mill. and*P. strobes* L. (Dwinell *et al.*, 1985; Muramoto and Dwinell, 1990; Correll *et al.*, 1991). Shoot dieback is reported as being common in *P. elliottii, P. taeda* L., *P. radiata* and *P. echinata* Mill. (Dwinell *et al.*, 1985; Correll *et al.*, 1991). In the USA, *F. circinatum* infection caused symptoms ranging from shoot die-back, canker and even death in some trees in Florida. The disease was further observed in other parts of USA, including Virginia and Texas affecting different pine species including *P. radiata, P. muricata* D. Don and *P. halepensis* Mill. (Kuhlman *et al.*, 1982; Dwinell *et al.*, 1985).

Figure 1.1 Symptoms caused by *F. circinatum* in pine seedlings and mature pines(A) Damping-off, wilting and drawn die-back of seedlings in nurseries. (B) Development of hyphae on a dead seedling stem. (C) Development of abundant resin exudates (pitch) beneath cankers (left), cankers that girdle branches and trunk (right) (Wright *et al.*1987). (D) Mortality of the terminal branches within the crown (Blakeslee and Oak, 1979; Wingfield *et al.*, 2008).

At early stages of seedling infection by *F. oxysporum* curled needles develop, followed by tip dieback and wilt symptoms. General wilt of the infected seedling is observed, leaves wilt and drop off, resulting in bare stems. Root development is affected and root rot is observed. Fruiting structures on the stem develops. *F. oxysporum* also causes damping-off that has been shown to increase following heat stress (Nelson, 1981; Axelrood *et al.*, 1995).

Symptoms observed following infection of pines with *D. pinea* range with age, with more severe damage observed in younger trees. *D. pinea* has been detected in trees not showing any symptoms and this is usually the case since symptoms usually develop under physiologically stressful conditions (Luchi *et al.*, 2007; Maresi *et al.*, 2007). The most prominent symptoms of *D. pinea* include discolouration of new shoots with short, brown needles. Small, tan to reddish-brown lesions of the lower regions of the needles are the first symptoms observed on

artificially infected young Australian, Scot and Ponderosa pine trees (Brookhouser and Peterson, 1971). Older trees become deformed, while crown dieback and reduced growth is observed upon repeated infection (Waterman, 1943). *D. pinea* can also cause yellowing and necrosis of needles and cones that kills most of the shoot (Brookhouser and Peterson, 1971; Stanosz *et al.*, 2007).

1.4 Global importance of the diseases and host

The first report of *F. circinatum* outside USA was made in the 1980s in Haiti where it affected *P. occidentalis* (Dwinell *et al.*, 1985). By 1986 *F. circinatum* was reported in California on *P. radiata*, *P. muricata* and *P. halepensis* (McCain *et al.*, 1987). Seedling wilt occurred in Japan in the early 1990s causing shoot dieback (Muramoto and Dwinell, 1990). It was then reported in South Africa on *P. patula* seedlings (Viljoen *et al.*, 1994). In the late 1990s it was reported to have spread to other countries including Mexico and Chile (Britz *et al.*, 2001; Wingfield *et al.*, 2002a).

The first report of *F. circinatum* in Europe was in northern Spain where it infected *P. pinaster* Aiton and *P. sylvestris* L., both in nurseries and plantations (Landeras *et al.*, 2005). In northern Spain, *F. circinatum* was found in different geological areas where it was characterised, based on morphological and genomic features (Lievens and Thomma, 2005; Perez-Sierra *et al.*, 2007). The susceptibility and response of conifer species of the Great Lake of North America to *F. circinatum* has been evaluated. Three-year old seedlings of *P. resinosa* Aiton, *P. banksiana Lamb,. P. strobus L,. P. sylvestris* and *P. nigra* J.F.Arnold were inoculated by introducing a drop of *F. circinatum* conidia at a site where the needle fascicle was removed. The mortality rate was recorded twelve weeks after inoculation. Most resistance was observed in *P. nigra* and *P. resinosa*, while *P. banksiana, P. strobus* and *P. sylvestris* were more susceptible (Enebak and Stanosz, 2003). A similar susceptibility pattern was observed for *P. banksiana, P. sylvestris* and *P. nigra* in California (Clark and Gordon, 1998). In the southern United States, *P. virginiana* Mill. was reported to be more susceptible than *P. echinata* (Dwinell, 1978). The pathogen has been reported in Italy where it attacks *P. halepensis* (Carlucci *et al.*, 2007).

In South Africa it has been observed that *F. circinatum* can cause severe damage to many species of pine: slash (*Pinus elliottii* Engelm.), loblolly (*P. taeda* L.), shortleaf (*P. echinata* Mill.) and virginia (*P. virginiana*) pines (Porter *et al.*, 2009). It is believed that *F. circinatum* was introduced to South Africa by infected seed from Mexico (Britz *et al.*, 2001). Initially *F. circinatum* was only found in nursery plantations and affected *P. patula* (Viljoen *et al.*, 1995; Wingfield *et al.*, 2002a; Wingfield *et al.*, 2002a). Following this outbreak, the disease became established in nurseries throughout the country, targeting different *Pinus* species and causing root and collar rot in seedlings (Viljoen *et al.*, 1994). The first outbreak of pine pitch canker in mature trees was found in mature *P. radiata* in the Western Cape in 2007 (Coutinho *et al.*, 2007). Transmission of the pathogen in South Africa has been associated with adult beetles, i.e. *Pissodes nemorensis* that can carry pitch canker conidia (Gebeyehu and Wingfield, 2003).

In south Florida pitch canker was observed on slash (*P. elliottii*), eastern white (*P. strobes* L.), longleaf (*P. palustris* Mill.), table mountain (*P. pungens* Lamb.) and pitch (*P. rigida* Mill.) pines (Kuhlman *et al.*, 1982; Landeras *et al.*, 2005). The disease usually becomes epidemic and leads to great economical losses, both in pine plantations and seed orchards. This is because infected seedlings become morphologically and physically impaired, and when planted in the field, they usually grow poorly or die (Smith and Snyder, 1975). In Chile *F. circinatum* was discovered in nurseries infecting pine seedlings, but it has not been reported on adult trees in plantations, which is a similar case with South African infections (Viljoen *et al.*, 1994; Wingfield *et al.*, 2002b).

Fusarium oxysporum is a soil borne pathogen that can be found in all parts of the world. The importance of *F. oxysporum* has been reported in pine nurseries throughout western North America. Economic losses have been reported on several *Pinus* species including *P. monticola* Douglas ex D. Don and *P. ponderosa* Douglas ex C. Lawson, which developed severe root rot and wilting symptoms due to infection with *F. oxysporum* (Nelson, 1981). Damping off and root rot caused by *F. oxysporum* were reported as some of the most common diseases in forest nurseries in New Zealand. This pathogen has been commonly isolated from roots of *P. radiata* and soils in a number of nurseries in New Zealand (Nelson, 1981; Dick and Dobbie, 2002). *F. oxysporum* has been reported in Argentina and south-western Europe attacking *P. monticola*

Douglas ex D.Don, *P. radiata*, *P. elliottii* Engelm. And*P. taeda* L.(Nelson, 1981; Homechin *et al*., 1986; Stewart *et al*., 2012).

Diplodia pinea has been reported in New Zealand, South Africa, Australia and Mexico, (Brookhouser and Peterson, 1971; Wingfield and Knox-Davies, 1980; Juhasova *et al.*, 2006). The health of *Pinus nigra* became a major concern since 2005 due to a number of parasitic fungi and *D. pinea* was found to be amongst these pathogens in Australia (Juhasova *et al.*, 2006). *Diplodia pinea* damages numerous pine species including *P. nigra* from Australia, *P. sylvestris* from Scotland and *P. ponderosa* from Ponderosa (Brookhouser and Peterson, 1971; Juhasova *et al.*, 2006).

1.5 Importance of the diseases caused by *F. circinatum, F. oxysporum* and *D. pinea* in South Africa

Since the early 1990s the forestry industry has been rapidly growing in South Africa. It contributes greatly to the economy of the country through the production of pulp products and timber. In the country close to 1.5 million hectares is used for pine production, representing only 1.2% of the land area. Most of this land is available in Mpumalanga province where 0.6 million hectares is used for pine plantation.

Most important *Pinus* species planted in South Africa originate from North and Central America. These include *P. patula, P. elliottii, P. taeda and P. radiata*. The latter is grown only in the Western Cape. Porter *et al.* (2009) evaluated the susceptibility of South African native conifers to *F. circinatum* disease. This study was carried out by inoculating seedlings of *P. elongatus* (Ait.) L'Herit.ex Pers., *P. contorta* var. latifolia, *Widdringtonia schwarzii* (Marloth) Mast., *W. nodiflora* (L) Powrie, and *W. cedarbergensis* Marsh with a virulent strain of *F. circinatum*. Three weeks following inoculation with the pathogen all the *Pinus* species developed distinct lesions and after twelve weeks they were all dead. *Podocarpus* and *Widdringtonia* showed resistance to the pathogen. The pattern shown by the South African native conifers is also true for other countries (Barrows-Broaddus and Dwinell, 1985a; Barrows-Broaddus and Dwinell, 1985b).

Fusarium oxysporum is a common inhabitant of seedling nursery soils. Damping off caused by *F. oxysporum* is one of the common diseases affecting seedlings in forest nurseries. *Fusarium*

wilt and damping off cause economic losses in forestry in the Southern hemisphere by affecting *P. pinaster* Aiton and *P. radiata*. The susceptibility of these *Pinus* species was recorded to be much higher towards *F. circinatum* than that of *F. oxysporum*. Under normal nursery conditions damping off caused by *F. oxysporum* is uncommon, and it is likely that all the disease development is associated with *F. circinatum* (Homechin *et al.*, 1986).

In South Africa *D. pinea* can cause up to 28% loss of volume and 55% in potential production in *P. radiata* and *P. patula* following hail damage. *D. pinea* has been isolated from *P. radiata* and *P. patula* seedlings. It affects pine trees damagedby heavy rainfall (water stress), hail and drought (Zwolinski *et al.*, 1990).Smith *et al.* (1996) carried out a study that evaluated the occurrence and hosts for *D. pinea* in South Africa. Results from this study indicated that 50% of young green *P. patula* and 90% of *P. radiata* carried the pathogen, while it was absent from the cones of *P. elliottii* and *P. taeda*. These results were similar to those obtained by Swart and Wingfield (1991) in which the fungus was observed in *P. radiata*. *Diplodia pinea* and has also been detected in the wood of *P. sylvestris* (Petrini and Fisher, 1988).

1.6 Detection methods for plant pathogens

Fusarium circinatum is currently detected using the polymerase chain reaction (PCR), which is a reliable, sensitive and accurate method. A primer pairCIRC1A-CIRC4A that targets an ISG region of the nuclear ribosomal operon has been successfully used in most published studies on the detection of this fungus (Schweigkofler *et al.*, 2004; Perez-Sierra *et al.*, 2007). PCR is a useful method for detection of the fungus in asymptomatic plants and in distinguishing pathogens to the species level. The PCR method has been used for detection of a number of other fungal species including *Rhizoctonia solani* Kühn, *F. oxysporum* and *D. pinea* (Grimm and Geisen, 1998; Calderon *et al.*, 2002b; Budge *et al.*, 2009; Validov *et al.*, 2011). Monitoring specific symptom development is also one of the commonly used methods for the detection of *F. circinatum* in seedling nurseries. Culturing methods and microscopic examination is the standard method for detection of *F. circinatum*. Antibody based detection systems that use an enzyme linked immunosorbent assay (ELISA) have been prepared for detection of *Fusarium* species (Kitagawa *et al.*, 1989; Gan *et al.*, 1997; Hayashi *et al.*, 1998; Lievens *et al.*, 2008). Dip-sticks for detection of *Fusarium* mycotoxins (T-2 toxin) have also been prepared using monoclonal antibodies (de Saeger and van Peteghem, 1996). Tube-like

immuno-assays for the detection of *Botrytis, Aspergillus* and *Penicillium* on grapes have been developed (Dewey and Meyer, 2004).

There has been great effort in the development of more specific and sensitive diagnostic methods such as the use of antibody-based techniques, biosensors and those that are based on evaluating nucleotides (Ward *et al.*, 2004). In the early 1990s the use of antibody-based detection systems dominated publications and received similar attention to that of nucleotide-based assays. From the early 1990s to 2007 there was a 100% increase in the number of papers published on plant detection methods using polymerase chain reaction (PCR) assays and this is expected to have doubled by 2010 going to 2013 (Skottrup *et al.*, 2008). Little focus has been placed on the use of biosensors (Figure 1.2).

Detection methods for plant pathogens must be able to discriminate between different pathogens and be able to detect the presence of the inoculum before the development of symptoms. There are a number of validation steps that need to be carried out before a new detection technique enters the market. These include sensitivity, specificity and reproducibility, accuracy of results, consistency and reliability of detection method (Lievens *et al*., 2008). Samples from different areas should be tested and blind tests should be carried out. Cost issues need to be considered but this may depend on the importance of the disease and the crop affected (Lievens and Thomma, 2005; Lievens *et al.*, 2008). Other issues that need to be considered include multiplexing, expertise and validation. Multiplexing involves screening a large number of samples simultaneously or in a short period of time. This is much more efficient than performing multiple simplex reactions. This can be achieved by using a 96 well ELISA plate in which multiple pathogens can be evaluated in a single assay. Common methods used for identification of fungal pathogens depend on culturing methods and evaluation of morphological features. Such diagnosis requires an expert with strong taxonomical knowledge that may take years of education to acquire. Recent developments in detection systems have the potential to provide diagnostic tools that can be easily interpreted by the nursery staff, or technicians who do not have specific expertise (Faria *et al.*, 2012). With some detection systems more specific assays such as PCR may be required for validation of the identity of the pathogen.

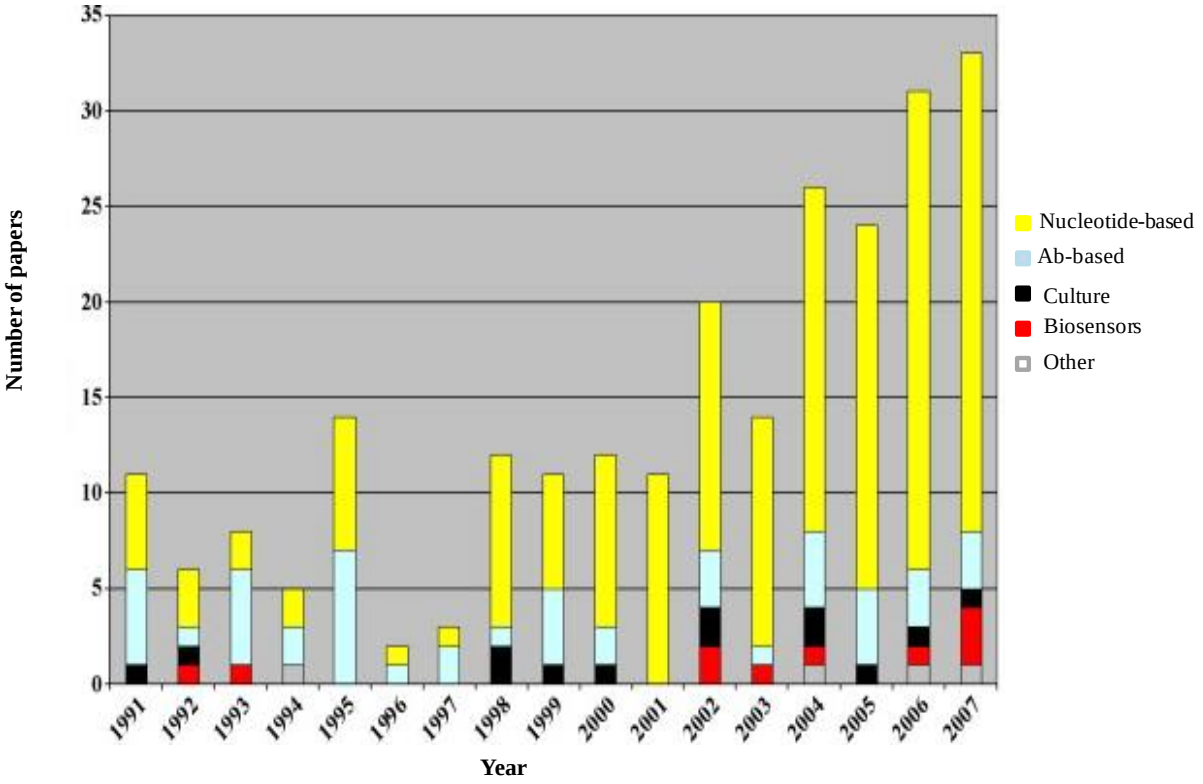


Figure 1.2 Published papers that focused on the detection systems for plant pathogens. This was carried out by searching for "plant pathogen, detection" on the ISI Web of knowledge. The category 'other' includes detection systems such as mass spectrometry and infrared-based detectors (Skottrup *et al.*, 2008).

Diagnostic methods for detecting the presence of *Fusarium* species and other fungal pathogens used to be mainly based on culturing plant material from seedlings suspected of infection, using selective media, and visualising the growth pattern, especially morphological features (Lievens and Thomma, 2005). ELISA based tests are currently available for the quantification of *F. culmorum*, *F. graminearum* and *F. avenaceum* (Gan *et al.*, 1997; Iyer and Cousin, 2003). In general such tests are unable to differentiate the infectious pathogen to the species level but they can be used for quantification across different physiological stages (Beyer *et al.*, 1993). PCR methods have been successfully used for the detection of a number of fungal pathogens such as *F. circinatum*, *D. pinea*, *F. oxysporum*, *Septoria tritici* blotch (Grimm and Geisen, 1998; O'Donnell *et al.*, 1998; Fraaije *et al.*, 1999; Weiland and Sundsbak, 2000; Schweigkofler *et al.*, 2004; Luchi *et al.*, 2007).

1.6.1 Traditional methods used for detection of plant pathogens and their limitations

Symptoms such as seedling wilt and dieback of terminal branches that may develop following fungal infection are occasionally similar to those caused by other factors such as drought

stress, excessive use of fertilizers, exhaustion of soil nutrients and pH shift (Ward *et al.*, 2004). Multiple infection of one seedling by different pathogens and other wilting pathogens, such as *F. oxysporum*, which are normally found in nurseries, can also affect the detection based on symptom development. The similarity of symptoms and mode of infection of *F. circinatum* and *D. pinea* makes it difficult to distinguish them from one another once they have been well established in the field (Skottrup *et al.*, 2008). Some plants only show symptoms at late stages of infection or under stress conditions (Bloomberg, 1981).

Growing pathogens on nutrient agar and inspecting their colony morphology, mycelium shape, pigmentation, analysis of the secondary metabolites and viewing under the microscope are the standard methods for diagnosis (Peltonen, 1995). These methods may be influenced by a number of factors such as incubation conditions and variation in the mycelium formation. In some cases there is a need to distinguish between populations of the same pathogen based on virulence, resistance to antibiotics and production of toxins. For such diagnostics traditional methods are not satisfactory.

1.7 Immunochemical detection methods

Antibodies may be raised against viruses, bacteria, specific proteins, hormones and fungi. One of the main limitations to raising antibodies against more complex organisms such as bacteria and fungi is the fact that these organisms may have different morphological features and may express different structural proteins at each stage of their life cycle. Obtaining species-specific antibodies has been one of the challenges that plant pathologists face. Most polyclonal antibodies raised against fungi are only specific to the genus level (Arie *et al.*, 1991; Gan *et al.*, 1997). This is due to the fact that most intact fungi possess proteins and carbohydrates epitopes that are arranged in a similar manner throughout the genus. Immuno-assays that have been developed over the years have been limited to soluble and homogeneous suspensions of antigens (Arie *et al.*, 1991; Arie *et al.*, 1995; Gan *et al.*, 1997; Hayashi *et al.*, 1998). Such limits have made the detection of fungal species difficult because these consist of insoluble mycelia, which may be difficult to convert into soluble suspensions (Kitagawa *et al.*, 1989; Grimm and Geisen, 1998).

Antigens from fungal pathogens are commonly prepared by growing fungi in liquid medium. The resulting filtrate contains exo-antigens. Mycelium pads are then re-suspended in buffer,

homogenised and centrifuged. The supernatant obtained contains mycelium-soluble antigens while the pellet contains mycelium fragments that can be directly used in immunisation as whole cell antigens (Dewey *et al.*, 1990; Brill *et al.*, 1994; Gan *et al.*, 1997; Hitchcock *et al.*, 1997; Hayashi *et al.*, 1998). Latex antigens are those that are directly isolated from a plant tissue infected with a specific fungus, and have previously been used for raising antibodies. Selection of antigens to use when raising antibodies is specific to each individual study (Table1.1).

Detection systems based on the use of antibodies are only as good as the antibody produced. But most importantly the preparation of the antigen plays a vital role, since any impurities may limit the antibody specificity. For instance, injecting crude fungal culture, filtrates or the whole mycelium will result in antibodies that recognise numerous antigens, making it difficult to characterise the resulting antibody-specificity (Gan *et al.*, 1997). Various antigen preparation methods from fungal pathogens have been used to improve the specificity of antibodies obtained. These include immunising with partially purified cell walls and glycoproteins and using specific proteins produced by pathogens during the infection process (Glancy *et al.*, 1990; Arie *et al.*, 1991; Gan *et al.*, 1997). From these preparations problems with cross-reactivity and non-specific detection of antigens have been observed. In most studies antigen isolation from mycelium samples includes re-suspending mycelium in buffer, homogenisation and pelleting the resulting debris by centrifugation. The clear supernatant with a known concentration is used as the antigens for performing the ELISA (Arie *et al.*, 1991; Gan *et al.*, 1997; Thornton and Gilligan, 1999). Preparation of antigens for immunisation from

Table 1.1Fungal antigen preparations and their corresponding antibodies used in immunological detection methods

Antigen preparation	Study by:	Antibody and immunochemical assay	Comments
Whole cell antigens	Hitchcock *et al*., 1997	Monoclonal antibodies (anti-*Trichoderma*) used in ELISA and immunofluorescence (IF) assay	Cross-reactive with species from other genera More specific than antibodies to mycelium-soluble antigens.
	Arie *et al*., 1991	Monoclonal antibodies (anti-*F. oxysporum*) used in dot immunobinding assay	Specific to the genus, *Fusarium*
Mycelium-soluble antigens	Hitchcock *et al*., 1997	Monoclonal antibodies (anti-*Trichoderma*) used in ELISA and immunofluorescence (IF) assays	70% and 123% cross-reactivity with species from other genera.
	Holtz *et al*., 1994	Polyclonal antibodies (anti-*Thielaviolpsis basicola*) used in ELISA, IF and western blotting	Cross-reactive with other fungi commonly found in the same environment with *Thielaviopsis basicola* Berk. & Br.
	Dewey and Meyer, 2004	Monoclonal antibodies (anti-*Aspergillus* and anti- *Penicillium*) used in ELISA	Reactivity obtained to genus level
	Gan *et al*., 1997	Polyclonal antibodies used (anti-*Fusarium*) in ELISA and western blotting	Cross-reacted with antigens from other genera
Latex antigens	Hitchcock *et al*., 1997	Monoclonal antibodies (anti-*Trichoderma*) used in ELISA and immunofluorescence (IF) assay	ELISA was species-specific to *Trichoderma* and in IF cross-reactivity was observed
	Karpovich-tate and Dewey, 2001	Monoclonal antibodies (anti-*Ulocladium atrum*)used in ELISA	Antibodies were specific to genus level
Antigens from spore suspension	Wright *et al*., 1987	Monoclonal (anti-mycorrhizal) antibodies used in ELISA	Species-specific antibodies obtained to conidia and hyphae of *Glomus occultum* C. Walker.
Exo-antigens	Gan *et al*., 1997	Polyclonal antibodies (anti-*Fusarium*) used in ELISA and western blotting	Genus-specific antibodies for some *Fusarium* antigens species-specificity obtained

mycelium debris brings about significant cross-reactivity, because they contain cell wall material such as chitin and other polysaccharides that are common between different species.

1.7.1 Antibody production and isolation

Two routes for the production of antibodies have been developed namely, polyclonal and monoclonal (Ward *et al.*, 2004). Experimental animals used for antibody-production include horses, goats, donkeys, guinea pigs, rabbits and mice. For isolation of antibodies from these experimental animals, collection of blood is required. For the production of monoclonal antibodies mice is commonly used, but this may be limiting because some antigens are not immunogenic in mice (Mojca, 2003). Like mammals, chickens make antibodies in response to challenge with antigens. Production of polyclonal antibodies using chickens provides an alternative, convenient and inexpensive method for antibody production. While the egg is still in the ovary, immunoglobulin receptor mediated endocytosis transfers large amounts of the serum immunoglobulin Y (IgY) into the yolk. The concentration of IgY in the yolk is equivalent to that found in the serum. However, IgY in the yolk is easier to purify and can be extracted in larger amounts, because the yolk is free of impurities such as IgM and IgA found in the serum (Schade and Erhard, 2001). Chickens lay approximately one egg per day. The total antibody (IgY) per egg varies from 40-80 mg, depending on the age of the laying hen (Pauly *et al.*, 2011). Antibodies obtained from one egg are equivalent to that obtained from 30 ml of blood from rabbits. However, only approximately 5 ml of blood can be collected per day. The ability of chickens to produce high yields is advantageous because it reduces the number of animals used in each study (Michael *et al.*, 2010). Larger animals such as horses and donkeys can produce more antibodies than chickens but these are expensive to feed and difficult to handle. Raising antibodies in laying hens is a favoured system compared to others, because chickens are easy to handle and antibodies are packaged in their eggs, which presents a more humane way of collecting antibodies than bleeding of mammals. IgY is also advantageous over the use of mammalian antibodies, because it can be used in a number of immunochemical assays without loss of specificity and sensitivity (da Silva and Tambourgi, 2010). IgY is extracted from the egg yolk by means of a precipitation procedure that uses polyethylene glycol (PEG) (Polson *et al.*, 1980). In this procedure, the first important step is to

remove lipids from the egg yolk, followed by precipitation of the total IgY from the supernatant free of the lipids. The purity of polyclonal antibodies obtained is around 80% (Pauly *et al.*, 2011). Polyclonal antibodies can recognise multiple epitopes on the antigen surface and these have been used successfully in detection of plant pathogens including *F. circinatum, F. oxysporum* and *Serpula lacrymans (Wulfen) P. Karst*. (Arie *et al.*, 1995; Gan *et al.*, 1997).

Gan *et al.* (1997) characterised polyclonal antibodies raised against *Fusarium* species [*F. sporotrichioides* Sherb., *F. poae* Peck Wollenw. and *F. graminearum* (Schwein.)Petch.)]using an ELISA and immunoblotting. In their study chickens were immunised with two sets of immunogens. The first set was obtained from within the mycelia and these were referred to as mycelia-soluble antigens. Exo-antigens were used as the second set of immunogens. These were concentrated via a series of steps including freeze-drying. This method for preparation of antigens from fungal pathogens is commonly used by most researchers (Arie *et al.*, 1991; Holtz *et al.*, 1994). Low antigen concentration was one of the main problems in these studies. Both mycelium-soluble and exo-antigens were analysed on SDS-PAGE and some protein bands were not observed when a silver stain was used. These protein bands were only visible after the SDS-PAGE was used for protein transfer onto nitrocellulose membrane. This membrane was used in a western blot technique to determine antigenic proteins. Western blotting indicated that immunodominant antigens were all above 28 kDa in size for these *Fusarium* species. The antibodies against mycelia-soluble antigens showed cross-reactivity with the antigens from other genera such as *Aspergillus* and *Penicillium*, whereas the antisera against exo-antigens were generally more specific when compared to those against mycelium-soluble antigens. Species-specific antibodies were only obtained from antisera against exo-antigens from *F. poae (Peck) Wollenw*. Like Gan *et al.* (1997), Brill *et al.* (1994) reported that polyclonal antibodies raised to culture filtrate were more specific but these were less reactive than those raised against mycelia extracts. A study carried out by Holtz *et al.* (1994) using antigens prepared from *T. basicola* indicated that there is no significant difference in the antibodies obtained using both sets of antigens (soluble antigens and those obtained from mycelium fractions).

Monoclonal antibodies (API9-2) for *F. oxysporum* were produced with mycelium-soluble antigens (Arie *et al.*, 1991). Antibody producing cells were analysed by dot immunobinding assay. The cell lines secreting monoclonal antibodies prepared in this study produced antibodies that were specific to the genus *Fusarium* and did not react with fungi from other genera but no species-specific antibody was found.

A study to detect *Phytophthora* species on roots of dark-rooted wood plants (*Chamaecyparis*) was conducted. Polyclonal antibodies were used to develop an indirect dot-immunobinding assay (DIBA). The drenching method for artificial inoculation with 1 x 10^5 zooconidia was used. Symptoms where only observed six weeks post-inoculation. For isolation of antigens seedlings were collected, rinsed under running tap water, sectioned, homogenised and centrifuged. The supernatant was then used in the assay (Hahn and Werres, 1997). Similar work has been reported before (Parent *et al.*, 1985; Kimishima and Kobayashi, 1990). These studies reported the problem of non-specific reaction, in which detection on non-infected seedlings was observed. A series of optimisation steps had to be carried out to minimise the background reactions in western blot.

1.7.2 Different methods for determining antibody reactivity and specificity

Both monoclonal and polyclonal antibodies have been used in a number of *Fusarium* detection systems with great success. Immunofluorescence, immunoblotting, enzyme-linked immunosorbent assays (ELISA), modified ELISA, chromatographic assays and dip-stick assays have been developed (Kitagawa *et al.*, 1989; Arie *et al.*, 1995; Gan *et al.*, 1997). ELISA is a method that was introduced early in 1970 (Clark and Adam, 1977). It requires small volumes of reagents and multiple tests can be performed in one plate (Clausen, 1997).

ELISA has proved to have wide-spread application in the plant pathology due to the ability to identify and quantify different fungal pathogens. ELISA formats such as indirect ELISA or double antibody sandwich (DAS) ELISA can allow quantification of fungi in plant tissue and complex mixtures such as soil and plant extracts (Figure 1.3). In the indirect ELISA, antigen is first bound to a solid support. Primary antibody followed by a secondary or detection antibody labelled with an enzyme is added. Addition of a substrate solution gives a colour change that can be measured to quantify the amount of antigen present in a sample (Figure 1.3 A). In the DAS-ELISA, antibody (capture antibody) is immobilized on a solid support; usually a

polystyrene microtitre plate is used. Antigen is then added which complexes with the antibody. A detector antibody (secondary antibody) bound to biotin is added, followed by avidin complexed with an enzyme [horseradish peroxidase or alkaline phosphatise (HRPO) or alkaline phosphatase (AP)]. Alternatively, the secondary antibody could be labelled with an enzyme (HRPO or AP).Substrate solution is added which then reacts with the enzyme bound to the detector antibody or avidin. This produces a coloured product that indicates the amount of antigen present in the samples (Figure 1.3 B). Between each step unbound protein is removed with washes using a mild washing reagent that comprises buffer and detergent such as Tween 20.

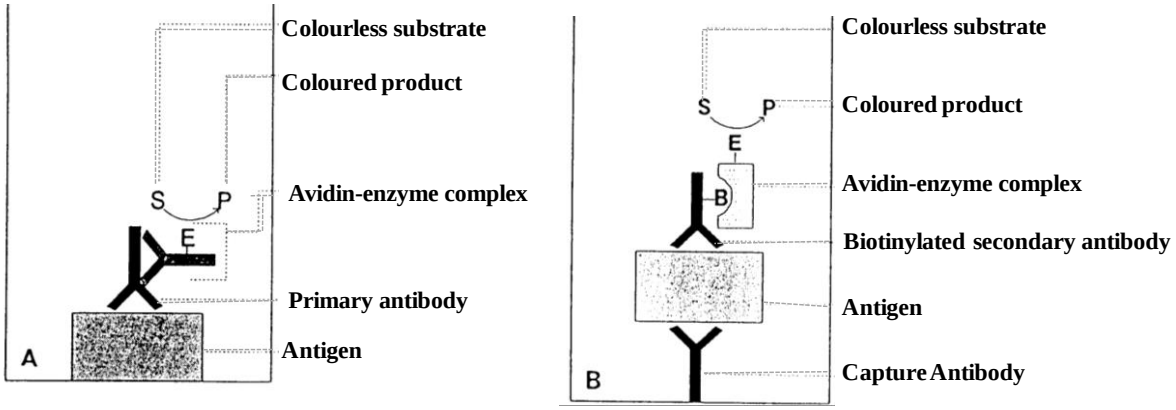


Figure 1.3 Common ELISA formats used in the development of *Fusarium* detection methods. (A) Indirect ELISA format. (B) Double antibody sandwich ELISA (Brill *et al.*, 1994).

Brill *et al.* (1994) showed that between the indirect and double sandwich ELISA the latter provides increased levels of specificity. More than a 100-fold increase in sensitivity was observed with sandwich ELISA and consistent results between different polyclonal antibodies were obtained. Antibodies raised against Strain F504 of *F. oxysporum* were used in DAS-ELISA in which the solid supports (amino-Dylark balls) were first coated with diluted anti-*F. oxysporum* antibodies. Cell fragments of strain F504 of *F. oxysporum* were added, and for detection, a secondary antibody was used. Little cross-reactivity was observed when other *Fusarium* species and even strains closely related to Strain 504 of *F. oxysporum*, such as Strain 501 were used in ELISA (Kitagawa *et al.*, 1989).

Some fungal pathogens, such as *R. solani*, require prolonged incubation periods to increase production of antigens before they can be effectively used in ELISA. The importance of antigen preparation for optimal sensitivity of ELISA has been evaluated (Hitchcock *et al.*,

1997). Contaminants such as organic compounds used during antibody labelling and insoluble debris can reduce the sensitivity of ELISA. This is a result of the contaminants competing with antigens for binding sites on the micro-titre plate well during the antigen coating stage of the assay. Thornton and Gilligan (1999) reduced the levels of contaminants by diluting the prepared antigens from *R. solani* with fresh buffer before loading the sample into the micro-titre plate wells.

Proteins secreted by a pathogen for disease development can serve as a good tool for detection of the causal agent (Arie *et al.*, 1998; Grimm and Geisen, 1998). Polyclonal antibodies were developed in mice for detection of endopolygalacturonase (PG) protein. This protein is produced by *F. oxysporum* for disease development. *F. oxysporumf.sp.lycopersici* Race 2 was maintained on potato dextrose medium (PDA) and this was used for PG purification. Purified protein was used for immunisation. Production of antibodies was confirmed using western blotting analysis. This analysis indicated that PG protein has a size of approximately 40 kDa, which is in line with the reports by Gan *et al.* (1997) that *Fusarium* immunodominant proteins are larger than 28 kDa. Direct tissue-blotted immunobinding was used to detect PG protein on infected tomato stems. In this experiment infected and non-infected stems were directly placed on the nitrocellulose membrane. This membrane was then immersed in anti-PG antibodies. Colour development was an indication for a positive test, which was only observed with infected stems and not with non-infected stems (Arie *et al.*, 1998). Other studies have looked at raising antibodies against fungal pathogens such as *R. solani* to evaluate growth dynamics and hyperparasitism in biological controls. In one study the level of antigen isolated from an infected plant was compared in the presence and absence of a biological control agent (*Trichoderma harzianum* Rifai.). There was a direct correlation between the amount of mycelium and the levels of ELISA readings obtained (Thornton and Gilligan, 1999). Where there was no control agent higher absorbance readings were obtained. This was an indication that ELISA can be applied in studies where the levels of infection need to be detected. However, other studies have reported the use of antibodies as not an ideal system for specific and quantitative detection of fungal antigens from infected wood (Clausen, 1997).

Other methods have improved on the basic principles of ELISA to allow for on-site detection rather than carrying samples to the laboratory. These methods can be used by farmers in the

greenhouses and in the field. Detection systems based on the dip-stick principle for detection of human and some plant diseases are well established (Cahill and Hardham, 1994; Dewey and Meyer, 2004). The binding of antibodies to the membrane during the preparation of a dip-stick is achieved through hydrophobic, ionic or electrostatic interactions. The use of membranes for antibody binding has a number of advantages; these include the large surface area available for proteins to bind due to the porosity of the membrane. The porous nature allows for the liquid carrying the antigen (test sample) to move quickly through the membrane to interact with the specific site on the surface (Kaur *et al.*, 2007; Skottrup *et al.*, 2008; Zhang and Wang, 2008). ELISA dip-stick can be used with little training as the results are based on the development of a colour reaction (Cahill and Hardham, 1994). In addition there is no need for the use of expensive equipment such as spectrophotometers and micro-titre plates.

In 1994 the efficiency of an ELISA dip-stick method was evaluated in the detection of *Phytophthora cinnamomi* Rands. zoospores in soil using monoclonal antibodies. Zoospores in a suspension were allowed to react with the membrane and these were then separated, based on the principles of chemotaxis and electrotaxis. When all particles stopped moving on the membrane, based on their individual chemical properties, cysts formed. The cyst point was detected using immuno-assays. Enzyme-linked monoclonal antibodies were used for detection and the colour development was visible with the naked eye. Using this method a total of 40 zoospores ml^{-1}were detected in less than 45 min (Cahill and Hardham, 1994). This showed that ELISA dip-stick is a quick method to use for onsite detection. Dip-stick assays have been successfully used in other studies that looked at the *Fusarium* mycotoxins (T-2 toxin) (de Saeger and van Peteghem, 1996).

Fungal antigens can be located within plant tissues using labelled antibodies; this is useful since it allows the pathogen to be monitored as it moves or grows within the tissue. In this system a specific antibody is conjugated with a florescence dye. As the labelled antibody binds to the pathogen it is viewed under a microscope. This method is widely used in research but remains unsuitable for the screening of large numbers of samples since the antigen binding site can be hidden. In addition it needs to be carried out by trained personnel, and expensive equipment such as a fluorescence microscope is needed. A study carried out by Arie *et al.* (1995) raised monoclonal antibodies to *F. oxysporum* soluble antigens. These antibodies were

classified as genus specific and were used to detect *Fusarium* species in plant tissue. Immunofluorescence assays (IFA) and direct tissue-blotted immunobinding assay (DT-IBA) detection systems were developed. IFA allows for *in situ* detection, localization and monitoring of behavior of the pathogen. In this assay plant tissue (fresh roots, crown or stem) were sectioned into thin slices and applied to a microscope slide and fixed. For detection, binding of anti-*Fusarium* monoclonal antibody conjugated to a fluorescent dye was monitored under the microscope. The binding site for these monoclonal antibodies was only observed on the surfaces of mycelia and no signal was detected on any type of conidia. IFA has been used in other studies to identify plant pathogen conidia on microscope slides (Kennedy *et al.*, 1999). Arie *et al.* (1995) reported that a tissue-blotted immunobinding assay was an ideal system to use to detect seedlings infected with *Fusarium*. In this system cross sections with a plane cut-surface of infected and non-infected plant tissues were put on a nitrocellulose membrane that had been previously immersed in buffer. For detection antibodies were added and allowed to react with the membrane. Infection in stems were detected within four hours. This system was also successful in the detection of molecules excreted by fungal pathogen (*F. oxysporum*) during the infection process (Arie *et al.*, 1998).

In the current study an ELISA for detection and discrimination of *F. circinatum* from *F. oxysporum* and *D. pinea* was optimised. Firstly, antigens from these pathogens were analysed on SDS-PAGE, to evaluate and select specific protein unique for each. These specific proteins were used for immunisation in attempt to produce of more specific antibodies. This method has not been reported in the previous detection studies that focused on *Fusarium* pathogens. Mycelium-soluble antigens and exo-antigens were also be used for immunisation.

Polyclonal antibodies were raised in laying hens, which is a favoured system compared to using other experimental animals because antibodies are packaged in eggs, which presents a more humane way of collecting antibodies than bleeding of mammals and provides increased antibody yields. ELISA was selected because it can be used in complex mixtures such as those of plant extracts and soil mixtures without any purification steps needed. This is an important element, as *F. circinatum* is commonly found in complex mixtures such as soil and plant extract. In the current study seedlings were infected with different conidia concentrations, ranging from one conidium ml^{-1} to $1x\ 10^{6}$ ml^{-1} to detect antibody detection limit. This was not

evaluated in the previous studies that focused on the detection of fungal antigens isolated from plant material. The goal was to develop an ELISA test that could be used directly in the field, and results could be easily interpreted by nursery staff. Cross-reactivity was expected to some extent. Since all three pathogens were included into one detection system, specificity could be indicated by a more intense colour, indicating the presence of a specific pathogen compared to others. The principle used for dip-stick and tube-like immuno-assay mirrors that used in indirect ELISAs (Dewey and Meyer, 2004). ELISA prepared in this study will serve as the first step towards preparation of ELISA dip-stick kit for field detection for *F. circinatum*.

1.8 Nucleic acid-based detection methods

Molecular techniques that make use of PCR assays are generally more accurate, faster, specific and do not require extensive taxonomical knowledge (Lievens *et al.*, 2008; Loos *et al.*, 2009). These systems allow for the detection of non-culturable micro-organisms. Some of the important parameters in this assay include using an appropriate DNA extraction method and selection of primers that are specific for amplification of the gene of interest. Real-time PCR is considered as one of the best methods to use for detection of plant pathogens. This assay allows the monitoring of the reaction after each amplification cycle. The quantity measured after each cycle can be used to generate a standard curve with known amounts of target copies. This can be used to accurately quantify the target pathogen and monitor the progress of the disease in an infected plant (Garrido *et al.*, 2009; Lunchi *et al.*, 2011). Real-time PCR has an increased sensitivity and specificity compared to conventional PCR assays.

1.8.1 PCR specificity and application

The advantage of using a PCR assay is its power to distinguish between closely related species. In the detection of fungal diseases this is exploited by targeting conserved genes. These base pairs are usually targeted for detection using specific PCR primers, detector oligonucleotides and hybridization probes (Livak, 1999; Consolandi *et al.*, 2001; Papp *et al.*, 2003). To enhance specificity a detection system that can recognise a number of different genes in one reaction has an advantage.

One of the frequently targeted regions for fungal detection is the nuclear ribosomal DNA (rDNA) present in high copy number in all fungal organisms. This region has been exclusively

studied and characterized in studies that involve molecular phylogenetic analyses (McCartney *et al.*, 2003; Inami *et al.*, 2010; Validov *et al.*, 2011). Public databases for rDNA are available, allowing for sequence comparisons between different fungal species, and this aids the development of highly specific diagnostic methods (Xue *et al.*, 1992).

Fungal rDNA consists of three subunits separated by internal transcribed spacers (ITS). It is within these spacers that alternating regions of high conservation and variation occur (Grimm and Geisen, 1998; Weiland and Sundsbak, 2000). The region of variation allows for discrimination of one genus from another, and even to species level (Xue *et al.*, 1992; Baldwin *et al.*, 1995; Budge *et al.*, 2009). The conserved regions allow for primers from one species to be used to detect other similar species from the same genus or family. Internal transcribed spacers may not always contain sufficient variation for separating certain species (Kong *et al.*, 2004). Since it is very important to discriminate between different species, more research developments have focused on other conserved genes that show regions of variation. Conserved genes that vary between different fungal species such elongation factor 1-apha, β-tubulin and actin have been extensively studied as targets for discriminating between closely related fungal pathogens (O'Donnell *et al.*, 1998; Fraaije *et al.*, 1999; Weiland and Sundsbak, 2000).

Schweigkofler *et al.* (2004) introduced a method for the detection of *F. circinatum* both in air (conidia floating in the air) and plants. In this study fungal conidia were trapped onto filter paper and the primers were synthesized from the variable regions of ITS. Another method of trapping conidia has been used in other studies in which the conidia were trapped onto a transparent tape and analysed by PCR (Calderon *et al.*, 2002b; Calderon *et al.*, 2002a). *F. circinatum* contains a species-specific primer pair referred to as CIRC1A-CIRC4A that can amplify a 360 base-pair DNA fragment. This fragment is found in the intergenic space region of the nuclear ribosomal operon. The trapped conidia were selected, purified and prepared for real-time PCR and in the presence of the specific primer pair (CIRC1A-CIRC4A) and a positive test was obtained. The use of the filter paper was much more effective then the use of Petri dishes with selective media to trap the conidia. This study shows that not only are the nucleic based methods specific and accurate but they can be used in the quantification of the number of conidia present in the air. The latter was carried out by preparing a calibration

curve using the threshold values of standards of the known number of *F. circinatum* conidia. The threshold values of the unknown sample were then compared to those of known ones. Real-time PCR was also used for the detection of fungal pathogens, such as the occurrence of *D. pinea* in asymptomatic pine shoots. In one study shoots from healthy trees (*P. nigra*) were collected and used for DNA extraction. Using real-time PCR, the fungus was detected in the asymptomatic shoots where numbers of DNA fragments were relatively low (Luchi *et al.*, 2007).

For detection of *F. circinatum* at very low concentrations in seeds a biomass enrichment step has been introduced. This is achieved by culturing a number of seeds suspected of infection in potato dextrose broth for 72 hours. From this incubation enough DNA can be extracted for detection using real-time PCR. This method has proved to be effective for the detection of one infected seed per thousand. This methodology was more specific and sensitive than the conventional PCR test previously developed for 420 pine seed DNA extracts (Loos *et al.*, 2009). Abd-Elsalam *et al.* (2006) introduced a detection system for the presence of a wilting pathogen in cotton, *F. oxysporum f.sp. vasinfectum* (FOV). Noviello & W.C. Snyder. The primers used in this study were only specific for FOV and no other cotton pathogens. These were present in the internal transcribed spacer between 18S, 5.8S and 28S ribosomal DNA.

Primers 5'CCCCTGTGAACATACCTTACT-3' and 5'-ACCAGTAACGAGGGTTTTACT-3'were designed and were able to amplify a 400 base pair DNA fragment for FOV from different parts of the world. A control study consisting of a universal primer that can amplify generic fungal DNA was used to study a number of different cotton pathogens. These primers could amply a fragment of 200 base pairs of all fungi associated with cotton (*F. moniliforme* J. Sheld., *Verticillium albo-atrum* E.B. Himelick.,*V. dahliae* Kleb.,*Aspergillus* species Johann Heinrich Friedrich Link, *Fusarium oxysporum*, *F. sambucinum* (Schwein.) Petch, and *F. solani*). This control served as an indicator that DNA from other fungi was amplifiable and it indicated the specificity of the FOV primers (Nazar *et al.*, 1991).

In the mid 1990s PCR assays were used to detect *F. culmorium, F. graminearum and F. avenaceum*. In one study less than 10^{-12} fungal DNA base pairs was detected, which indicated very high sensitivity. This assay included screening for sequence differences between these species, which was achieved by random amplification of polymorphic DNA. Unique

fragments were cloned and sequenced. Based on the sequences obtained specific primers were designed. These were then used to amplify single DNA fragments. No cross-reactivity was observed since each primer was specific for one species. These primers were successfully used to detect the presence of the inoculum directly from tissue extracts with multiple infections (Schilling *et al.*, 1996). Similar studies on the detection of *F. verticillioides* (Sacc.) Nirenberg using PCR assays have been reported (Faria *et al.*, 2012).

Some of the drawbacks to using PCR assays include the issue of cost. A well equipped laboratory is required to carry out PCR assays. This might be a big issue for developing countries. Secondly, some fungal species such as *F. oxysporum*, *F. solani* and *R. solani* comprise of pathogenic, non-pathogenic and even beneficial strains (Recorbet *et al.*, 2003). In such instances general sequence databases comparing different species cannot be used. Instead genes linked to pathogenicity need to be compared (Johnson *et al.*, 2000). This can be hampered by the lack of adequate sequence information. The reproducibility and reliability of PCR assays may also be reduced by the possibility that some of the nucleic acids are co-extracted with other naturally occurring phenolics such as fulvic acids during extraction. This can be circumvented by the optimisation of extraction kits (Lievens and Thomma, 2005).

1.9 Comparisons between immunochemical and nucleic-acid based detection systems

Immunochemical and nucleic-acid based techniques are more accurate and definitive than the traditional methods for fungal identification. Selection of either ELISA or PCR is based on the application and the availability of equipment, money, time and trained staff. The body of research previously carried out on the particular pathogens may direct the choice of detection method. For instance, the availability of highly specific sequence information can make the use of a PCR approach more appropriate. However,the availability of information on the pathogencity and proteins that directly attack the host, favours the use of immunochemical systems. In some detection studies of fungi both these systems have been combined to increase the specificity (Grimm and Geisen, 1998).A more detailed comparison between the two systems in given in Table 1.2.

Table 1.2A comparison between immunochemical-detection systems and nucleic acid-based detection systems for detection of fungal pathogens in plants.

Sampling	Immunochemical-detection system	Nucleic acid-based detection system
Ease of development	Involves an animal system. Immunising material can be the specific protein for the pathogen, chitin, cell-surface glycoproteins, and carbohydrates.	Easy to find sequence differences between species if sequence information is already available. System for detection of specific markers are available (RAPDs).
Sample preparation	Depends on the plant material and factors that may alter the antigen exposure for good antigen-antibody interaction.	Minimal, may obtain DNA from crude sample or may require DNA purification. Extraction and purification kits are available.
Procedure	Detection can be obtained in 15 min when using kits (ELISA dip-stick). May be complex when carried out in the laboratory for quantification (ELISA, Western blotting).	Requires technical specialist to perform since it requires specialised equipment and proper measurements of reagents (primers, nucleotides).
Portability	Can be used on site or in the field. Can monitor aerial pathogens in greenhouses over time.	Can only be carried out in the laboratory.
Specificity	Cross-reactivity between fungal species of the same genus, but good with viruses.	Very specific. Can distinguish between different species from the same genus based on the specific primers used.
Sensitivity	Depends on the concentration, exposure, possible morphological changes of the antigen.	Very sensitive, biomass may be increased by growing on solid/liquid medium. Multiple copies of the target DNA which corresponds to a single cell can be obtained following DNA sequencing.
Quantification	Quantified using ELISA.	Quantification may be difficult; real-time orquantitative PCR may be required.
Detection	Difficult to detect more than one pathogen; can detect both viable material and denatured proteins.	Can detect more than one pathogen using multiplex PCR. Detection can be carried out on dead material.
Cost	Relatively cheap, allows for testing multiple reactions simultaneously	Expensive, especially real-time PCR.

1.10 Conclusion

In conclusion, both immunochemical and nucleic-acid based detection systems have important roles in plant pathogen diagnostics. These have proved to be more effective in terms of specificity, accuracy and time efficiency than traditional plant pathogen detection methods such as culturing on selective media, and monitoring of symptom development. The use of antibodies allows for *insitu* studies of the pathogen, which is not possible with the traditional detection methods. This gives a better understanding of the antigen behaviour and the host-pathogen interaction. However, the specificity of antibodies is not always satisfactory, especially for fungal pathogens. Nucleic-acid based detection systems are playing a major role in plant pathology research because of the highly specific technique they offer. More studies and the use of these detection methods can reduce cost issues due to more products being available and more manufacturers to choose from. Efficient sampling strategies have to be developed that can quickly identify pathogens in seeds and asymptomatic trees. The consensus today is that one needs a combination of classical, DNA and immuno-based detection systems, for accurate pathogen identification. For speed and efficiency in the field ELISA is superior. The objective of the current study was to raise antibodies that can be used to develop ELISA tests for the discriminatory detection of *F. circinatum*, *D. pinea* and *F. oxysporum* in pine seedlings.

CHAPTER 2

Development and characterisation of chicken antibodies raised against *Fusarium circinatum*, *Fusarium oxysporum* and *Diplodia pinea* for use in Enzyme-Linked Immunosorbent Assays (ELISA) or western blotting

Abstract

Fusarium circinatum is a causal agent of pine pitch canker on mature trees and terminal seedling wilt on pine seedling. Currently the detection of *F. circinatum* is largely dependent on the diagnosis of disease symptoms. However, other pathogens or abiotic factors result to the development of the same set of symptoms. In this study, an ELISA was developed for quick, accurate and low cost detection of *F. circinatum* in the field. Separate groups of chickens were immunised with soluble antigens from *D. pinea*, *F. circinatum* and *F. oxysporum* mycelia and exo-antigens from *F. circinatum*. A 34 kDa protein purified from SDS-PAGE specific for *D. pinea* was also used for immunisation. Western blot analysis indicated that immuno-dominant antigens for *F. circinatum* were larger than 35 kDa and their reactivity were not the same between different isolates. Anti-*D.pinea* antibodies showed minimal cross-reactivity with antigens from *F. circinatum* and *F. oxysporum*. Cross-reactivity between the two *Fusarium* species was observed when using ELISA and western blotting technique. However, this ELISA can be used for discrimination of *F. circinatum* from other common pine infecting fungi. A significant difference ($P < 0.001$) in the reactivity of these antibodies was observed when they were incubated with other fungal pathogens. There is still a need to investigate the reactivity of these antibodies in the field on artificially inoculated seedlings, followed by full scale field tests ratified by PCR assays.

2.1 Introduction

Fusarium circinatum Nireberg and O'Donnell [=*F. subglutinans* (Wollenw. and Reinking) Nelson *et al. f.sp. pini* Corell *et al*]is a fungal pathogen that causes two sets of pine diseases: pitch canker of mature pines and seedling wilt in nurseries. Seedling wilt has serious impact on pine production in South Africa and other countries throughout the world, leading to serious losses in the pine industry (Wingfield *et al.*, 2008). Recently, *F. circinatum* has been reported to occur in countries such as Spain, Haiti, Chile, South Africa and Japan (Dwinell, 1978; Viljoen *et al.*, 1994; Viljoen *et al.*, 1995; Wingfield *et al.*, 2002a; Perez-Sierra *et al.*, 2007). Some of the susceptible pine species include *Pinuspatula* Schldl. et Cham, *P. elliottii* Engelm., *P. radiate* D. Don and other non-pine species such as *Pseudotsu menziesii* (Mirb) (Douglas-fir) (Carey and Kelley, 1994; Clark and Gordon, 1998; Gordon *et al.*, 1998; Carlucci *et al.*, 2007). In South Africa and other countries in the Southern hemisphere *P. radiata* and *P.*

patula are commonly used for timber and soft wood production respectively and that greatly contributes to the national economicy (Dwinell, 1978; Porter *et al.*, 2009). Terminal wilt, root and collar rot are some of the symptoms observed in seedlings. On mature trees fading and wilting of the needles constitute the first set of symptoms (Gordon *et al.*, 1998).

Interpretation of visible symptoms, use of selective media for *Fusarium* and microscopic analysis are some of the standard methods used for identification of *F. circinatum*. These methods can be slow, require additional confirmation steps and their specificity is often impaired by the presence of taxonomically closely related pathogens and those that cause similar symptoms such as *F. oxysporum* Schlecht and *Diplodia pinea* (Fr) Dyko & B. Sutton, respectively (Britz *et al.*, 2001). The use of nucleic acid based detection systems using PCR has been reported for the detection of different fungi including *F. circinatum* (Calderon *et al.*, 2002b; Calderon *et al.*, 2002a; Schweigkofler *et al.*, 2004; Luchi *et al.*, 2007; Loos *et al.*, 2009). PCR-based detection methods have been accepted as a highly specific and sensitive method in plant pathogenicity studies, because it uses specific primers to target and amplify DNA sequences for each fungus (Homechin *et al.*, 1986; Xue *et al.*, 1992; Livak, 1999; Consolandi *et al.*, 2001; Papp *et al.*, 2003). However, PCR diagnostic tests are not ideal to use in the field and require trained personnel.

Antibody based detection systems using enzyme-linked immunosorbent assay (ELISA) are quicker and easier to conduct compared to PCR, and can be used in the field. ELISAs have a widespread application in plant pathology, where they are used for detection and identification purposes of different fungal and viral pathogens. This is because some formats such as double-antibody sandwich (DAS) ELISA can be used for quantification of fungi in plant tissue, complex mixtures such as soil samples and plant extracts. Antibodies used in ELISA tests commonly target the proteins (antigens) that distinguish different pathogen species. This makes ELISA tests most suitable to use in the detection of a specific fungal antigen in complex mixtures, and multiple tests can be conducted simultaneously.

Antibodies have been developed to whole cell extract antigens (prepared from freeze-dried fungal mycelia that has been re-suspended in buffer and homogenised), mycelium-soluble antigens (collected from the supernatant following centrifugation of whole cell antigens) and particulate antigens (isolated from plant material infected with a specific fungus) isolated from

Mycena galopus (Pers) P. Kumm. Specificity of these antibodies was analysed using ELISA and indirect immunofluorescence tests. Antibodies to whole cell extracts- and mycelium soluble antigens were specific to genus level, and those to whole cell extracts cross-reacted with fungi from other genera, whereas antibodies to particulate antigens were species-specific (Hitchcock *et al.*, 1997). Antibodies raised against mycelium-soluble – and whole cell extract antigens were used in ELISA for the detection of *Thielaviopsis basicola* (Berk. & Broome) Ferraris. These antibodies were specific to *T. basicola* but also showed minimal cross-reactivity with fungi from other genera (Holtz *et al.*, 1994). In other studies, antibodies were developed using mycelium-soluble antigens from *F. culmorum* (Wm.G. Sm.) Sacc, *F. graminearum* (Schwein.)Petch., *Fusarium oxysporum* Schlecht and *F. avenaceum* (Fr.) Sacc.When ELISA tests were used for the detection, specificity to genus level was observed (Kitagawa *et al.*, 1989; Beyer *et al.*, 1993; Arie *et al.*, 1995).

Antigenic determinants for *Fusarium* pathogens were characterised using a genus-specific monoclonal antibody. Analysis on ELISA indicated that antigens existed as exo-antigens. However, it was believed that these antigens were on the mycelial surface and could be easily released into the environment (Hayashi *et al.*, 1998). Other studies reported the antigenic determinants do exist on the surface of mycelia for *Fusarium* species, *T. basicola* and *Ulocladium atrum*, as shown by immunofluorescence assays (Holtz *et al.*, 1994; Arie *et al.*, 1995; Karpovich-tate and Dewey, 2001). Further analysis of the exo-antigens characterised for *Fusarium* pathogens indicated that they were heat and proteinase-K resistant, leading to a conclusion that these were carbohydrate in nature (Hayashi *et al.*, 1998). A similar study characterised extracellular antigens isolated from *Phytophthora megasperma f. sp. glycinea* (Wycoff and Ayers, 1990). These antigens were first treated with periodate, alpha-mannosidase and endo-beta-N-acetylglucosaminidase and then analysed by western blots and ELISA. Using monoclonal antibodies against *P. megasperma f. sp. glycinea* the extracellular antigens were also characterised as carbohydrate in nature (Wycoff and Ayers, 1990). Development of multiple bands on the western blots indicated that the *P. megasperma f. sp. glycinea* proteins may have single or multiple types of attached carbohydrates antigens (Wycoff et al., 1987; Wycoff and Ayers, 1990). Immunofluorescence tests using monoclonal antibodies indicated that antibody binding was more directed against an unidentified chitin and polysaccharide for *Mycena galopus* (Hitchcock *et al.*, 1997). This binding site was also

reported for the *Fusaria* genera when monoclonal antibodies to *M. galopus* cross-reacted with the *Fusarium* pathogens and when anti-*Fusarium* antibodies were used for detection (Arie *et al.*, 1995; Hayashi *et al.*, 1998). The presence of similar cell wall constituents such as chitin and polysaccharides between different fungal groups makes it difficult to obtain species-specific antibodies and to avoid cross-reactivity with fungi from other genera (Kitagawa *et al.*, 1989).

Monoclonal antibodies raised against *F. oxysporum* 860929 in mice using mycelium-soluble antigens were specific to the genus, *Fusarium* and no species-specificity was observed (Arie *et al.*, 1991). Results obtained from this study were similar to those obtained by Gan *et al.*, (1997) where the genus (*Fusarium*) specific antibodies were obtained. In the latter study, polyclonal antibodies were raised in chickens using exo-antigens and mycelium-soluble antigens prepared from three *Fusarium* species (*F. sporotrichioides*, *F. poae* and *F. graminearum*). Antibody reactivity was evaluated with ELISA and immuno-blotting assay. The latter indicated that *Fusaria* immuno-dominant proteins were larger than 28 kDa and common between three *Fusarium* species. In another study immuno-dominant proteins of three strains of *F. verticillioides* were reported to run at 67 kDa and 113 kDa on reducing SDS-PAGE (Biazon *et al.*, 2006). This indicated that fungal pathogens contain multiple antigenic proteins, and a possibility of protein degradation or the presence of pre-cursor proteins. This was also reported for *P. megasperma f. sp. glycinea* (Wycoff *et al.*, 1987; Wycoff and Ayers, 1990).

The objective of the work described here was to use antibodies developed in chickens to develop an ELISA that can be used to detect *F. circinatum* in infected pine seedlings. This ELISA needs to be specific enough to discriminate *F. circinatum* from *D. pinea* and *F. oxysporum* which are common pine infecting fungal pathogens. Antibodies reported in this study were developed in chickens using exo- and mycelium-soluble antigens as well as a protein purified from reducing SDS-PAGE gel in which a crude sample of mycelium-soluble antigens of *D. pinea* was separated.

2.2 Materials and method

2.2.1 Fungal cultures

Different isolates of *Diplodia pinea*, *Fusarium circinatum* and *Fusarium oxysporum* were kindly provided and their identity confirmed by Prof. Coutinho from the Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria South Africa. For *D. pinea* three isolates were obtained including *D. pinea* 16, *D. pinea* 15 and *D. pinea* 31. For *Fusarium* species, isolates used included *F. circinatum* 3578, *F. circinatum* 3580, *F. circinatum* 3579, *F. circinatum*1219, *F. circinatum* 1217, *F. oxysporum* 776, *F. oxysporum* 1861, *F. oxysporum* 1858 and *F. circinatum*. Other *Fusarium* species used were *F. solani*, *F. graminearum* and *F. culmorum* obtained from the Plant Protection Research Institute (PPRI) (Mycology Diagnostic Services, Pretoria, South Africa). Two storage methods were used to store these isolates. In the first method isolates were cultured on potato dextrose agar (PDA) and culture-plugs were cut out of the plates and placed in sterile 2 ml microfuge tubes and these were sealed and kept at ambient temperature. In the second method isolates were cultured in sterilized soil and these were stored at ambient temperature.

2.2.2 Preparation of antigens from fungal cultures

The method used in this study for antigen preparation was adopted from a study by Gan *et al.* (1997) and modified. *D. pinea*, *F. circinatum* and *F. oxysporum* were first cultured on PDA (Merck) for 14 days at 28°C. Culture-plugs were cut out of the plates and placed in 100 ml sterile nutrient broth [(1% (w/v) yeast extract and 1% (w/v) dextrose)] in 250 ml conical flasks and incubated at 28°C for 14 days, under constant agitation. Mycelia from the nutrient broth cultures were filtered out using cheese cloth and the filtrate (exo-antigens) was stored at -20°C. Mycelial pads were then washed five times with distilled water to remove any remaining traces of exo-antigens. Mycelium pads were scraped-off the cheese cloth and placed into 2 ml microfuge tubes and then re-suspended in phosphate buffered saline [(PBS: 0.8% (w/v) NaCl; 0.02% (w/v) KCl; 0.115% (w/v) $Na_2HPO_4.2H_2O$; 0.02% (w/v) KH_2PO_4)]. These were homogenised for 5 min using a mini-bead-beater (Biospec Bartlesville, USA), sonicated and centrifuged at 14 000 xg for 5 min at ambient temperature using a neofuge 15R centrifuge (Vacutec California, USA). Clear supernatant containing mycelium-soluble-antigens was

collected and stored at -20°C. Exo-antigens were freeze-dried, re-suspended in PBS and sonicated. This was then dialyzed overnight against de-ionized water (3 x changes) in dialysis tubing with a molecular cut-off of 12 – 14 kDa. The next day the dialysis tubing was placed in PEG 20000 for 15 - 20 min to concentrate the protein. The resulting samples were stored at -20°C.

Protein concentration of the exo- and mycelium-soluble antigens was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). The working reagent of the BCA™ Protein Assay Kit contains bicinchoninic acid that is used to monitor cuprous ions produced in the reaction of protein with alkaline Cu^{2+}. A stable purple colour is produced, which is directly proportional to the protein concentration. Amino acid residues such as cysteine, tyrosine and tryptophan are important for the colour formation (Smith *et al.*, 1985). For the determination of protein concentration in test samples a standard curve was constructed using bovine serum albumin (BSA) of known concentration (0.025 – 2 $mgml^{-1}$) (Figure 2.1). Samples were mixed with the working reagent (sample to working reagent ratio = 1:20) and placed in a Nunc®(Nunc Roskilde, Denmark) 96 well plate. The plate was covered and incubated at 37°C for 30 min. Colour change was measured at 562 nm using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

2.2.3 Preparation of antigen from SDS-PAGE separated proteins

Analysis and selection of unique protein bands for each pathogen was carried out using reducing SDS-PAGE gels. Three isolates of each fungal pathogen were selected, including *D. pinea* (*D. pinea 16*, *D. pinea 15* and *D. pinea 31*). Three out of five *Fusarium* isolates were selected: *F. circinatum* (*F. circinatum 3578*, *F. circinatum 3580* and *F. circinatum 3579*) and *F. oxysporum* (*F. oxysporum 776*, *F. oxysporum 1861* and *F. oxysporum 1858*). Both exo- and mycelium-soluble antigens were analysed on 10 % SDS-PAGE gels (Laemmli, 1970). Equal volumes of each sample to a final concentration of approximately 20 μgml^{-1}(at lower concentration, faint protein bands developed) and reducing sample buffer [(125 mM Tris-HCl; 4 % (w/v) SDS; 20 % (v/v) glycerol; 10 % (v/v) 2-mercaptoethanol, pH 6.8)] were heated at 100 °C for 5 min.

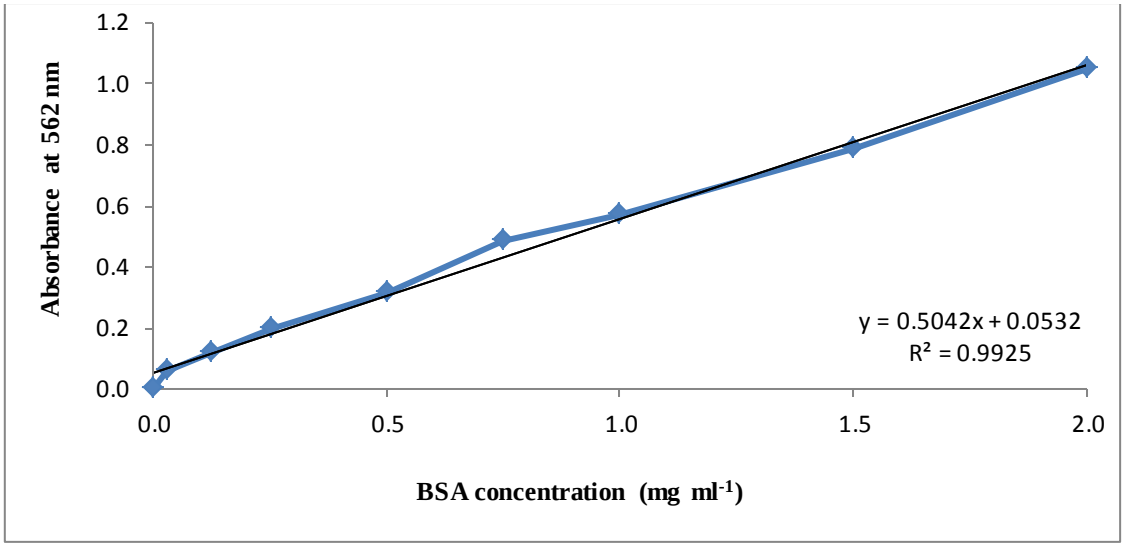


Figure 2.1Standard curve for BCA protein quantification assay. The standard curve was constructed using BSA of known concentration (0.025 – 2 mg.ml^{-1}), mixed together with a working reagent and the colour development was measured at 562 nm. The values indicate the means of two samples.

This sample treatment allows for 2-mercaptoethanol to reduce the disulfide bonds of the protein. SDS then interacts with the denatured protein and masks it with the negative charges. In the presence of an electric field the negatively charged protein migrates based on the size of the protein. Protein migration in the gel is inversely proportional to the molecular weight (Weber and Osborn, 1969). Approximately 20 µg of sample was then loaded into each well and separated at constant current (18 mA per gel) until the loading dye reached the bottom of the gel. The first well of the gel was used to load the molecular weight marker [bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase, (30 kDa) and lysozyme, (14 kDa) unless otherwise stated]. The gel was then stained with Coomassie Blue [(0.125% (w/v) Coomassie Blue R-250; 50% (v/v) methanol; 10% (v/v) acetic acid)] for four hours. After de-staining the gels using de-stain I [(50% (v/v) methanol; 10% (v/v) acetic acid) and de-stain Π (7% (v/v) acetic acid; 5% (v/v) methanol)], they were analysed for unique protein by using a Gene Tool from Syngene (Syngene Bio-imaging Private Ltd, India). In this system molecular weights of each of the protein bands on the gel are calculated using the molecular weight marker as a reference. The sizes of each of the protein bands between isolates were analysed and compared to those from the other fungal pathogens studied. A unique band that was consistent between different runs was observed at approximately 34 kDa in three *D. pinea* isolates and this band was selected for purification.

Protein-purification was carried out by running a series of gels as previously described in this section. A Coomassie Blue staining solution consisting of 0.5% (w/v) Coomassie Blue in acetic acid-isopropyl alcohol-water in a ratio 1:3:6 was used to stain the gel for 15-20 min. Gels were destained with a de-staining solution [acetic acid: methanol: water (50:165:785)]. Bands of interest were cut out using a scalpel. These gel slices were placed into a microfuge tube and soaked in defixing buffer [50 mM Tricine; 25 mM Tris; 0.2% (w/v) SDS; 5 mM dithiothreitol (DTT)] for 30 min at ambient temperature. Following incubation, gel slices were equilibrated in electrophoresis buffer [(50 mM Tricin; 25 mM Tris; 1.73 mM SDS, pH 8.5)] for 5 min. In this buffer gel slices were cut into smaller blocks and placed into an electro-elution glass tube. Elution was carried out at 80 V overnight at ambient temperature using a ECU-040 Electro-Eluter concentrator (C.B.S. Scientific Co. Inc. Solana Beach, California). Termination of the electro-elution step was indicated by a colour change of the small gel slices from blue to clear, indicating that the blue coloured protein had been eluted. The concentrated protein was collected from the collection chamber and analysed by reducing SDS-PAGE for purity. To determine the concentration of the electro-eluted protein, BSA samples of 1, 0.5, 0.25 and 0.125 $mgml^{-1}$ were prepared as standard protein. These were loaded into wells of a 10 % SDS-PAGE gel alongside the electro-eluted protein. Protein band intensity of the standards was compared to that of the electro-eluted protein to estimate the concentration of the protein.

2.2.4 Immunisation and antibody isolation

A total of 10 laying hens were used. Before immunisation one egg from each chicken was collected and used for isolation of pre-immune antibodies. Two chickens were used for each antigen and a total of five antigens were used to raise antibodies. Protein concentration was adjusted to a final concentration of 250 μgml^{-1} for mycelium-soluble antigens from *D. pinea* 31, *F. circinatum* 3578 and *F. oxysporum* 776, and for exo-antigens from *F. circinatum* 3578. Protein concentration for *D. pinea*-34 kDa electro-eluted protein was adjusted to 50 μgml^{-1}. Each antigen was mixed with an equal volume of Freund's complete adjuvant for the first injection. For booster injections Freund's incomplete adjuvant was used. Booster injections were administered at two week intervals for four weeks. A PEG precipitation method was used for antibody isolation (Polson *et al.*, 1980; Polson *et al.*, 1985). In this method egg white was separated from the egg yolk and carefully washed under tap water to remove any traces of

albumin. The yolk was punctured into a measuring cylinder and the initial volume of the yolk was noted. A volume of isolation buffer [1.56% (w/v) $NaH_2PO_4.2H_2O$; 0.02% (w/v) NaN3 titrated to pH 7.6 with NaOH] equal to twice the yolk volume was added. To this mixture 3.5% (w/v) solid PEG (M_r 6000) was added and fully dissolved by stirring. This was centrifuged (4420 xg, 30 min RT) to precipitate the vitellin fraction. The supernatant fluid was filtered through an absorbent cotton wool plug to remove the lipid fraction. To the filtrate, 8.5 % (w/v) PEG was added, fully dissolved, and centrifuged (12000 xg, 10 min RT). The supernatant was discarded and the pellet was re-suspended in an isolation buffer volume equal to the original egg yolk volume, 12% (w/v) PEG was added, fully dissolved and centrifuged (12 000 xg, 10 min RT). The supernatant was discarded and the pellet containing IgY was re-suspended in isolation buffer, in a volume equal to 1/6 th of the yolk volume. Sodium azide (10 % (w/v) solution was added to a final concentration of 0.1 % (w/v). This was stored at 4℃. ELISA was used to monitor antibody production over time.

2.2.5 Preparation of ELISA and cross-reactivity test

An indirect ELISA was used for evaluation of antibody production and in cross-reactivity tests (see Chapter 1, Figure 1.3). The micro-titre plates (Nunc Maxisorb®) were coated with 100 µl of antigen (100 $\mu g.ml^{-1}$) in PBS and incubated at 37℃ for 2 hours in an incubator followed by incubation at 4℃ overnight in a refrigerator. This was followed by blocking unoccupied sites in wells with 200 µl BSA (0.5%, w/v in PBS) for one hour at 37 ℃ in an incubator. Three washes with PBS containing 0.1% (v/v) Tween 20 (PBS-Tween) was carried out. Primary antibody (10 μgml^{-1} unless otherwise stated) in BSA (0.5%, w/v in PBS) specific for each pathogen was added and incubated at 37℃for 2 hours. The plates were then washed 2 times with PBS-Tween. Rabbit anti-chicken IgY-horse radish peroxidase (HRPO) conjugate (1 in 20000) was added to the wells and incubated for 1 hour at 37℃. The plates were washed 3 times with PBS-Tween. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate solution containing H_2O_2 [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer, pH 5.0] was added and the plates were incubated in the dark at ambient temperature for ten min. This time period had to be increased if sufficient colour change was not obtained. The absorbance was measured at 405 nm using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

Antibody cross-reactivity was evaluated using indirect ELISA. A final concentration of 10 $\mu g ml^{-1}$was used for each antibody. Five micro-titre plates were set out for this test and in each plate, one set of antigen was used for coating. The plates were treated as previously mentioned. For each antigen, all five antibodies were used and the colour change after the addition of ABTS substrate solution was measured and compared at 405 nm.

2.2.6 Data analysis

Data was analysed using GenStat® Executable release 14th Edition Statistical Analysis Software. Analysis of variance (ANOVA) was used to analyse the reactivity of the five different antibodies against five isolates of *F. circinatum* and *F. oxysporum* and three isolates of *D. pinea*. Significant differences between treatments were determined using Duncan test. In this test the absorbance values obtained from each of the experiments conducted where each antibody reacted with the respective antigen were analysed. In this test the antibody and antigen combination (treatment) that showed the most reactivity indicated by the highest absorbance mean value get allocated its own latter or latters. These letters served to indicate the significant difference between the treatments, where the treatments that share a latter had no significant difference.

2.2.7 Western blotting for analysis of antigen reactivity

Mycelium-soluble antigens for *D pinea* 16, 15 and 31, *F. circinatum* 3578, 3580 and *3579* and *F. oxysporum* 776, 1861 and 1858 were separated on reducing SDS-PAGE gels (section 2.2.3). Duplicate gels were prepared and one was stained in Coomassie Blue to view the original protein pattern, the second gel was used for protein transfer to nitrocellulose membrane. Nitrocellulose membrane and three filter papers cut into rectangular shapes of the same size as that of the gel were immersed into blotting buffer (0.6% (w/v) Tris-HCl; 1.44% (w/v) glycine; 20% (v/v) methanol). Prior to use 4.5 ml of 10 % SDS in distilled water was added to 450 ml of the blotting buffer. The three pieces of filter paper were placed on the surface and then the nitrocellulose membrane, in the presence of the blotting buffer. The gel was placed on top of the nitrocellulose membrane and additional 3 filter paper sheets were then placed on top of the gel. This was done carefully, avoiding any bubbles especially between the gel and the nitrocellulose membrane (Towbin *et al.*, 1979). Blotting was carried at 40 mA for 16 hours using a wet blotter (BioRad Hercules, CA, USA) in transfer buffer [(45

mM Tris-HCl; 173 mM glycine; 0.1% (w/v) SDS)]. The membrane was transiently stained with Ponceau S [(0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid)] until protein bands were visible. Protein bands of the molecular weight marker were marked with a pencil and the nitrocellulose membrane was de-stained with distilled water. The nitrocellulose membrane was then air dried, and blocked for 1 hour with 5% (w/v) low fat milk powder in Tris buffered saline (TBS) [(0.224% (w/v) Tris; 1.16% (w/v) NaCl and pH adjusted to pH 7.4 with HCl)]. It was then washed in TBS 3 x 5 min and incubated with the primary antibody (10 µg ml^{-1}) in 0.5 % (w/v) BSA-TBS overnight at ambient temperature, with gentle agitation. Membranes were washed twice with TBS for 5 min per wash, followed by incubation at ambient temperature for 1 hour in secondary antibody (rabbit anti-chicken IgY-horse radish peroxidase (HRPO) conjugate) (1 in 10000 dilution) in 0.5% (w/v) BSA-TBS. Thereafter, membranes were washed 3 times with TBS for 5 min per wash. The membrane was then immersed in 4-chloro-1-naphthol-H_2O_2 substrate solution [(0.06 % (w/v) 4-chloro-1-naphthol; 0.1% (v/v) methanol; 0.0015 % (v/v) H_2O_2 in TBS)] and kept in the dark until bands were clearly visible. The membrane was removed from the substrate solution and washed in distilled water and air dried for 1 hour.

2.3 Results

2.3.1 SDS-PAGE analysis of different fungal isolates and antigens

Analysis on SDS-PAGE was based on evaluating the protein banding profile, where similar protein bands and unique bands were noted as similarity or differences of pathogens, respectively. When mycelium-soluble antigens were analysed, a similar protein banding profile was observed between three *D. pinea* isolates (Figure 2.2, lanes 2-4). A unique and prominent protein band was observed at approximately 34 kDa and 39 kDa for *D. pinea* (Figure 2.2, lanes 2-4) which was not present in lanes containing *F. circinatum* and *F. oxysporum* (Figure 2.2, lanes 5-10). When protein bands for *F. circinatum* and *F.oxysporum* were compared, a unique protein band running at approximately 20 kDa was observed only for *F. oxysporum*. Between the two pathogens protein content was very similar and a prominent protein band running at approximately 45 kDa was observed in all isolates (Figure 2.2, lane 5-10). Antigen expression was not consistent between different preparations and during storage some of the antigens seem to get degraded. This was indicated by some of the bands that were

initially observed but no longer visible after storage. Even after storage the unique protein band at approximately 34 kDa for *D. pinea* was observed in all *D. pinea* preparations while the 45 kDa and 68 kDa bands were observed for all the *Fusarium* species. Three isolates of *D. pinea* and *F. oxysporum* showed similar protein bands and those of *F. circinatum* showed different protein banding with the largest number and most prominent bands observed for *F. circinatum* 3578 (Figure 2.2, lane 5). Analysis of exo-antigens showed no band development even when more sensitive stains (silver stain and aureole stain) were used (result not shown).

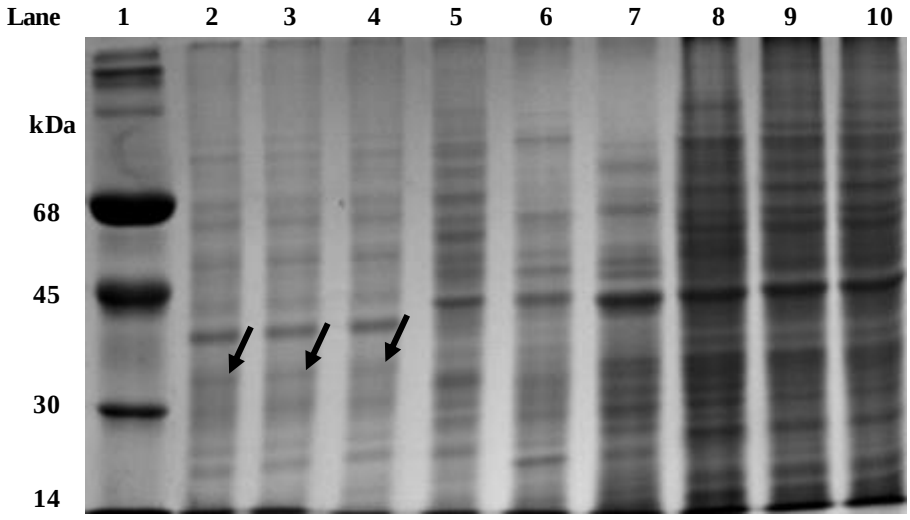


Figure 2.2Comparison of protein content from different fungal pathogens.Three isolates of *D. pinea, F. circinatum* and *F. oxysporum* were cultured in nutrient broth and mycelium-soluble antigens were separated from the exo-antigens by filtration, homogenised, sonicated and centrifuged. Supernatant containing soluble antigens was analysed by 10 % reducing SDS-PAGE Approximately 20 µg of sample was loaded into each well. Lane 1, MWM; lane 2, *D. pinea* 16; lane 3, *D. pinea 15*, lane 4, *D. pinea* 31; lane 5, *F. circinatum* 3578; lane 6, *F. circinatum* 3580; lane 7, *F. circinatum* 3579; lane 8, *F. oxysporum 776*; lane 9, *F. oxysporum 1861*; lane 10, *F. oxysporum 1858*. Arrow points to bands of interest that was further purified.

2.3.2 Purification of *D. pinea* 34 kDa protein band from SDS-PAGE

Following evaluation of protein content for each pathogen, a protein band at approximately 34 kDa that was unique to *D.pinea* was selected for gel-purification for antibody production. The unique protein band observed at approximately 20 kDa in *F. oxysporum* samples was not expressed in all the different cultures prepared; therefore it was not selected for gel-purification. To test for the purity of the *D. pinea*-34 kDa protein, one isolate (*D. pinea 16, F. circinatum 3578* and *F. oxysporum 776*) from each pathogen was separated on SDS-PAGE along with the purified protein. A pure protein was obtained indicated by one band at the expected size (indicated by an arrow in lane 5 of Figure 2.3 A). However, some contaminating bands were also observed at approximately 68 kDa (Figure 2.3, A, lane 5). To determine the

concentration of the purified protein, BSA samples of known concentrations (1 to 0.125 $mgml^{-1}$) were used. By comparing the protein band intensity of a BSA sample with a known concentration to that of the purified protein the final concentration of the purified protein was determined to be approximately 50 μgml^{-1} (Figure 2.3 B, lane 8).

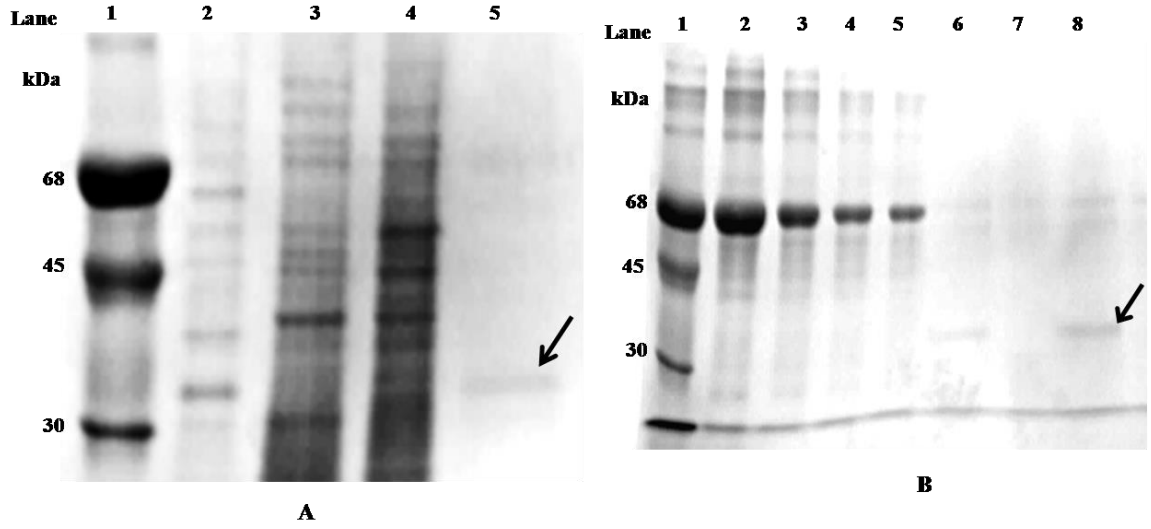


Figure 2.3Determining purity and concentration of *D. pinea*-34 kDa protein.(A). Unique protein for *D. pinea*at 34 kDa was purified from an SDS-PAGE gel by electro-elution. Purity of the protein was analysedby crude samples of pathogens. Lane 1, MWM; lane 2, *D. pinea 16*; lane 3, *F. circinatum 3578*; lane 4, *F. oxysporum 776*; lane 5, *D. pinea 34* kDa protein. (B) Concentration of purified protein was determined by running BSA samples with known concentrations alongside purified protein; lane 1, MWM; Lane 2, BSA 1 $mgml^{-1}$; lane 3, BSA 0.5 $mgml^{-1}$; lane 4, BSA 0.25 $mgml^{-1}$; lane 5, BSA 0.125 $mgml^{-1}$; lane 6, *D. pinea* 34 kDa protein first purification; lane 7, *D. pinea* 34 kDa protein second purification; lane 8, *D. pinea* 34 kDa protein third purification.

2.3.3 Monitoring antibody production and reactivity

An increase in antibody production was observed from the third week after the first immunisation and this was common between different sets of antibodies. Figure 2.4 shows the reactivity of anti-*F. oxysporum* antibodies where at Week 6 post-inoculation absorbance values reached 0.95. Anti-*F.oxysporum* antibodies were generally more reactive indicated by similar and high absorbance readings between Weeks 4 to 6. Pre-immune antibodies showed the lowest absorbance readings.

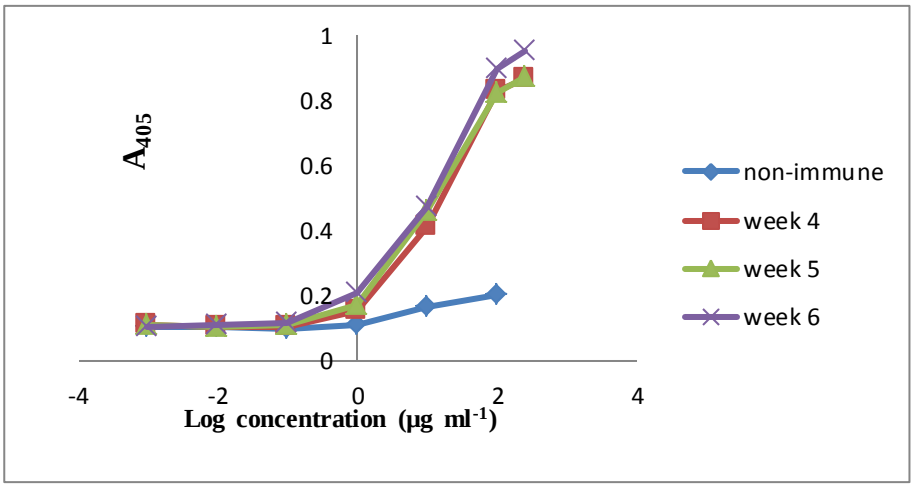


Figure 2.4 Evaluation of anti-*F.oxysporum*antibody production and reactivity with antigens from fungal pathogen over time. Micro-titre plates were coated with *F. oxysporum* mycelium-soluble antigens. Anti-*F.oxysporum* antibodies from Week 4 to Week 6 were added to a final concentration ranging from 250 to 0.001 μgml^{-1} in a micro-titre plate. Primary antibody binding was detected by rabbit anti-chicken IgY-horse radish peroxidase (HRPO) conjugate followed by ABTS/H_2O_2 chromogen substrate solution, and the absorbance reading at 405 nm using a FLUORStar Optima Spectrophotometer. Absorbance values plotted are the average of duplicate samples (the same procedure was used to evaluate the reactivity of anti- *D. pinea*, anti- *F. circinatum*, anti- *F. circinatum*-exo and anti- *D. pinea* 34 kDa antibodies).

2.3.4 Antibody reactivity towards antigens from different fungal isolates

During chicken immunisation only one isolate from *D. pinea, F. circinatum* and *F. oxysporum* was used. Selection of these isolates was based on the number of protein bands obtained on SDS-PAGE (the isolate with the most number of protein bands was selected) and the consistency of the protein banding pattern observed with different antigen preparations, a method similar to that used by Biazon *et al.* (2006). *D. pinea 31*, *F. circinatum 3578* and *F. oxysporum 776* were thus selected for antibody production. Antibody reactivity with other isolates was evaluated to confirm if reactivity is the same across different isolates. This included two more isolates of each of the *Fusarium* pathogens for a broader spectrum of isolates. When three isolates of *D. pinea* were evaluated more reactivity towards *D. pinea 16* (A1) was observed, but this was only less than one absorbance unit higher than with the other two isolates [(*D. pinea 31*) (A2) and (*D. pinea 15*) (A3)]. Similar reactivity between *D. pinea 15* and *D. pinea 31* was obtained (Figure 2.5, plots A2 and A3), but the reactivity of anti-*D. pinea* antibodies was relatively low since the highest absorbance value obtained was 0.650 (Figure 2.5, plot A1). Five isolates for *F. circinatum* were evaluated for their reactivity towards anti-*F. circinatum* antibodies. More reactivity was observed towards *F. circinatum*

1217 and *F. circinatum 1219* (Figure 2.5, plots B4, B5). Absorbance readings observed between different isolates ranged from 0.510 to 0.868. Anti-*F.oxysporum* antibodies reactivity was relatively similar with five different isolates for *F. oxysporum*, but least reactivity was observed for *F. oxysporum* 776 (b) (Figure 2.5, C4) with the absorbance value of 0.703. Anti-*F. oxysporum* antibodies were the most reactive antibodies as indicated by the highest absorbance values obtained. The reactivity of each of the isolates with the non-immune antibodies was lower than those with their corresponding antibodies (Figure 2.5, plots D1 – D5).

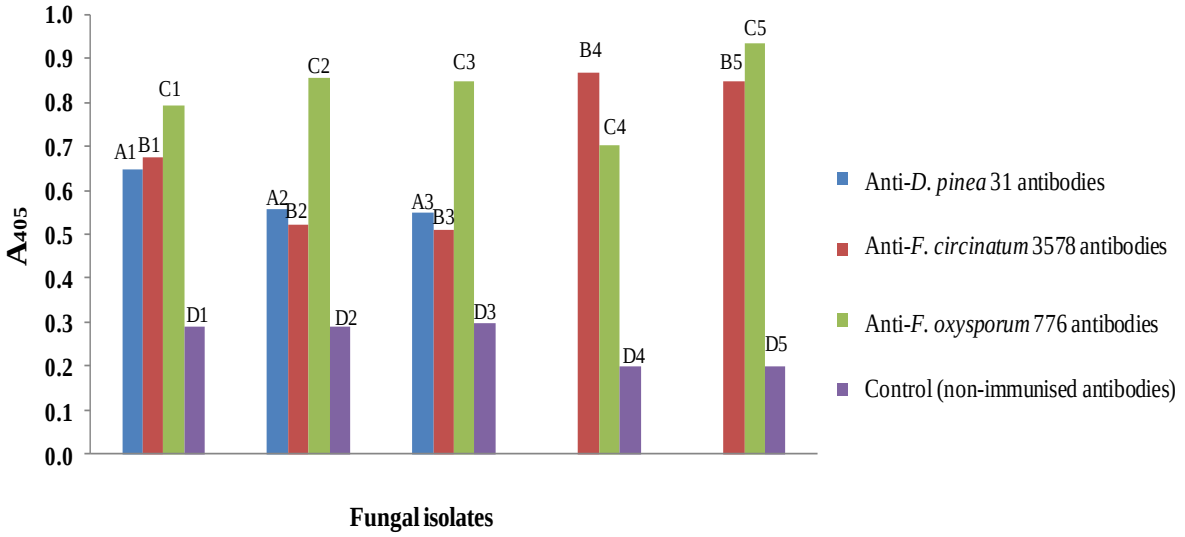


Figure 2.5 Evaluation of anti-*D. pinea* 31, anti-*F. circinatum* 3578 and anti-*F. oxysporum* 776 antibody reactivity with other isolates for the corresponding pathogen. Antigens (100 µgml^{-1}) isolated from three isolates for *D. pinea* (A1 – A3 *D. pinea*16, 15 and 31, respectively), five isolates for *F. circinatum* (B1 – B5 *F. circinatum* 3578, 3580, 3579, 1217 and 1219, respectively) and five isolates for *F. oxysporum* (C1 – C5 *F. oxysporum* 776, 1861, 1858, 776 (b) and 778, respectively were used for coating wells of the micro-titre plate, followed by incubation with primary antibody (10 µgml^{-1}) with its corresponding antigen. The reaction of the primary antibody was detected by incubation with rabbit anti-chicken HRPO conjugate, followed by ABTS/H_2O_2 chromogen substrate solution. Plots are the average absorbance values at 405 nm of two replicates.

2.3.5 Antibody cross-reactivity test

Antibodies developed against mycelium-soluble antigens for *D. pinea*, *F. circinatum* and *F. oxysporum* showed more reactive and specificity than anti-*F. circinatum* exo- and anti-*D. pinea* 34 kDa antibodies. Each of these antibodies was then selected to evaluate cross-reactivity with other fungal pathogens. Cross-reactivity of anti-*D.pinea* antibodies with antigens from other fungal species was observed, but was minimal (Figure 2.6). In this experiment micro-titre plates were coated with mycelium-soluble antigens isolated from *D.*

pinea, F. circinatum, *F.oxysporum, F. solani, F. graminearum* and *F. culmorum*, respectively and incubated with anti-*D. pinea* antibodies. The anti-*D.pinea* antibodies reactivity towards *D. pinea* antigens was higher than that observed towards the other fungal antigens (Figure 2.6). Anti-*F.circinatum* antibodies were more reactive towards antigens isolated from *F. circinatum*, but cross-reactivity was observed with other fungal antigens with most observed for *F. solani, F. culmorum*, *F. graminearum* and *F. oxysporum*, respectively (Figure 2.6). Positive reactions gave absorbance readings that are high enough to separate them from the cross-reactive reaction. With further developments where more cross-reactivity tests will be evaluated cut-off values for each antibody specificity can be determined. On average the absorbance reading for the detection of *F. circinatum* was 0.278 higher than those of non-specific reactions. A similar trend was observed for anti-*F. oxysporum* antibodies where more reactivity was observed towards *F. oxysporum* antigens. Cross-reactivity was relatively high towards *F. solani, F. circinatum* and *F. culmorum*, but the absorbance readings for the specific antigen detected was 0.303 units higher than that of non-specific antigens.

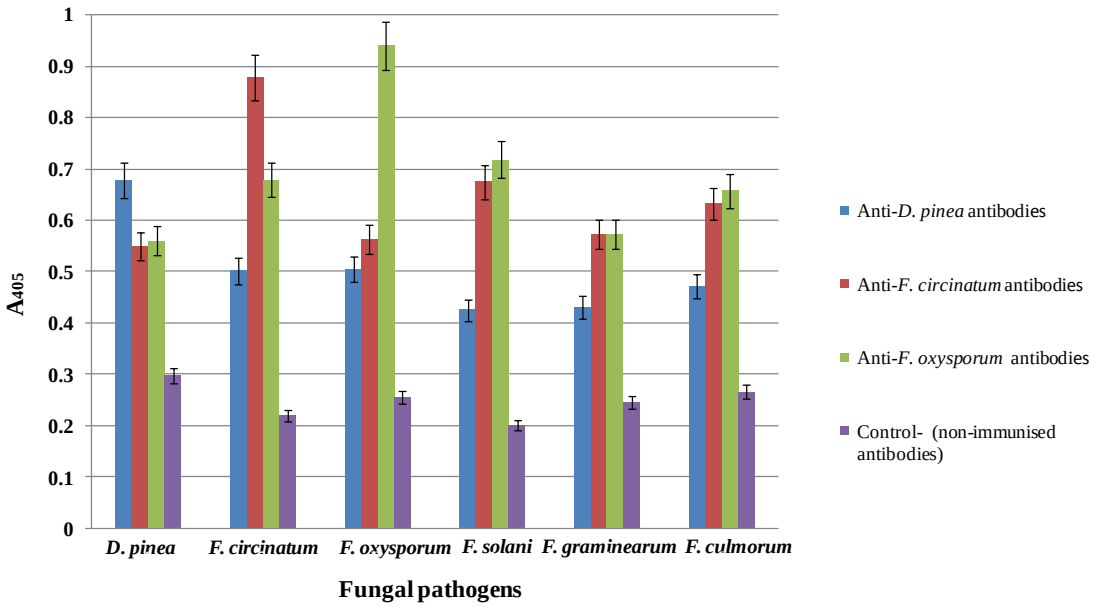


Figure 2.6 Evaluation of anti-*D. pinea*, anti-*F. circinatum* and anti-*F. oxysporum* antibody cross-reactivity using indirect ELISA. In each micro-titre plate five different fungal pathogens (*D. pinea*, *F. circinatum*, *F. oxysporum*, *F. solani*, *F. culmorum* and *F. graminearum*) to a final concentration of 100 µgml^{-1} were used for coating the wells. In each micro-titre plate primary antibody (10 µg ml^{-1}) was incubated followed by incubation with rabbit anti-chicken IgY-HRPO conjugate to detect the reactivity of the primary antibody. ABTS/H_2O_2 chromogen substrate solution was incubated for 30 min. Absorbance values at 405 nm are the average of duplicate samples.

This was calculated by subtracting the average reading of a specific from that of a cross-reactive reaction. The answers were then averaged.

2.3.6 Statistical analysis of cross-reactivity of antibodies with antigens from other fungal species

Chickens were immunised with five sets of antigens and the antibodies obtained were anti-*D. pinea*, anti-*F. circinatum*, anti-*F. oxysporum*, anti-*F. circinatum* exo-antigens and anti-*D. pinea* 34 kDa antibodies. Reactivity and sensitivity of these antibodies was analysed. In this experiment reactivity of each of the antibodies with different isolates for *D. pinea*, *F. circinatum* and *F. oxysporum* was analysed. Whereas in the previous experiments (Figure 2.5 and Figure 2.6) only antibodies developed against mycelium-soluble antigens were used. This was conducted to evaluate the immunochemical similarities between Isolates of the same fungal pathogen as well as with the Isolates from other fungal pathogens. Secondly the results from this experiment were used to evaluate the set of antigens that produced more specific antibodies. Isolates were designated as Isolates 1-5 for *F. circinatum* 3578, 3580, 3579, 1217 and 1219, respectively. A significant difference ($P < 0.001$) was observed for the reactivity of the different antibodies with different *F. circinatum* isolates, indicating variation in the immunochemical composition of these isolates (Table 2.1). However, anti-*F. circinatum* antibodies were most reactive with Isolate 4 and Isolate 5, which is in agreement with the observations made in the ELISA shown in Figure 2.5. Moreover, other antibodies cross-reacted mostly with Isolate 4. *F. oxysporum* isolates were designated as Isolates 1-5 (*F. oxysporum* 776, 1861, 1858, 776 (b) and 778, respectively). There was a significant difference between the reactivity of antibodies with the five isolates as indicated by the P value of <0.001, indicating immunochemical differences between isolates of the same pathogen (Table 2.1). Anti-*F.oxysporum* antibodies were more reactive towards Isolate 5 which is in line with the observations made the in ELISA shown in Figure 2.5. There was no significant difference observed between the reactivity of five different antibodies with three isolates of *D. pinea* [*D. pinea* 16 (Isolate 1), *D. pinea* 15 (Isolate 2) and *D. pinea* 31 (Isolate 3)]. As expected, most reactivity was observed from anti-*D. pinea* antibodies with all three *D. pinea* isolates. Anti-*F. circinatum* exo- and anti-*D. pinea* 34 kDa antibodies were the least reactive antibodies and showed non-specific binding.

Table 2.1 Analysis of the antibody reactivity towards different fungal pathogens and isolates.

Treatments (isolate vs antibody)	*F. circinatum*		*F. oxysporum*		*D. pinea*	
Isolate 1 Anti-*D. pinea* 34 kDa	0.2976	a	0.4507	a	0.5640	ab
Isolate 2 Anti-*D. pinea*	0.3233	a	0.7874	jk	0.5866	b
Isolate 2 Anti-*F. circinatum* exo	0.3402	a	0.6444	efgh	0.4487	ab
Isolate 1 Anti-*D. pinea*	0.3591	ab	0.5670	bcde	0.6016	b
Isolate 3 Anti-*D. pinea* 34 kDa	0.3623	ab	0.5808	cdef	0.5460	ab
Isolate 1 Anti-*F. circinatum* exo	0.3752	abc	0.5057	abc	0.5141	ab
Isolate 3 Anti-*F. oxysporum*	0.3763	abc	0.8534	kl	0.4814	ab
Control 1 Antibody 6	0.3785	abc	0.4530	a	0.3495	a
Isolate 1 Anti-*F. oxysporum*	0.3794	abc	0.7930	jk	0.5297	ab
Isolate 3 Anti-*F. circinatum* exo	0.3818	abcd	0.6577	efgh	0.4662	ab
Isolate 2 Anti-*D. pinea* 34 kDa	0.3427	abcde	0.6200	defg	0.5611	ab
Isolate 2 Anti-*F. oxysporum*	0.4029	abcde	0.8986	lm	0.5506	ab
Isolate 3 Anti-*D. pinea*	0.4141	abcde	0.6594	efgh	0.6083	b
Isolate 5 Anti-*D. pinea* 34 kDa	0.4175	abcde	0.4758	ab		
Isolate 5 Anti-*F. circinatum* exo	0.4755	bcdef	0.5375	abcd		
Isolate 3 Anti-*F. circinatum*	0.4781	bcdef	0.7647	ijk	0.4603	ab
Isolate 5 Anti-*D. pinea*	0.5010	cdefg	0.5050	abc		
Isolate 1 Anti-*F. circinatum*	0.5111	defg	0.6767	fghi	0.6106	b
Isolate 5 Anti-*F. oxysporum*	0.5142	efg	0.9557	m		
Isolate 2 Anti-*F. circinatum*	0.5195	efg	0.7318	hij	0.5494	ab
Isolate 4 Anti-*D. pinea* 34 kDa	0.5235	efg	0.4893	abc		
Isolate 4 Anti-*F. circinatum* exo	0.5800	fgh	0.6735	fghi		
Isolate 4 Anti-*D. pinea*	0.6235	gh	0.5790	cdef		
Isolate 4 Anti-*F. oxysporum*	0.6670	h	0.7110	ghij		
Isolate 5 Anti-*F. circinatum*	0.6775	h	0.8059	jk		
Isolate 4 Anti-*F. circinatum*	0.9227	i	0.6444	efgh		
CV%	**12.400**		**2.4000**		**4.8000**	
Lsd	**0.1105**		**0.0875**		**0.1510**	
Sed	**0.0555**		**0.0436**		**0.0750**	
P	**<0.001**		**<0.001**		**<0.186**	

Experiments were repeated three times and data was analysed using GenStat® Executable release 14th Edition Statistical Analysis Software. Analysis of variance (ANOVA) was used to analyse the reactivity of the five different antibodies against five isolates of *F. circinatum* and *F. oxysporum* and three isolates of *D. pinea*. Same latters in different treatments indicate that there was no significant difference between the treatments when analysed using Duncan Multiple Range at P = 0.05. The bigger the latter the greater the reactivity obtained in that treatment.

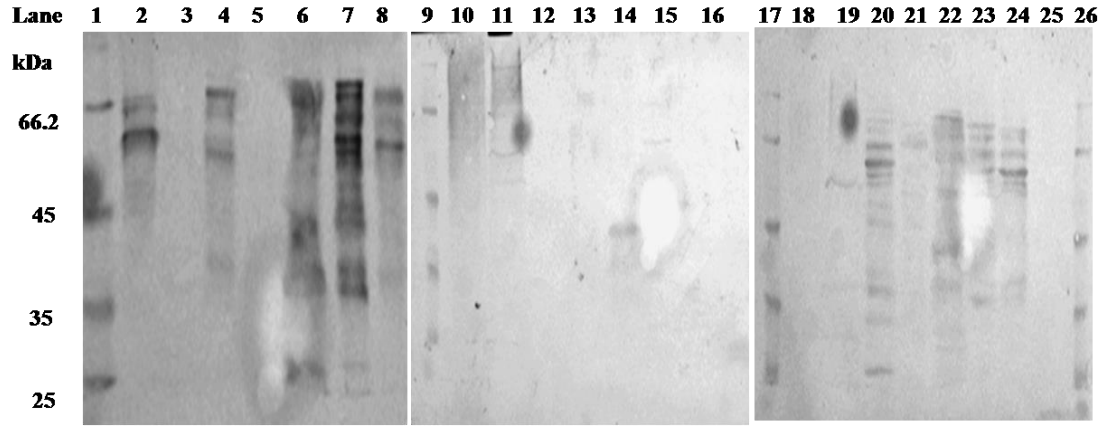
2.3.7 Analysis of antibody reactivity with different antigens blotted on nitrocellulose membrane

Western blot analysis indicated some common antigens between different fungal pathogens. When anti-*F. circinatum* antibodies were used for detection of different fungal pathogens, results indicated that they cross-react with antigens larger than 45 kDa for *D. pinea* and *F. oxysporum* (Figure 2.7). For *F. circinatum* isolates these antibodies reacted with antigens of molecular weight above 24 kDa, but the immuno-dominant antigens were observed from 35 kDa and above. Common antigen between the three *F. circinatum* isolates were observed at approximately 67 kDa, 58 kDa, 45 kDa, 34 kDa and kDa 35 kDa (Figure 2.7 lanes 6-8). However,a prominent antigen was observed at 68 kDa for only *F. circinatum* 3578 and *F. circinatum* 3580 (Figure 2.7 lanes 6 and 7). The common antigen between the *F. circinatum* isolates at 45 kDa might be the prominent protein observed on the SDS-PAGE in Figure 2.7 that was common between *F. circinatum* and *F. oxysporum*. However, anti-*F.circinatum* antibodies did not bind the 45 kDa protein from *F. oxysporum*. A common antigen at 67 kDa observed on the nitrocellulose membrane was also a common protein on the SDS-PAGE observed between the three *F. circinatum* isolates. The most reactive *F. circinatum* isolate indicated by the development of darker bands on the nitrocellulose membrane was *F. circinatum* 3580 followed by *F. circinatum* 3578 and *F. circinatum* 3579, respectively (Figure 2.7 A, lanes 6 – 8). However, this reactivity might be due to overloading of *F. circinatum* 3580. Cross-reactivity of anti- *F. circinatum* antibodies with *D. pinea 31* and *F. oxysporum 776* was observed in lanes 2 and 4, Figure 2.7, (A), most notably at 67 kDa (faint), 65 kDa and 58 kDa with *D. pinea* and at 67 kDa and 58 kDa (faint) with *F. oxysporum*. The antigens at 67 kDa and 58 kDa were common between the three pathogens when detected using anti-*F. circinatum* antibodies. No antigens from *D. pinea 16* and *F. oxysporum 1861* were recognised by anti-*F. circinatum* antibodies since no bands developed for these isolates (lane 3 and 5, Figure 2.7A).

Reactivity of anti-*D. pinea* antibodies was not as prominent as that observed for anti-*F. circinatum* antibodies. This is similar to the observations made in the ELISA shown in Figure 2.5 and Figure 2.6. A smear from 72 kDa to approximately 54 kDa was observed when anti-*D. pinea* antibodies reacted with *D. pinea 31* (lane 10, Figure 2.7 B). However, antigens at 68 kDa and 56 kDa were observed with the detection of *D. pinea* 16. The antigen at 56 kDa has

the same size as one of the proteins that were common between three *D. pinea* isolates indicated by the SDS-PAGE in Figure 2.2. Anti-*D.pinea* antibodies showed minimal cross-reactivity with *F. oxysporum*. A band at 59 kDa developed when these antibodies reacted with *F. oxysporum* 1861 (lane 15, Figure 2.7 B). A non-specific band developed at 45 kDa during the detection of *F. oxysporum* 776 with anti-*D. pinea* antibodies. It should be noted that this band might be correlated with the protein band at 45 kDa observed in the SDS-PAGE in Figure 2.2. Anti-*D.pinea* antigens did not show cross-reactivity with *F. circinatum* and *F. oxysporum* 1858.

Anti-*F. oxysporum* antibodies reacted strongly with all isolates of *F. oxysporum* and also indicated that the common antigens between the three *F. oxysporum* are at 60 kDa, 58 kDa and 53 kDa (lanes 22 – 24, Figure 2.7 C). It should be noted that the antigen at 58 kDa was also detected in *F. circinatum* but not with *D. pinea* as it was previously observed when using anti-*F. circinatum* antibodies (lane 20, Figure 2.7 C). It can be concluded that the antigen at 58 kDa is common between the two *Fusarium* species studied as it was also detected by anti-*F. circinatum* antibodies. Multiple bands of different sizes developed for the detection of *F. circinatum* 3578. The reactivity of anti-*F. oxysporum* antibodies with *F. circinatum* 3578 indicated by the development of a band at 25 kDa in lane 20, Figure 2.7 can be reported as a non-specific reactivity since this was not observed for *F. oxysporum* isolates which are specific for the antibody tested. Anti-*F. oxysporum* antibodies showed minimal cross-reactivity with *D. pinea*, indicated by a development of a faint band at 51 kDa (lane 19, Figure 2.7 C).



A. Detection using anti-*F. circinatum* antibodies **B.** Detection using anti-*D. pinea* antibodies **C.** Detection using anti-*F. oxysporum* antibodies

Figure 2.7 Evaluation of cross-reactivity of different antibodies raised to mycelium soluble-antigens from *D. pinea*, *F. circinatum* and *F. oxysporum* shown by western blotting. Approximately 20 µg of protein from *D. pinea*, *F. circinatum* and *F. oxysporum* was separated on SDS-PAGE gel and blotted onto nitrocellulose membrane then membranes and reacted with primary antibody (10 μgml^{-1}) to evaluate cross-reactivity, followed by incubation with rabbit anti-chicken IgY-HRPO conjugate and 4-chloro-1-naphthol H_2O_2 substrate solution. (A). Detection using anti-*F. circinatum* antibodies; lane 1, MWM; lane 2, *D. pinea* 31; lane 3, *D. pinea* 16; lane 4, *F. oxysporum* 776; lane 5, *F. oxysporum* 1861; lane 6, *F. circinatum* 3578; lane 7, *F. circinatum* 3580; lane 8, *F. circinatum* 3579.(B). Detection using anti-*D. pinea* antibodies, Lane 9, MWM; lane, 10 *D. pinea* 31; lane 11, *D. pinea* 16; lane 12, *F. circinatum* 3578; lane 13, *F. circinatum* 3580; lane 14, *F. oxysporum* 776; lane 15, *F. oxysporum* 1861; lane 16, *F. oxysporum* 1858. (C). Detection using anti-*F. oxysporum* antibodies; lane 17, MWM; lane 18, *D. pinea* 31; lane 19, *D. pinea* 16; lane 20, *F. circinatum* 3578; lane 21, *F. circinatum* 3580; lane 22, *F. oxysporum*776; lane 23, *F. oxysporum* 1861; lane 24, *F. oxysporum* 1858; lane 25, contains only nutrient broth as control; lane 26, MWM.

2.4 Discussion

The work described in this study demonstrates an assay that can be used as an alternative method for detection and discrimination of *F. circinatum* from other common fungi that infect pine seedlings. Other methods for detection of fungi currently available were not investigated in the present study, but judging from the complications that previous research have reported, this assay should serve as a better method for the detection of fungal pathogen compared to the visualization of symptom development that is commonly used. The ELISA optimized in this study showed promise in the detection of and discrimination between *F. circinatum*, *F. oxysporum* and *D. pinea*.

Gan *et al.* (1997) reported that more specific antibodies against fungal pathogens can be obtained by selecting unique proteins for each pathogen and using these proteins for immunisation, or by the selection of unique monoclonal antibodies. In the current study analysis of the protein profile for *D. pinea*, *F. circinatum* and *F. oxysporum* showed that the protein banding profile was different amongst these pathogens but some proteins had similar sizes. The wide spread in the molecular sizes observed for each pathogen and the presence of

common protein bands amongst these pathogens is not surprising because crude samples were used in this experiment that contain the whole spectrum of mycelium protein. The occurrence of proteins of the same size between different fungal pathogens has been reported for other *Fusarium* pathogens and strains of *Thielaviopsis basicola* (Kitagawa *et al.*, 1989; Holtz *et al.*, 1994; Gan *et al.*, 1997). In this study different isolates for each pathogen were analysed and protein bands of the same size and colour intensity were observed amongst isolates from the same pathogen, especially for *D. pinea* and *F. oxysporum*, indicating that these proteins might be the same protein, but can only be confirmed by N-terminal protein sequencing. In a study by Biazon *et al.* (2006) analysing different *F. verticillioides* strains, variability was shown in the protein profile among the eight strains evaluated, protein bands with the same molecular weight were further analysed and then identified as common antigens among these different strains. In the current study analysis of three isolates of *F. circinatum* also showed variability in the protein banding profile which indicated the presence of different antigens between these isolates. No unique protein bands were observed between the two *Fusarium* pathogens indicating that further research is needed to characterise the protein content between the different fungi such as the use of iso-electric focusing and two dimensional gel electrophoresis that allows for improved separation of proteins.

The level of virulence between fungal isolates may vary and different isolates of the pathogen may cause the development of disease. It is therefore important that the detection method developed is able to detect across different isolates for each fungi. In this study each of the antibodies developed were able to preferentially detect isolates of the corresponding fungal species. However, varying detection levels between isolates of the same pathogen were observed indicating that the antigens between fungal isolates vary, especially amongst the five isolates of *F. circinatum*. This variation was also confirmed by the significant differences observed in the cross-reactivity test, where different antibodies were incubated with the different isolates for each pathogen and showed different reactivity patterns in both ELISA tests and western blots.

Different levels of specificity have been reported for antisera obtained from immunisation with soluble-antigens, exo-antigens and cell wall fragments of fungalmycelia (Dewey *et al.*, 1990; Brill *et al.*, 1994; Gan *et al.*, 1997; Hitchcock *et al.*, 1997; Hayashi *et al.*, 1998). In the

present study three sets of antigens were used for immunisation to optimise the ELISA tests, i.e. exo- and mycelium-soluble antigens as well as a protein purified from reducing SDS-PAGE gel in which a crude sample of mycelium-soluble antigens of *D. pinea* was separated. However, antibodies against mycelium-soluble antigens were the most immuno-reactive. Previous reports have showed that immunising with mycelium-soluble antigens result in antibodies that are more reactive but less specific than those developed against exo-antigens, however, other researchers have reported increased specificity with the former (Brill *et al.*, 1994; Gan *et al.*, 1997; Hitchcock *et al.*, 1997; Hayashi *et al.*, 1998). In this study reactivity obtained with anti-*F. circinatum* exo-antigen antibodies showed no significant difference when these antibodies reacted with other *Fusarium* species and no increased specificity was observed which is in contrast with the observations made by Gan *et al.* (1997). This informed the selection of antibodies to use for cross-reactivity tests in ELISA and western blotting.

Comparing the cross-reactivity amongst anti-*D. pinea*, anti-*F. circinatum* and anti-*F. oxysporum* antibodies prepared in this study showed that anti-*D. pinea* antibodies were less reactive with other pathogens when analysed with both ELISA and western blots. Antibodies against *F. circinatum* can be characterised as genus-specific antibodies since these showed strong reactivity with other fungi of the same genus. However, these antibodies were more reactive towards their corresponding antigen indicated by the higher absorbance reading and development of multiple bands on the western blot when detecting *F. circinatum* isolates, in contrast with the few bands that developed for the detection of *D. pinea* and *F. oxysporum* isolates with the same antibodies. Reactivity of anti-*F. oxysporum* antibodies were more directed to *F. oxysporum* antigens as expected, but the specificity of these antibodies can also be charecterised as genus specific. Such antibody specificity has also been observed in other studies that used both monoclonal and polyclonal antibodies for fungal detection (Arie *et al.*, 1991; Bossi and Dewey, 1992; Thornton *et al.*, 2002; Schmechel *et al.*, 2003; Schmechel *et al.*, 2005). In a study reported by Chen *et al.* (2000), laying hens were immunised with exo-antigens from *Aspergillus flavus* Link and *A. ochrecuis* Wilhelm. ELISA was used to evaluate cross-reactivity of these antibodies and some were found to be species-specific (anti-*A. ochrecuis* antibodies) while the others were only specific to genus level. Antibodies against exo-antigens and mycelium-soluble antigens of *F. sporotrichioides, F. poea* and *F. graminearum* were produced and species-specific antibodies were only reported for *F. poea*

(Gan *et al.*, 1997). Whereas with the other *Fusarium* species polyclonal antibodies obtained were genus-specific when analysed with western blots and ELISA. In the same study, 63% cross-reactivity was observed between *Fusarium* species and 55% was observed with pathogens from other genera (*Aspergillus* and *Penicillium*) when antibodies raised to mycelium-soluble antigens, were used, whereas antibodies raised to exo-antigens showed 0–4% cross-reactivity. Other studies have reported similar trend of antibody reactivity where it was concluded that more cross-reactivity occurs between fungi from the same genera because of common epitopes present within the same genus as was also observed in the current study (Biazon *et al.*, 2006).

In this study western blotting was used to analyse cross-reactivity and common antigens amongst different fungal pathogens. The development of smears and multiple bands observed in western blots in this study has previously been reported, where it served as an indication of cross-reactivity and multiple antigens (Wycoff *et al.*, 1987). Most of the antigens observed on the western blots in the present study had the same size as the proteins on SDS-PAGE, indicating that these might be the same proteins present across different isolates and fungal pathogens. The presence of smears has previously been correlated with protein degradation and denaturation (Wycoff *et al.*, 1987; Gan *et al.*, 1997; Biazon *et al.*, 2006). Development of multiple bands of different sizes on a nitrocellulose membrane may be due to other factors such as glycosylation of proteins. The broad spectrum covered by the banding profile for *F. circinatum* and *F. oxysporum* in the present study indicated that, even though there are some of the antigens common between fungal pathogens, there is a variety of antigens within each pathogen. Secondly, it might be due to common protein domains between fungal pathogens or that may be generated during proteolytic cleavage of the protein during their processing or cleavage of a precursor protein. There is also a possibility of these proteins being glycosylated in an alternative manner that then results in multiple banding observed (Wycoff *et al.*, 1987; Wycoff *et al.*, 1990).

It has been reported that some fungi in the same genus demonstrate serological similarities but some of the species may not express reactive antigens (Brill *et al.*, 1994). This might explain observations made in the present study on nitrocellulose membrane, in which the banding profile was not the same amongst the isolates of the three pathogens studied. Hayashi *et al.*

(1998) observed the development of multiple bands on nitrocellulose membrane when *F. oxysporum* was detected using monoclonal antibodies. Amongst these bands a major 55 kDa band was noted which was also observed in the current study for *F. oxysporum*. The use of immuno-blotting technique indicated that immuno-dominant antigens for *F. sporotrichioides*, *F. poae* and *F. graminearum* were greater than 28 kDa in size (Gan *et al.*, 1997). In this study immuno-dominant proteins for *F. circinatum* were larger than 35 kDa. The difference in the level of cross-reactivity observed when ELISA and western blotting was used may be a result of some of the epitopes being denatured by SDS and no longer recognised by the antibodies because of the change in conformation. Also, some of the denatured proteins may not be properly blotted onto the nitrocellulose membrane during the transfer stage.

Some of the main components within the fungal mycelium that are antigenic determinants and those that promote cross-reactivity have been evaluated. Monoclonal antibodies raised to *Fusarium* species were shown by immunoflorescence to be directed to the mycelium surface. It was suggested that these targeted surface antigens could easily be released and become part of exo-antigens (Arie *et al.*, 1995; Hahn and Werres, 1997; Hayashi *et al.*, 1998). This might explain some of the observations made in the present study such as the reactivity between anti-*F. circinatum* exo-antigen antibodies and mycelium-soluble antigens from *F. circinatum*. This reactivity indicated that some of the soluble-antigens are similar to those on the mycelia surfaces or that the antigens on the mycelium surface were released and became part of the exo-antigens used during the culturing period. Analysis of the relationship between fungal antigenic sites by Hayashi *et al.* (1998) showed that the antigenic site for *F. oxysporum* was not denatured at high temperatures and in the presence of proteinase K. This indicated that the antigenic site for *F. oxysporum* is not a protein or lipid. Additional tests indicated that it is carbohydrate in nature (Hayashi *et al.*, 1998). Some of the carbohydrates detected were β-1,3 linked terminal glycosyl residues and those that were non-terminal. Gerik *et al.* (1987) prepared a serological staining procedure that monitored antigens for *Verticillium dahlia* using polyclonal antibodies prepared from cell wall material. Results indicated that binding was directed towards chitin and other cell wall polysaccharides that were common in different fungal pathogens (*Fasarium,Ulocladium atrum* and *Phytophthora)* (Gerik *et al.*, 1987; Karpovich-tate and Dewey, 2001). Antigens produced by different mould species were evaluated and results indicated that 44 strains of *Penicillium* and 12 strains of *Aspergillus*

contained antigens that were extracellular polysaccharides in nature. These were identified as immunochemically related to an antigen that is genera specific for *Mucor* and *Fusarium* pathogens (Notermans and Soentoro, 1986). The strong relationship and common nature of the antigenic determinants between different fungal groups can be used to explain the cross-reactivity observed in the present study.

Antibodies obtained in this study were not species-specific; they showed cross-reactivity with pathogens from other genera, however, these antibodies were more reactive towards their corresponding antigens indicating that they can be used for detection of *D. pinea, F. circinatum* and *F. oxysporum* followed by more validating tests such as PCR, where necessary. It should be noted that some antigens are only expressed and more pronounced when the pathogen is in contact with its natural host such as plant tissue (Hitchcock *et al.*, 1997; Karpovich-tate and Dewey, 2001). This indicates the need to evaluate antigens isolated from infected seedlings given that the antigens evaluated in the present study were cultured in PDA media. In the next chapter results are presented of pine seedlings artificially inoculated with different fungal pathogens for the isolation of antigens for each of the parent pathogens used for infection. These antigens were allowed to react with the different antibodies developed in the present study and the ELISA test was optimized for field detection and discrimination between *D. pinea*, *F. circinatum* and *F. oxysporum*.

CHAPTER 3

Discriminatory detection of *Fusarium circinatum* from *Fusarium oxysporum* and *Diplodia pinea* in infected pine seedlings using Enzyme-Linked Immunosorbent Assay (ELISA)

Abstract

In the present study an ELISA for quick detection of *F. circinatum* in pine seedlings was developed using antibodies raised against mycelium-soluble antigens of this fungus. To validate the specificity of this assay, antibodies to *D. pinea* and *F. oxysporum* were also developed. Antigens for use in the ELISA were produced by inoculation of seedlings with conidia from each pathogen. Infection was monitored using scanning electron microscopy and the development of symptoms. The antigens were isolated from different plant parts. The ELISA was sufficiently sensitive to detect infection caused by a conidia concentration as low as approximately 1conidiumml^{-1}two weeks post inoculation. Cross-reactivity was observed, but a positive reaction for the detection of *F. circinatum* and *F. oxysporum* was indicated by an optical density above 0.8. Fungal identity was confirmed by PCR using primer pairsfor amplification of a 70, 89 or 360 bp fragment for *F. oxysporum, D. pinea* and *F. circinatum* respectively. Validation of each pathogen was obtained and no cross-infection between seedlings was detected. This indicated that the cross-reactivity of the ELISA was due to other factors, such as common epitopes. With further development an ELISA dip-stick or ELISA using these antibodies should provide an easy, fast field test to identify infections of pine, discriminating between *F. circinatum, F. oxysporum* and *D. pinea.*

3.1 Introduction

Fusarium circinatum Nireberg and O'Donnell [=*F. subglutinans* (Wollenw. and Reinking) Nelson *et al. f.sp. pini* Corell *et al*]is an economically important pathogen of pines. It causes pitch canker of mature pines and seedling wilt in nurseries. The latter is the most important pine disease in South Africa, hence this study focuses on seedling wilt caused by *F. circinatum*. A number of *Pinus* species are susceptible to this fungus. Seedling wilt causes reduced yields and high levels of seedling mortality resulting in great economic losses (Viljoen *et al.,* 1994; Wingfield *et al.,* 2008). In nurseries, infection results in seedling damping-off, stem lesions, reddish-brown discoloration, lesions on the roots, resin exudates and terminal wilt that leads to seedling death (Correll *et al.,* 1991; Carey and Kelley, 1994; Viljoen *et al.,* 1994; Landeras *et al.,* 2005; Perez-Sierra *et al.,* 2007). The first report of *F. circinatum* in the Southern hemisphere was made in the Mpumalanga region on *Pinus patula* where it caused root-rot in nursery cuttings in the early 1990s (Viljoen *et al.,* 1994).

In South African nurseries new infections result from using contaminated water and seeds. *F. circinatum* can be isolated from decaying infected branches in the upper crown and dead needles (Barrows-Broaddus and Dwinell, 1985a). The fungus has also been isolated from asymptomatic trees (Correll *et al.*, 1991). There are conditions in which the fungus can enter an endophytic state, where it will not induce any symptoms in the infected tree or seedling (Correll *et al.*, 1991; Lunchi *et al.*, 2011). Once the asymptomatic seedlings have been planted out in the field, the fungus can occasionally switch from a latent to an active state and start causing wilt. The mechanism for this switch is not known, but it has resulted in even greater losses in the field (Storer *et al.*, 1998; Luchi *et al.*, 2007).

During infection *Fusarium* conidia recognize certain substratum characteristics on the roots and adhere immediately after landing (Pascholati *et al.*, 2002). Conidia first form germ tubes and this is followed by the formation of appressoria, which then give rise to hyphae (Kuo and Hoch, 1996). Once the hyphae have developed, they enter the roots directly where they penetrate the host epidermal cell walls and colonise the cortex by intra- and intercellular growth (Nicholson and Kunoh, 1995; Carver *et al.*, 1996). The hyphal growth continues and reaches the vascular tissue, where the hyphae quickly spread upward through the xylem vessels. This results in clogging of the water ducts, resulting in plant wilting. Levels of mycelial growth have been reported to remain relatively low in these ducts even at advanced stages of disease. Despite limited mycelial growth, the flow rate of water in the xylem vessels may be reduced by up to 88% to that of the uninfected tree (Nicholson and Kunoh, 1995; Vidhyasekaran, 2008). In infected plants mycelium can be detected from early stages of infection before symptom development (Correll *et al.*, 1991; Lunchi *et al.*, 2011).

Development of accurate and sensitive detection systems for plant pathogens is one of the most important strategies for controlling disease spread, and slowing its introduction into sites where the pathogen is not present. In South African nurseries *F. circinatum* is identified by analyses of symptom development, growth on selective media and microscopy (Lievens and Thomma, 2005). However, these identification methods are often impaired by the presence of other fungal pathogens such as *Diplodia pinea* (Fr) Dyko & B. Sutton that causes similar wilting symptoms. Taxonomically closely related fungal species such as *Fusarium oxysporum* Schlecht, impairs the use of selective media and microscopy for identification of *F.*

circinatum. Polymerase chain reaction (PCR) methods have been developed for the detection of different plant pathogens, such as *Leptosphaeria maculans* Lmb, *Rhizoctonia solani*, *F. circinatum*, *F. oxysporum* and *D. pinea* (Xue *et al.*, 1992; Grimm and Geisen, 1998; Edel *et al.*, 2000; Weiland and Sundsbak, 2000; Calderon *et al.*, 2002b; Schweigkofler *et al.*, 2004; Abd-Elsalam *et al.*, 2006; Godoy-Lutz *et al.*, 2008; Budge *et al.*, 2009; Validov *et al.*, 2011).For the detection of *F. circinatum*, a primer pair CIRC1A-CIRC4A that targets an ISG region of the nuclear ribosomal operon has been successfully used in most of the popular detection studies (Schweigkofler *et al.*, 2004; Perez-Sierra *et al.*, 2007). The PCR product is then analysed on an agarose gel where positive results will be indicated by the presence of an amplified specific DNA at the expected size. Polymerase chain reaction is useful in the detection of diseases in asymptomatic plants, in identifying pathogens to the species level and investigating non-culturable pathogens. The sensitivity of PCR assays may be reduced when the DNA is directly isolated from soil or plant tissue due to inhibitors such as plant phenolics from these samples. There is also a problem of false positive results in the presence of DNA contaminants.

Immunochemical systems for the identification of *F. circinatum* that focus on the use of ELISAs have been well documented. These have been reported as much easier and quicker to use for quantification and detection of fungi, both in the laboratory and in the field (Lievens and Thomma, 2005; Lievens *et al.*, 2008). Enzyme linked immunosorbent assays are ideal methods to use in complex mixtures such as plant extracts which is not true for PCR assays. Also, ELISA tests have an advantage over the other PCR assays and the use of selective media and microscopy because they allow for the detection of multiple samples simultaneously. However, with antigens isolated from plant extracts, non-specific reactions due to normal plant constituents need to be considered.

Previous studies have shown that raising antibodies using extracts from infected plant tissue tend to generate more specific monoclonal antibodies (Dewey *et al.*, 1992; Meyer and Dewey, 2000; Kaur *et al.*, 2007). However, other studies have indicated that such antibodies tend to react with plant proteins. Similar reactivity patterns have been observed with antibodies raised to soluble fungal antigens (Ricker *et al.*, 1991). In some cases antibody binding may be more directed towards plant cell walls than those of fungi when antibodies against extracts of

infected plants are used for detection. This is because some of the proteins may be expressed to higher levels than those of the invading fungi (Meyer and Dewey, 2000). The problem of antibody cross-reactivity between closely related fungal species should be considered when developing identification tools for fungi, based on antibodies. This is because fungal pathogens, especially those that are closely related taxonomically, generally comprise similar glycoprotein scaffolds, carbohydrate epitopes and cell wall material such as chitin common amongst different fungal pathogens (Wycoff *et al.*, 1987; De-Bernardis *et al.*, 1994; Hitchcock *et al.*, 1997).

In the current study a detection system for *F. circinatum* in pine seedlings was developed. Antibodies that were previously prepared (see Chapter 2) against mycelium-soluble antigens of *F. circinatum* were evaluated for reactivity with antigens isolated from infected pine seedlings. The detection limit of these antibodies was evaluated. Cross-reactivity with other common pine infecting fungi such as *D. pinea* and *F. oxysporum* was determined. The future goal of this research was to prepare a detection system that is simple enough for semi-skilled nursery staff to use in the field to test seedlings suspected of being infected.

3.2 Materials and methods

3.2.1 Preparation of pathogens and artificial inoculation

Fungal pathogens (*F. circinatum*, *F. oxysporum* and *D. pinea*) used in this study were obtained from the Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI),University of Pretoria. Four month old pine seedlings were obtained from SAPPI Southern Africa (Pty) Ltd. Seedlings were transplanted in pots and grown in greenhouse in a composted pine bark potting mix. Greenhouse temperatures fluctuated between 25 – 30°C. *D. pinea 16*, *F. circinatum 3578* and *F. oxysporum 776* were cultured on potato dextrose agar (PDA) (Merck) for 14 days at 28°C. To promote sporulation these cultures were exposed to UV light for two weeks. Conidia were washed off the plate surface using 50 ml sterile water. These were then filtered to remove mycelium components using cheese cloth. Conidia were counted using a haemocytometer. Serial dilutions were carried out to reach different conidia concentrations including 1×10^6, 1×10^3, 1×10^2, 1×10^1 and 1 conidia ml^{-1}. Seedlings were inoculated two weeks after transplanting. A drenching method was used for inoculation (Kimishima and Kobayashi, 1990; Hahn and Werres, 1997).

Each spore concentration was prepared in duplicate and inoculation was carried out twice onto five different seedlings and the experiment was repeated three times. The conidia count method did not work for *D. pinea* because there were very few spores observed and hence *D. pinea* was cultured on PDA again. To promote sporulation the cultures were exposed to UV light for two weeks. A method adapted from a study carried out by Stanosz *et al.* (2007) was used, in which plugs were cut off from the sporulated agar plates and used for inoculation. During inoculation the agar plugs were placed on a seedling shoot that had been wounded. In this method mycelium from the plugs grows directly into the plant trough the wound and the spores that make contact with the wound also germinate and penetrate into the seedling. This inoculation was also carried out twice onto five different seedlings and the experiment was repeated three times.

3.2.2 Scanning electron microscopy for monitoring infection

Two weeks post-inoculation, two seedlings from each inoculation were collected, including the non-inoculated seedlings that served as controls. These were rinsed with tap water and cut into three sections i.e. leaves, stems and roots. From each section longitudinal cuttings were performed to expose the plant interior. The samples were fixed overnight in 2% buffered osmium tetroxide [50% (v/v) 4% OsO_4; 25% (v/v) 0.2 M sodium cacodylate buffer pH 6.5]. This was followed by immersing the samples in 0.2 M sodium cacodylate buffer pH 6.5 for 2 x 30 min. Samples were dehydrated by 10 min dips into a series of ethanol dilutions including 30%, 50%, 70%, 80% and 90% ethanol. The final stage of dehydration was carried out in 100% ethanol for 3 x 10 min in a fume cupboard. Dried plant material were then transferred into a critical point drier baskets under 100% ethanol and placed in a pre-cooled HCP-2 critical point drier (Hitachi Gauteng, South Africa). Following critical point drying, samples were coated with gold palladium sputter coating, ESEM, coating unit E5100 (Polaron Equipment Limited Arizona, USA). Samples were viewed in an environmental scanning electron microscope (ESEM), Philips, FEI XL 30(Holland South Holland, Netherlands), Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Pietermaritzburg at an accelerated voltage of 15 KeV. Viewing of the interior longitudinal side of each section was carried out and multiple pictures of each were taken.

3.2.3 Detection of antigens using indirect ELISA

Two, four, six and eight weeks post-artificial inoculation, two seedlings from treatment with each conidia concentration were collected. These were used to evaluate the sensitivity of the ELISA developed. However, to detect levels of antigens from different plant parts, seedlings infected with 1 x 10^3 conidia ml^{-1} for each pathogen were used. These seedlings were selected because they showed the expected symptoms that gradually increased with time for each of the respective pathogens and the same behaviour was observed over different inoculations. Whereas seedlings inoculated with other spore concentration (1 x 10^6, 1 x 10^2, 1 x 10^1 and 1 conidia ml^{-1}) showed some inconsistencies between the replicates. Seedlings were first rinsed with tap water and transversely cut to separate into leaves, stem and roots. Each section was further cut into smaller pieces using a scalpel. These were placed into 2 ml microfuge tubes, enough to fill half of the tube, the remaining seedling materials were stored at -80 ℃. Phosphate buffered saline [(PBS: 0.8% (w/v) NaCl; 0.02% (w/v) KCl; 0.115% (w/v) $Na_2HPO_4.2H_2O$; 0.02% (w/v) KH_2PO_4)] was added and two beads were added. Using a Mini-bead-beater(Biospec Bartlesville, USA), the samples were homogenized for five min. The suspension obtained was centrifuged (15 000 xg, 5 min, RT). Protein concentration in the supernatant was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Antigen (200 µg ml^{-1}) was used in the indirect ELISA. The micro-titre plates (Nunc Roskilde, Denmark) were coated with 100 µl of antigen in PBS and incubated overnight at 4℃. This was followed by blocking non-specific binding with 200 µl BSA (0.5%, w/v in PBS) for one hour at 37℃. Three washes with PBS containing 0.1% (v/v) Tween 20 (PBS-Tween) was carried out. Primary antibody (100 µg ml^{-1}) in BSA [0.5% (w/v) in PBS] specific for each pathogen (anti-*D. pinea*, anti-*F. circinatum* and anti-*F. oxysporum* antibodies) was added and incubated for one hour at 37℃. The plates were then washed 2 times with PBS-Tween. Rabbit anti-chicken IgY-horse radish peroxidase (HRPO) conjugate (Sigma, St Louis, MO, USA) (1 in 20,000) was added to the wells and incubated for one hour at 37℃. The plates were washed three times with PBS-Tween. 2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS) chromogen/H_2O_2substrate solution [0.15 M citrate-phosphate buffer, pH 5.0 containing0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2] was added and the plates were incubated in the dark at ambient temperature for 10 min. This period was increased where sufficient colour change was not obtained. Absorbance was measured at 405

nm using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany). The optimal reactivity of the antibodies raised was found to be at 100 µg ml^{-1} for indirect ELISA. To evaluate antibody sensitivity seedlings infected with each conidia concentration were prepared in the same manner mentioned above. The ELISA was carried out in the same manner andnon-infected seedlings and non-immunised antibodies were used as controls.

3.2.4 Preparation of horse radish peroxidase (HRPO) linked detection antibodies

To increase assay sensitivity and specificity, a sandwich ELISA was developed. The first step involved conjugating primary antibodies developed in chickens against mycelium-soluble antigens from *D. pinea, F. circinatum* and *F. oxysporum* to HRPO to use them as the detector antibodies. For conjugation 4 mg of HRPO (Jackson Immuno Research West Grove, PA, USA) was dissolved in distilled water. A freshly prepared solution of 100 mM sodium periodate was added and stirred for 20 min at ambient temperature. This was placed in dialysis tubing with a molecular weight cut-off of 12 kDa and dialysed against four changes of a 1 mM Na-acetate buffer, pH 4.4 at 4℃ overnight. The pH of the solution was raised to pH 9 – 9.5 by addition of 200 mM Na_2CO_3 buffer, pH 9.5. Following the change in pH, IgY was immediately added to a final concentration of 8 $mg.ml^{-1}$ and this was incubated for two hours at ambient temperature . Free enzyme was reduced by addition of freshly prepared 4 $mgml^{-1}$ Na-borohydride solution and incubation at 4℃ for 2 hours. This was dialysed against four changes of 100 mM Na-borate buffer, pH 7.4, overnight at 4℃. Finally an equal volume of 60% (v/v) glycerol in Na-borate buffer, pH 7.4 was added and the conjugate was stored at 4℃(Hudson and Hay, 1980).

The prepared conjugated antibody was not subjected to purification (affinity purification) and therefore antibody dilutions as high as those of the affinity purified commercial anti-species detection antibody [(Rabbit anti-chicken IgY-horse radish peroxidase (HRPO) conjugate (1 in 20000 dilution)] could not be used. The dilution of conjugate to be used was established in a checkerboard ELISA. Various conjugated antibody dilutions were prepared ranging from 1 in 500 to 1 in 10. Brill *et al.* (1994) reported a 100-fold increase in sensitivity when using sandwich ELISA compared to indirect ELISA. In this study the concentration of the primary antibody to be used in the sandwich ELISA was evaluated, starting from the 100 $µgml^{-1}$ [used in the indirect ELISA (Section 3.2.3)] to 0.01 $µgml^{-1}$. Absorbance values were recorded at

various concentrations of the primary antibodies incubated with various dilutions of the HRPO-linked antibody. A 1 in 20 dilution of HRPO-linked antibody reacted optimally when incubated with its corresponding primary antibody at a final concentration of 0.2, 0.6 and 0.2 $\mu g ml^{-1}$ of anti-*D. pinea*, anti-*F. circinatum* or anti-*F. oxysporum* antibodies respectively.

3.2.5 Evaluation of antibody cross-reactivity using sandwich ELISA

The method used for sample preparation was similar to that used in the indirect ELISA (Section 3.2.1). Stems from seedlings infected with 1 x 10^3 conidia ml^{-1} were used for the purpose of checking antibody cross-reactivity. These were selected because symptom development was clearer in these seedlings within two weeks. In the indirect ELISA, plates were coated with 0.2, 0.6 and 0.2 $\mu g ml^{-1}$ of anti-*D. pinea*, anti-*F. circinatum* and anti-*F. oxysporum* antibodies in PBS, respectively. These were incubated at 4°C overnight, followed by blocking of unoccupied sites with200 µl of 0.5% (w/v) BSA in PBS for one hour at 37°C. Three washes with PBS containing 0.1% (v/v) Tween 20 (PBS-Tween) was carried out. Each antigen (200 $\mu g\ ml^{-1}$) was added into wells that were coated with the respective antibodies and incubated for 2 hours at 37°C. The plates were then washed two times with PBS-Tween. A 1 in 20 dilution of the respective HRPO-linked antibodies (that was the same as the primary antibody used for coating) was added to the respective wells and incubated for one hour at 37°C. Plates were washed three times with PBS-Tween and for detection ABTS/H_2O_2 chromogen substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in 50 mM citrate-phosphate buffer pH 5.0] was added. Plates were incubated in the dark at ambient temperature until enough colour change was obtained. This was measured at 405 nm using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany). Non-infected seedling samples and pre-immunisation antibodies were used as controls.

3.2.6 DNA isolation from pine seedlings

Polymerase chain reaction was used to confirm the identity of pathogens tested in the ELISA and to evaluate the possibility of any cross-infection that might have occurred in the greenhouse during the inoculation stage. Seedlings used for isolation of antigens were also used for DNA isolation using a hexadecyltrimethylammonium bromide (CTAB method) (Murray and Thompson, 1980). Stems from *D. pinea*, *F. circinatum* and *F. oxysporum* infected seedlings were collected from storage at –80°C and crushed in PBS in a 2 ml

microfuge tube. Warm extraction buffer [10% (v/v) 10 mM Tris-HCl, pH 8, 4% (v/v) 20 mM EDTA, 28% (v/v) 1.4 mM NaCl, 20% (v/v) 2% CTAB, 0.2% (v/v) 2-mercapto-ethanol] was added to each sample to a total volume of 750 µl. This was incubated at 65°C with shaking for an hour. A chloroform: isoamyl alcohol (C:IAA) (24:1) solution (500 µl) was added, followed by gentle shaking and centrifugation (12000 xg, 3 min, 4°C). The supernatant was transferred to a clean tube and 500 µl of cold isopropanol was added and centrifuged (12000 xg, 5 min, 4°C). The supernatant was discarded and 70% (v/v) ethanol was added to the pellet, which was then allowed to stand at ambient temperature for 20 min. This was centrifuged (12000 xg, 5 min, 4°C) and the DNA pellet was air-dried. Tris-EDTA (TE) buffer pH 8, was used to re-suspend the pellet first at ambient temperature for one hour followed by an overnight incubation at 4°C. Five hundred µl of 7.5 M NH_4OAc was added and for DNA extraction equal volumes of C:IAA was used and the supernatant was transferred to a clean tube. To precipitate DNA 100% (v/v) ethanol was added and the DNA was stored at -20°C overnight. DNA was collected from the pellet after centrifugation (12000 xg, 5 min, 4°C) and washed twice with 70% (v/v) ethanol and air dried for 2 hours. Two hundred µl of TE buffer was used to re-suspend the DNA, RNAse A was added and the sample incubated at 37°C for 2 hours. DNA concentration and purity was determined using the $A_{260/280}$ ratio determined using a NanodropND-1000 spectrophotometer (Inqaba Biotec Johannesburg, South Africa).

Using the DNA isolated directly from infected plant tissue in PCR necessitated the use of an alternative DNA source. The stems from *Fusarium*-infected seedlings were cut longitudinally and placed on *Fusarium* selective medium (FSM) [1.5% (w/v) peptone powder; 0.1% (w/v) KH_2PO_4; 0.05% (w/v) $MgSO_4$. 7 H_2O; 2% (w/v) bacterial agar; 1% (w/v) quintozene] with the interior surface touching the medium and incubated at 28°C. Some samples were cultured directly on PDA. In the case of *D. pinea* infected seedlings, the stem was placed on PDA with the interior surface touching the medium and incubated at 28°C. Mycelium growth was observed from one week post incubation. Mycelium was cut out and sub-cultured onto new PDA plates to grow a clean colony with enough mycelium. Using a scalpel, mycelia were scraped off the plates and crushed in liquid nitrogen. The powdered mycelium was used for DNA extraction using the CTAB method mentioned above.

3.2.7 DNA amplification

Primers specific for amplification of *D. pinea* DNA used in this study were previously developed by Luchi *et al.* (2011). These primers were designed to target specific fungal sequences in the mitochondrial small subunit ribosome gene (mt SSU rDNA) (Table 3.1). Confirmation of *F. circinatum* on seedlings was carried out using the primer pair CIRC1A-CIRC4A (Table 3.1). These are specific for amplification of a 360-bp DNA fragment in the intergenic spacer region of the nuclear ribosomal ISG region. For *F. oxysporum* confirmation a primer pair (PFO2 and PFO3) was used that targeted a 28S rDNA was used (Table 3.1) (Edel *et al.*, 2000).

Table 3.1 Primer sequences for the amplification of *D. pinea, F. circinatum* and *F. oxysporum* sequences

Pathogen	Primer name and primer sequence	Expected size (bp)	Reference
D. pinea	DpHRM_F-50-GCTACCTTGGAGTAAGGGACA-30 DpHRM_R-50- TTTCCATCTAGGAGCGAAAAT-30	89	Lunchi *et al*. (2011)
F. circinatum	CIRC1A -5' CTTGGCTCGAGAAGGG CIRC4A- 5' ACCTACCCTACACCTCTCACT	360	Schweigkofler *et al.*(2004)
F. oxysporum	PFO3-5'-CGGGGGATAAAGGCGG-3' PFO2-5'- CCCAGGGTATTACACGGT-3'	70	Edel *et al*. (2000)

Amplification was carried out for DNA isolated from both PDA and from plant tissue. PCR amplifications were performed in a total volume of 20 µl. This contained 5 µl of DNA (approximately 20 ng), 2 µl of 100 µM of forward and reverse primers, KAPA universal master mix (10 µl) and 1 µl of nuclease free water. The primer pair specific for each pathogen was used for the pathogen confirmation step. For evaluation of cross-infection, each primer pair was used for DNA amplification of each of the three pathogens. PCR cycling parameters were initiated by first heating the lid of G-storm thermocycler (Vacutec, California, USA) at 110℃. This was followed by the series of temperature cycles: hot start automatic 95℃ for 300 seconds, denaturation at 95℃ for 15 seconds, temperature step at 65℃ and 57℃ for 900 seconds for *Fusarium* pathogens and *D. pinea*, respectively. Elongation was carried out at 72℃ for 15 seconds and a final elongation step at 72℃ for 180 seconds. A total of 30 cycles

were carried out and the products were stored at 10°C.A 2 % (w/v) agarose gel was used to evaluate products obtained from all PCR amplifications products. The use of FSM and PDA for DNA isolation was selected to be the best method as no amplification was observed from DNA isolated directly from infected tissue.

3.3 Results

3.3.1 Evaluation of mycelial growth on pine seedling

Scanning electron microscopy was used to monitor the development of the fungal mycelium within each seedling. This was used to evaluate and select the best plant part to provide the highest antigen concentration to use in ELISA. Non-infected seedlings showed no mycelial growth in either the leaves or stem (Figure 3.1). While *D. pinea* infected seedlings showed massive growth of mycelium in the leaves, little was observed in the roots two weeks post inoculation. Leaves and stems were then selected as the best source for antigen isolation for *D. pinea*. At this point no symptom development was observed. Mycelium growth was distributed throughout each seedling, with more growth observed in the stem and root in the *F. circinatum* infected seedlings. Thus for testing on ELISA only the stem and roots were used. *F. oxysporum* infected seedling showed no mycelium growth in the leaves but mycelia were observed in the stem and root. No symptoms were observed in either of the *Fusarium* infected seedlings two weeks post inoculation.

3.3.2 Detection of fungal antigens from different seedling parts using ELISA

Indirect ELISA was used for the detection of fungal antigens directly isolated from infected pine seedlings. This experiment attempted to confirm whether mycelia observed under the microscope could be detected using ELISA and if the levels of mycelia observed under the scanning electron microscope correlated with the results obtained with ELISA. *D. pinea* infected seedlings showed more mycelia growth on the leaves than the stems under the scanning electron microscope, but the ELISA readings did not show as much of a difference between the levels of antigen detection for the two organs as expected. Scanning electron micrographs for *Fusarium* infected seedlings indicated similar mycelium growth patterns in the roots and stems (Figure 3.1).

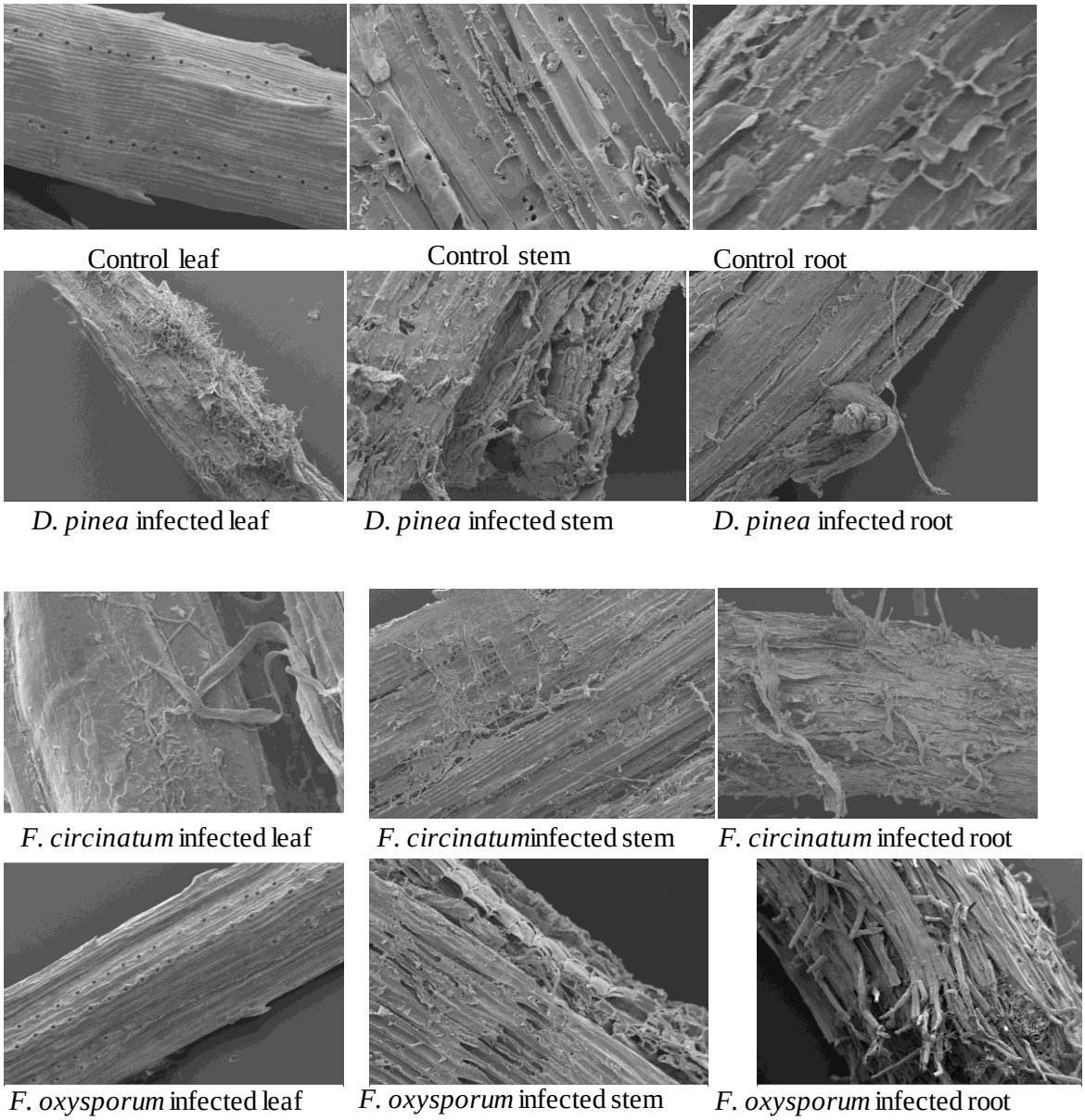


Figure 3.1 Electron micrographs showing different sections of pine seedlings artificially inoculated with different fungal pathogens. Two weeks post seedling inoculation with *D. pinea, F. circinatum* or *F. oxysporum*, each of the seedlings was sectioned into stem, roots and leaves. Mycelium growth on each plant part was viewed at an accelerated voltage of 15 KeV using an environmental scanning electron microscope (ESEM). Micrographs were selected from two repeats of each pathogen artificial inoculation.

However, the levels of antigen detected in the ELISA using the corresponding antibodies were much higher in the stems than in the roots. Higher antigenic levels from Week 2 to Week 8 post infection were detected more in the stems as compared to the other parts of the seedlings infected with *D. pinea*, *F. circinatum* or *F. oxysporum* (Figure 3.2).

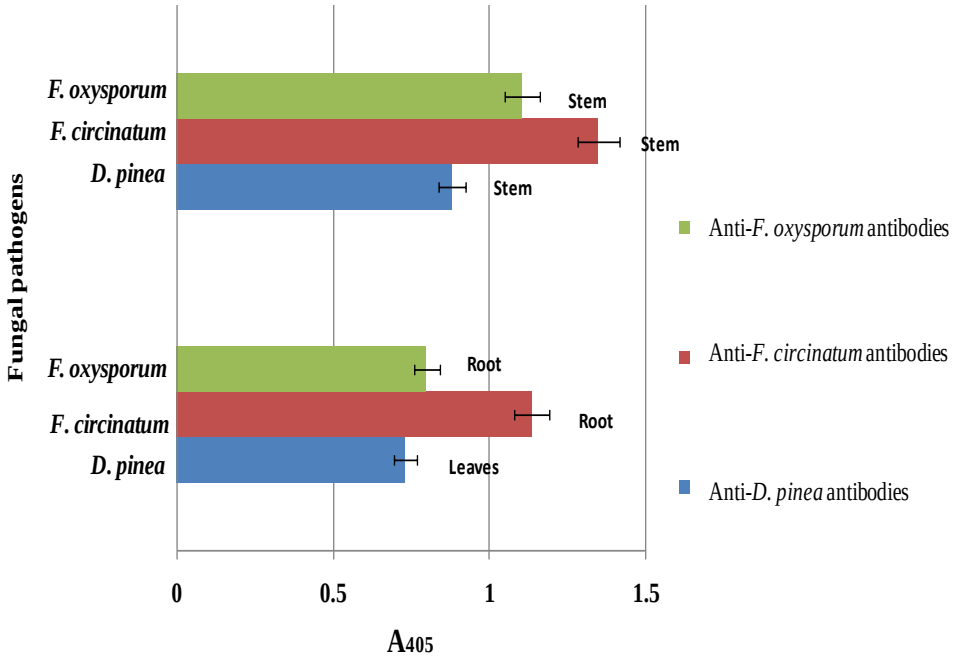


Figure 3.2 Detection using indirect ELISA of fungal growth on different parts of pine seedling artificially inoculated with fungal pathogen. Fungal antigens were isolated from different parts of the pine seedlings inoculated with *D. pinea*, *F. circinatum* or *F. oxysporum*. Antigens (200 µg ml^{-1}) were used for coating of micro-titre ELISA plates, followed by incubation with primary antibody (100 µg ml^{-1}). Binding of the primary antibody was detected with rabbit anti-chicken IgY-HRPO conjugate followed by ABTS/H_2O_2 chromogen substrate solution. Absorbance values at 405 nm are the average of duplicate samples. Non-infected pine seedling was used as a control.

3.3.3 Sensitivity of ELISA in the detection of fungi at different infection levels in pine seedlings

Detection levels obtained when using ELISA in seedlings infected with 1 x 10^6 – 1 x 10^2 conidiaml^{-1} were similar, absorbance readings obtained were all between 1 and 1.5. Absorbance values were lower at conidia concentrations of 1 x 10^1 – 1conidiaml^{-1} (Figure 3.3, A). The ELISA readings obtained for detection of *D. pinea* in seedlings were relatively low compared to the detection levels observed for *Fusarium* infected seedlings, indicating the difference in the antibody sensitivity levels. Also, there was not much of a difference between non-infected and *D. pinea* infected samples. This was indicated by the absorbance values of 0.44 and 0.48 for non-infected and *D. pinea* infected seedling, respectively.

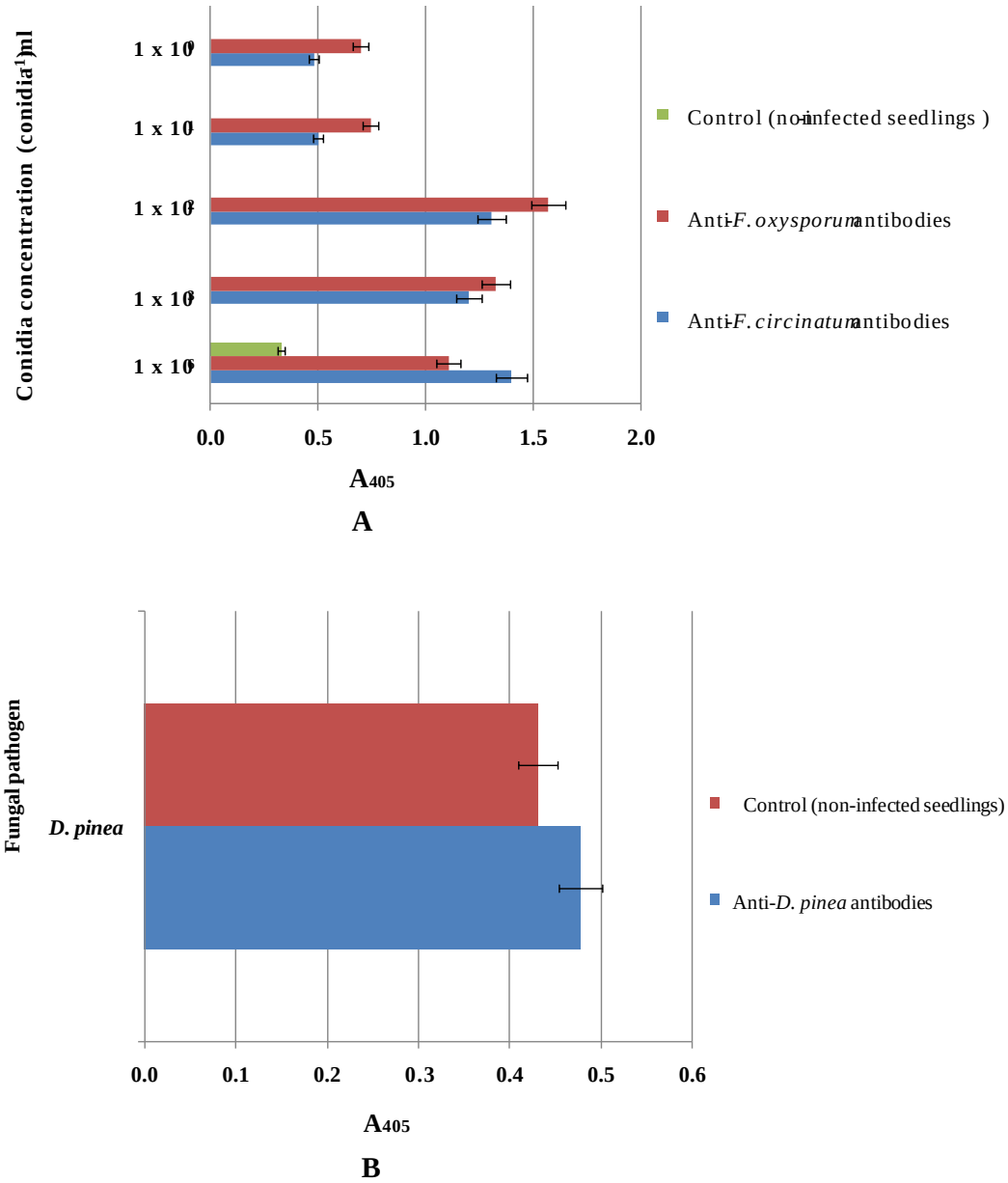


Figure 3.3 Evaluation of antibody sensitivity for detection of antigens isolated from infected seedlings. (A) Seedlings inoculated with fungal conidia (concentration of 1 to 1 x 10^6conidiaml^{-1}) from *F. circinatum* and *F. oxysporum* were used for isolation of antigens. Micro-titre ELISA plates were coated with the antigens followed by incubation with primary antibodies and detection with rabbit anti-chicken IgY-HRPO conjugate and ABTS/H_2O_2 chromogen/substrate. Absorbance values at 405 nm presented here are the average of duplicate samples. (B) Antigens isolated from pine seedlings inoculated with conidia from an agar block of *D. pinea* culture, directly placed on the seedlings. Each experiment was repeated two times.

3.3.4 Cross-reactivity test

Sandwich ELISA was used to evaluate cross-reactivity of anti-*D. pinea*, anti-*F. circinatum* and anti-*F. oxysporum* antibodies, with antigens isolated from plant extracts. When using a

sandwich ELISA format, similar reactivity with that of indirect ELISA was obtained at a primary antibody concentration less than 10 μgml^{-1}, whereas with indirect ELISA 100 μgml^{-1} of primary antibody was required to get a similar response (Figure 3.4). Anti-*D.pinea* antibodies were more reactive towards *D. pinea* antigens. Anti-*D.pinea* antibodies showed less cross-reactivity with *F. circinatum* than with *F. oxysporum*. Anti-*F.circinatum* antibodies were more reactive with the antigens isolated from *F. circinatum* infected seedlings. The anti-*F .circinatum* antibodies showed significant cross-reactivity with *F. oxysporum*. Anti-*F. oxysporum* antibodies were the most reactive antibodies indicated by the highest absorbance values obtained when they reacted with antigens obtained from *F. oxysporum* infected seedlings. These antibodies cross-reacted with the other two fungal pathogens studied (Figure 3.4). Anti-*F.oxysporum* antibodies discriminate between *F. oxysporum* and *D. pinea*. Cross-reactivity was obtained between fungal pathogens used in this study, however, following multiple ELISA tests carried out using both stem and root samples from *Fusarium* infected seedlings it was concluded that a reading above 0.8 indicated a positive reaction. Some variations were observed with that of *D. pinea*. Each of the antibodies developed showed some reactivity with plant constituents indicated by the ELISA reading of approximately 0.4 observed when they reacted with antigens isolated from non-infected seedlings (Figure 3.4). An absorbance of 0.4 is 50% of the cut-off value for the specific detection of *Fusarium* pathogens, and close to the cross-reactivity of the *D. pinea* with anti-*Fusarium* antibodies.

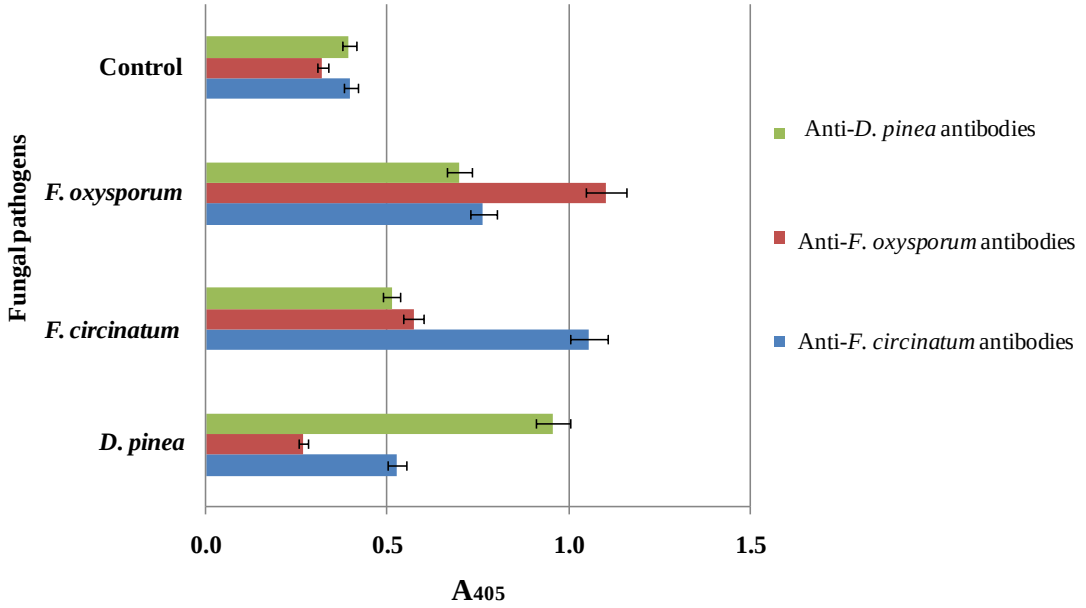


Figure 3.4Evaluation of antibody cross-reactivity with antigens isolated from artificially inoculated pine seedlings using a sandwich ELISA. Antigens were purified from pine seedling inoculated with *D. pinea*, *F. circinatum* and *F. oxysporum*. Micro-titre ELISA plates were coated with 0.2, 0.6 and 0.2 μgml^{-1} of anti-*D. pinea*, anti-*F. circinatum* and anti-*F. oxysporum*antibodies, respectively, followed by incubation with different antigens to test for antibody cross-reactivity. Secondary HRPO-linked antibody corresponding to the primary antibody was incubated followed by ABTS/H_2O_2 chromogen/substrate solution. Absorbance values at 405 nm are the average of duplicate samples.

3.3.5 Identification of fungal pathogens isolated from seedlings and evaluation of multiple infections

Seedlings were kept in the same green-house, therefore there was a possibility of cross-infection taking place. During incubation other opportunistic fungi might have infected the experimental seedlings, thus outgrowing the fungi of interest. Therefore PCR was used to confirm the identity of the pathogens detected by ELISA. DNA samples were initially directly isolated from infected seedling using the CTAB method. When this method was used no DNA amplification was observed for all pathogens, probably because of plant phenolics (Figure 3.5 A, lane 4, 5 and 7). To eliminate phenolics from the test material a selective medium was used for isolation of the *Fusarium* species from infected seedlings. Potato dextrose agar was used for isolation of *D. pinea*.

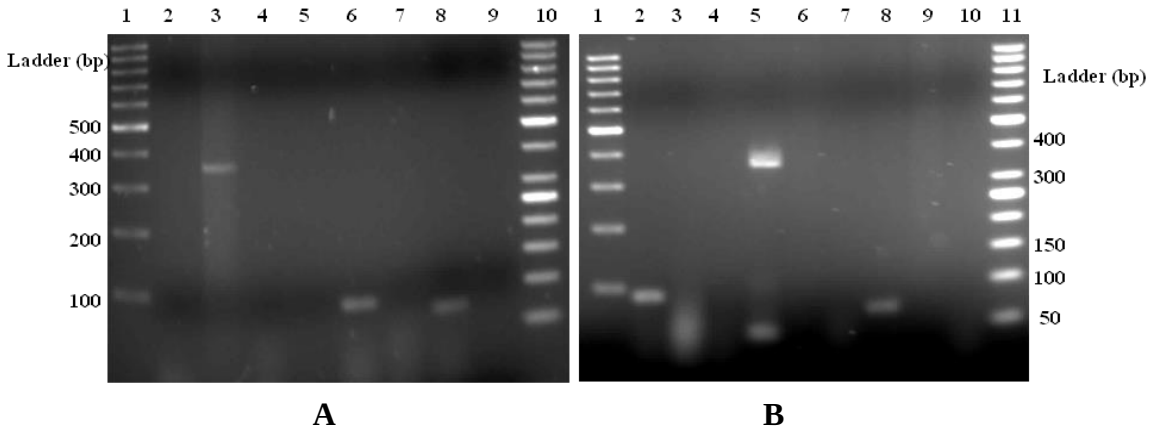


Figure 3.5 PCR amplification for confirmation of the fungal pathogen isolated from infected pine seedlings and evaluation of the possible multiple infections in each seedling. (A) Fungal pathogen isolated directly from an infected plant and those isolated using FSM and PDA: lane 1, 100 bp DNA marker; DNA isolated from- lane 2, control seedlings (non-infected); lane 3, *F. circinatum* infected seedling using FCM; lane 4, seedling infected with *F. circinatum*; lane 5, seedlings infected with *D. pinea;* lane 6, seedlings infected with *D. pinea* using PDA; lane 7 seedlings infected with *F. oxysporum*; lane 8, *F. oxysporum* using FSM; lane 9, distilled water control; lane 10, 50 bp DNA marker. (B) Evaluation of multiple infections; lane 1, 100 bp DNA marker; lanes 2, 3 and 4 DNA from *D. pinea*, *F. circinatum* and *F. oxysporum* infected seedlings, respectively, amplified with the DpHRM_F- DpHRM_R primer pair; lanes 5, 6 and 7 DNA isolated from *F. circinatum*, *D. pinea* and *F. oxysporum* infected pine seedlings, respectively, amplified using the CIR1A-CIR4A primer pair; lanes 8, 9 and 10, DNA isolated from *F. oxysporum*, *F. circinatum* and *D. pinea* infected seedling, respectively, amplified using the PFO3-PFO2 primer pair.

From these cultures DNA was isolated using a CTAB method. Using a primer pair specific for each fungus, amplification was observed as shown in Figure 3.5 A, Lanes 3, 6 and 8, where a 89, 360 and 70 bp product was observed indicating the infectious fungus for each seedling to be *D. pinea*, *F. circinatum* and *F. oxysporum*, respectively. DNA isolated from each seedling was subjected to amplification by all three primer pairs. Amplification was only obtained where a primer pair was specific for the DNA isolated. This indicated that cross-reactivity observed with ELISA was due to other factors such as common epitopes and not multiple infections. Primer pairs DpHRM_F-DpHRM_R, CIR1A-CIR4A and PFO3-PFO2 gave products of the expected sizes from the seedlings infected with *D. pinea*, *F. circinatum* and *F. oxysporum*, which were 89, 360 and 70 bp, respectively (Figure 3.5 B, Lanes 2, 5 and 8).

3.4 Discussion

In the present study an ELISA test was developed to detect fungal pathogens in pine seedlings with the aim of discriminating between *F. circinatum*, *F. oxysporum* and *D. pinea* infections. The antibodies used in the present study were developed from mycelium-soluble antigens of *D. pinea*, *F. circinatum* and *F. oxysporum* (Chapter 2). In the previous chapter results from a western blotting experiment indicated the presence of common immuno-dominant proteins

among the three fungal pathogens. Cross-reactivity was observed especially with pathogens of the same genus. In the current study reactivity of these antibodies was evaluated using *D. pinea*, *F. circinatum* and *F. oxysporum* infected seedlings.

In this study two methods for artificial inoculation of seedlings were used: a drenching method and scraping of mycelium and placing it on a mechanically created wound. This was followed by evaluation of mycelium growth using scanning electron microscopy which indicated that the fungus was able to spread throughout the plant within two weeks post inoculation. Most mycelium growth was observed in the leaves, evenly distributed throughout the plant and in the stem and roots for the seedlings infected with *D. pinea*, *F. circinatum* and *F. oxysporum*, respectively. The observations made in the present study especially for *F. oxysporum* infected seedlings where more mycelium growth was observed in the roots was expected for *Fusarium* species. This was similar to the previous observation made by Ricker *et al.* (1991) that some pathogens grow better in certain plant tissues. *Botrytis cinerea* was reported to grow better in tissues with high sugar levels, while *Fusarium* pathogens invade and colonise the roots (Nelson, 1981; Meyer and Dewey, 2000).

Pathogenicity tests were reported in Spain on eight month old seedlings of *P. pinaster*, *P. nigra* and *P. sylvestris*. These were inoculated with *F. circinatum* scraped off PDA plates and directly placed on a mechanically created wound on the stem just above the soil level. Symptoms developed within seven, eight and fifteen days post-innoculation on *P. nigra*, *P. pinaster* and *P. sylvestris*, respectively. By Day 29 all seedlings were dead (Perez-Sierra *et al.*, 2007). On Montery pines *Fusarium* pathogenicity was monitored and rated from Week 4 to Week 8 and at Week 8 seedling death was observed (Correll *et al.*, 1991). In the current study symptom development was observed from Week 2 and Week 3 (varied between seedlings) post-inoculation and seedling death was observed at 8 weeks post-inoculation.

The artificial method of inoculating plants by placing a pathogen directly on the wound has been used in previous studies, resulting in more rapid disease development. (Correll *et al.*, 1991; Meyer and Dewey, 2000; Perez-Sierra *et al.*, 2007). More rapid growth of *B. cinerea* in grapes was observed when inoculation was carried out using the wounding method than that of non-wounding (drenching method). This was indicated by higher ELISA absorbance readings observed when antigens detected were isolated from plants inoculated using the wounding

method than readings observed from plant extracts inoculated using the drenching method collected at the same time. These observations are in line with those from the current study. *D. pinea* inoculated seedlings showed increased ELISA absorbance readings when the wounding method was used. In the present study different plant tissues (leaves, stems, roots) were used to isolate antigens and these were tested using ELISA. It was observed that the most reactive antigens were from the stems for *Fusarium* infected seedlings.

The present study reported seedling inoculation with *Fusarium* species at various conidia levels. An increase in the level of antigen detection was observed with an increase in the conidia concentration. However, at high conidia concentration, detection levels remained relatively the same. It was also observed that absorbance values obtained from Week 2 to Week 8 post-inoculation were similar regardless of which antibody was used, but increasing disease severity was observed with time post-infection. However, some seedlings showed no symptom development but the presence of the fungus could still be detected by ELISA. This could be explained by previous reports where fungal pathogens were detected by ELISA and PCR on asymptomatic seedlings (Luchi *et al.*, 2007; Maresi *et al.*, 2007; Stanosz *et al.*, 2007). Other studies have reported the use of antibodies as not an ideal system for specific and quantitative detection of fungal antigen in infected wood samples (Clausen, 1997). Reports that low levels of mycelium growth will still clog the xylem vessels and cause seedling wilt might explain the similar ELISA readings observed in the present study despite varying disease severity. The low levels of mycelium reported to be efficient enough to clog the water ducts may explain detection at 1 conidium ml^{-1}. Secondly, this indicated that the ELISA test developed in the present study is sensitive enough to detect the pathogens at very low levels.

To evaluate if the ELISA tests prepared in this study could be used as a discriminatory detection method for pine-infecting pathogens, a cross-reactivity test was carried out. The most cross-reactivity was observed between pathogens from the same genus. However, a positive reaction was indicated by an ELISA reading above an OD of 0.8. Antibodies developed in the present study showed non-specific binding with different plant constituents because there was reactivity obtained with the non-infected seedlings. Also, the difference in the absorbance readings obtained from *D. pinea* infected seedlings indicated that the level of antigen expression (development of disease) was not the same between different seedlings.

The cross-reactivity observed between species from the same genus was not surprising because this has been previously reported for fungal pathogens such as *Fusarium, Aspergillus* and *Penicillium* isolated from plant extracts (Meyer and Dewey, 2000; Karpovich-tate and Dewey, 2001). Antibodies raised to *B. cinerea* isolated from artificially inoculated grapes cross-reacted with species of *Alternaria, Cladosporium*, *Stemphylium* and *Ulocladium* (Boss and Dewey, 1992). From the results observed from the cross-reactivity tests Boss and Dewey (1992) concluded that fungal pathogens produce similar cross-reactive immunodominant molecules *in planta* and that species-specific molecules were either not immunogenic, produced in the same quantities as those that are genera-specific or they are not water-soluble. The cross-reactivity reported by Boss and Dewey (1992) is similar to the one observed in the current study, where antibodies raised to *Fusarium* species cross-reacted with pathogen (*D. pinea*) from another genera. However, results obtained in the current study showed that more species-specific molecules were produced *in planta,* because the reactivity of each of the antibodies developed was more specific to its corresponding antigen from plant extracts when compared to that isolated from nutrient broth (Chapter 2). More specificity was also observed for *Mycena galopus* Pers antigens isolated from plant extracts when compared to whole-cell antigens and exo-antigens (Hitchcock *et al.*, 1997).

Given that in the present study all the seedlings were grown in the same greenhouse there was a possibility of cross-infection taking place. There was also a possibility of other opportunistic fungi outgrowing the fungi of interest. To address this, PCR using primers specific for each pathogen, was performed and confirmation of each fungus was obtained. Primers specific to *D. pinea*, *F. circinatum* and *F. oxysporum* only amplified DNA isolated from the seedlings that were inoculated with the corresponding fungus. The use of these primers also indicated that there was no cross-infection between seedlings; this suggested that the cross-reactivity observed was due to other factors such as common epitopes on fungal antigens.

Different researchers have explained the cross-reactivity as observed in the present study to be due to the common carbohydrate epitopes between some fungal antigens (Wycoff *et al.*, 1987; Hitchcock *et al.*, 1997). The common nature of the biochemical components that makeup the mycelium of most fungi is of concern in studies that aim to raise species-specific fungal antibodies. Most researchers have found antibody specificity to the genus level rather than to

the species level, where either monoclonal or polyclonal antibodies have been raised against fungal antigens (Arie *et al.*, 1991; Bossi and Dewey, 1992; Thornton *et al.*, 2002; Schmechel *et al.*, 2003; Schmechel *et al.*, 2005). Other studies including those reported in the previous chapter of the present study have reported the presence of common antigens between fungal species using analyses by ELISA and western blotting (Gan *et al.*, 1997). Reports from studies where immunofluorescence assays were used indicated that antibody binding to mycelium antigens is more directed to binding with chitin, hyphal tips and septa (Hitchcock *et al.*, 1997; Meyer and Dewey, 2000). To increase the level of specificity some researchers have proposed using antibodies from the first few weeks post-immunisation as these do not have high titre and cross-adsorbing antibodies (Ricker *et al.*, 1991). However, in the present study no increased antibody specificity was observed when antibodies produced during the first six weeks post the first immunisation were used for detection.

ELISA-based field tests for the detection of *Phytophthora, Pythium* and *Rhizoctonia* have been developed previously. These assays used sandwich ELISA (Ali-Shtayeh *et al.*, 1991). There is also a dip-stick kit available for the detection of *Fusarium* mycotoxins (T-2) (de Saeger and van Peteghem, 1996). The ELISA tests prepared in the present study were able to detect *F. circinatum*, *D. pinea* and *F. oxysporum* in infected pine seedlings, and discriminate between these pathogens. This can be used directly by nursery staff in the field where plates coated with primary antibodies can be provided in a kit, together with labeled secondary antibody, and the resulting reaction could be measured using a spectrophotometer. Use of an ELISA dip-stick or ELISA using these antibodies should provide an easy, fast field test to identify infections of pine, discriminating between *F. circinatum, F. oxysporum* and *D. pinea*.

DISSERTATION OVERVIEW

Detection of fungal infection using ELISA in infected pine seedlings was the main objective of the current study. One of the key objectives of this study was achieved, that *F. circinatum* could be discriminated from *D. pinea* and *F. oxysporum* in an ELISA. However, further developments are required to optimize this detection. The ELISA developed was cost-effective, and required little time to confirm the presence of *F. circinatum* in pine seedlings. Furthermore, a less time consuming, more cost-effective dip-stick method could be developed using antibodies produced in this study to confirm the presence of *F. circinatum* in pine seedlings.

ELISA tests are currently available for the detection of *Fusarium poae, F. graminearum, F. sporotrichides, F. oxysporum, F. culmorum, F. graminearum, Thielaviopsis basicola* and *F. avenaceum* (Kitagawa *et al.*, 1989; Beyer *et al.*, 1993; de Saeger and van Peteghem, 1996). In these studies, researchers have looked at using different methods for antigen preparation in attempts to raise more specific antibodies. Some researchers have reported antibodies obtained from immunising with antigens isolated from infected plant material to be specific to a species level, for the detection of *Trichoderma* (Hitchcock *et al.*, 1997). Others have obtained specificity to a genus level, e.g. for the detection of *Ulocladium atrum*(Karpovich-tate and Dewey, 2001). Increased specificity has been reported when mycelium-soluble antigens and exo-antigens were used for immunisation rather than the use of mycelium fragments (Dewey *et al.*, 1990; Brill *et al.*, 1994; Gan *et al.*, 1997; Hitchcock *et al.*, 1997; Hayashi *et al.*, 1998). Other researchers have reported the opposite or that there was no difference between the three sets of antigens (EI-Nashaar *et al.*, 1986; Kitagawa *et al.*, 1989).

The problem of cross-reactivity between closely related fungal species has been a problem in fungal diagnostics, regardless of the set of antigens used during immunisation. The use of antibodies linked to fluorescent dyes has indicated that the antibodybinding site is usually associated with the carbohydrates epitopes and uncharacterised proteins within the hyphal cell walls, chitin, hyphal tips and septa (Hitchcock *et al.*, 1997; Meyer and Dewey, 2000). Fungal mycelia across different genera are composed of cell wall materials common between different fungal pathogens (De-Bernardis *et al.*, 1994). This might explain the cross-reactivity that has

been observed by many researchers. However, some specificity has been observed, indicating that there are some unique proteins within each fungal group, and that there is variation in the cell wall materials used by different groups, as well as differences that exist between the sugar patterns found on the mycelia of fungal groups (Gan *et al.*, 1997; Karpovich-tate and Dewey, 2001).

In the study reported here, three fungal pathogens that are commonly found in pine seedling nurseries were used to raise antibodies. This was carried out to produce antibodies that would be used to develop ELISA tests that have the ability to discriminate between *F. circinatum, D. pinea* and *F. oxysporum*. This study aimed to incorporate the detection of all three pathogens into one ELISA plate to make it is easy to detect *F. circinatum* from the other two fungal pathogens, taking into account cross-reactivity. Since detection is based on colour change, incorporating all three pathogens made it easy to detect the specific fungus, by the development of a more intense colour that can be observed with the naked eye or measured at 405 nm using a spectrophotometer.

The three fungal pathogens were first analysed on SDS-PAGE and a number of similar proteins bands were observed. Such a phenomenon was previously reported for other different *Fusarium* species, *Thielaviopsis basicola* (Berk. & Br.) Ferraris and *Phomopsis longicolla* Hobbs (Kitagawa *et al.*, 1989; Ali-Shtayeh *et al.*, 1991; Brill *et al.*, 1994; Holtz *et al.*, 1994). The similarity of antigens observed between different isolates for each pathogen observed in the present study, indicated that one diagnostic assay can be used across different sites, as was previously reported by Biazon *et al.* (2006). During immunisation different sets of antigens (mycelium-soluble antigens, exo-antigens and a purified34 kDa protein purified from SDS-PAGE separated mycelium-soluble antigens) were used in an attempt to increase antibody specificity. This was not a successful method, since reactivity was only obtained from antibodies prepared using mycelium-soluble antigens. The lack of reactivity of antibodies obtained from a purified34 kDa protein might have been due to a low protein concentration used during immunisation. Secondly, this purified protein was used in its denatured form during immunisation, which might have resulted in antibodies that recognize a protein in a different conformation to that in the fungus.

In the present study antibody reactivity with antigens prepared in the laboratory and those isolated from artificially infected pine seedlings was analysed. The latter was more important for this study since the ELISA tests will be used to test for infection in pine seedlings. When using antigens isolated from nutrient broth western blot analysis indicated that there are multiple immuno-dominant common antigens for different fungal pathogens. Development of multiple bands and smears on the nitrocellulose membrane was an indication of either protein degradation, multiple antigens present in each fungal pathogen or change in structural conformation during protein the denaturation that was carried out during sample preparation for SDS-PAGE gels, where mercapto-ethanol and boiling at 100 ℃ was used. Such protein degradation was previously observed for *Fusarium* and *Phytophthora* species (Wycoff *et al.*, 1987; Gan *et al.*, 1997; Biazon *et al.*, 2006). Multiple band formation on the western blot might have been due to antibodies targeting common carbohydrate groups found on the different proteins. Western blotting indicated cross-reactivity between different pathogens, especially those from the same genus.

Cross-reactivity has been observed previously on immuno-dominant antigens of *F. sporotrichioides*, *F. poae* and *F. graminearum* analysed using western blotting (Gan *et al.*, 1997). In the current study analysis of antibody reactivity using ELISA indicated cross-reactivity similar to that observed using western blotting. What was achieved with analysis of antibody reactivity with "*in vitro* antigens" was

- A clear understanding of the multiple immuno-dominant proteins present in each pathogen.
- That cross-reactivity was observed between antigens from different fungal pathogens
- That each pathogen produces more than one immuno-dominant antigen.
- That fungi of the same genus and isolate from the same pathogen may demonstrate serological similarities but some of the species and isolates may not express all the same reactive antigens (Brill *et al.*, 1994).

This study was also carried out under greenhouse conditions, which mimicked the natural environment for the infection process. Seedlings were inoculated using a drenching method. Some of the important successes achieved in this study were:

- The finding that extraction of antigens from an infected seedling was a simple process that only involved crushing plant material in buffer and using the suspension directly in ELISA,
- Detection of the fungus before development of symptoms.
- Detection of the fungus on seedlings infected with approximately1conidium ml^{-1}.
- Detection that discriminated *F. circinatum* from *D. pinea* and *F. oxysporum*.
- Similar detection levels with different infection levels over time and that each of the fungi detected with ELISA was successfully confirmed with PCR.

This study resulted in the development of an ELISA detection method that can be used to confirm the presence of *F. circinatum* in pine seedlings that are expressing characteristic symptoms of infection with *F. circinatum*, such as seedling wilting and damping off.

The current study also aimed to optimize the ELISA tests developed for the three pathogens, to increase specificity and sensitivity of the detection system prepared. This was achieved by using a sandwich ELISA. Greater reactivity of the antigens isolated from artificially inoculated seedlings was observed than that of antigens prepared in nutrient broth. This observation is in line with previous reports, that some antigens may be expressed at higher levels or more pronounced when the pathogen is in contact with its natural host (Hitchcock *et al.*, 1997; Karpovich-tate and Dewey, 2001). The fact that antigens are better expressed when the fungus is in its natural host compared to when they are cultured in nutrient broth is to the current project's advantage since it will enhance field performance of the detection system developed.

The ELISA tests developed in this study could be used to detect the presence of the fungus on plant extracts following little processing. However, PCR amplification was not obtained on DNA directly isolated from plant extracts and sub-culturing prior to PCR was required. This indicates that ELISA has an advantage over PCR for the detection of *F. circinatum* infection in seedlings. Evaluating antigens isolated from plant extracts showed cross-reactivity between different fungal pathogens. This was expected, because of the common nature of fungal antigens previously observed on SDS-PAGE and western blotting. Pathogens from different genera have been reported to produce the same cross-reactive immuno-dominant molecules in their natural host such as plant tissue (Meyer and Dewey, 2000). Specificity to a genus-level,

rather than species-level has been a common phenomenon for most researchers when both monoclonal and polyclonal antibodies are raised to fungal antigens (Arie *et al.*, 1991; Bossi and Dewey, 1992; Thornton *et al.*, 2002; Schmechel *et al.*, 2003; Schmechel *et al.*, 2005). However, cross-reactivity was quantified which allowed for detection of specific pathogens.

There is still a need to evaluate the ELISA developed in this study on more samples collected from different seedling nurseries. There is also a need to do a number of blind tests. In future studies, evaluation of infection from day one post-inoculation may be useful in ensuring that the detection limit of this ELISA is clearly understood. There is still a need to simplify the ELISA so that it is user friendly for nursery staff. It is also possible to develop a new ELISA, based on the greater specificity that was reported for antibodies that are prepared to antigens isolated from infected plant material (Hitchcock *et al.*, 1997).

REFERENCES

Abd-Elsalam, K.A., Asran-Amal, A., Schnieder, F., Mighel, Q., Verreet, J.A. (2006)Molecular detection of *Fusarium oxysporum f.sp vasinfectum* in cotton roots by PCR and real-time PCR assay. *Journal of Plant Diseases and Protection***113**:14-19.

Ali-Shtayeh, M.S., MacDonald, J.D., Kabashima, J. (1991) A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. *Plant Disease* **75**:305-311.

Arie, T., Gouthu, S., Shimazaki, S., Kamakura, T., Kimura, M., Inoue, M., Takio, K., Ozaki, A., Yoneyama, K., Yamaguchi, I. (1998) Immunological detection of endo polygalacturonase secretion by *Fusarium oxysporum* in plant tissue and sequencing of its encoding gene. *Annals of the Phytopathological Society of Japan* **64**:7-15.

Arie, T., Hayashi, Y., Nagatani, A., Furuya, M., Yamagichi, I. (1991) Production and partial characterization of monoclonal antibodies against *Fusarium oxysporum* 86091a.*Annual Phytopathology Society of Japan***57**:696-701.

Arie, T., Hayashi, Y., Nagatani, K.Y.A., Furuya, M., Yamagichi, I. (1995) Detection of *Fusarium spp*. in plants with monoclonal antibody. *Annals of the Phytopathologicial Society of Japan* **61**:311-317.

Axelrood, P.E., Neumann, M., Trotter, D., Radley, R., Shrimpton, G., Dennis, J. (1995) Seedborne *Fusarium* on Douglas-fir: pathogenicity and seed stratification method to decrease *Fusarium* contamination. *New Forests* **9**:35-51.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue, M.J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on *Angiosperm Phylogeny*. *Annals of the Missouri Botanical Garden* **82**:247-277.

Barnard, E.L., Blakeslee, G.M. (1980) Pitch canker of slash pine seedlings: a new disease in forest tree nurseries. *Plant Disease* **64**:695-696.

Barrows-Broaddus, J., Dwinell, L.D. (1985a) Branch dieback and cone and seed infection caused by *Fusarium moniliforme var. subglutinans* in loblolly pine seed orchard in South Carolina.*Phytopathology* **75**:1104-1108.

Barrows-Broaddus, J.B., Dwinell, L.D. (1985b) Variation in susceptibility to the pitch canker fungus among half-sib and full-sib families of *Virginia pine* (*Pinus virginiana*) inoculated with *Fusarium moniliforme var. subglutinans*. *Phytopathology* **74**:438-444.

Beyer, W., Hoxter, H., Medaner, T., Sander, E., Geiger, H.H. (1993) Indirect ELISA for quantitative assessment of *Fusarium spp*. in rye. *Journal of Plant Diseases and Plant Protection* **100**:278-284.

Biazon, L., Meirelle, P.G., Ono, M.A., Itano, E.N., Taniwaki, M.H., Sugiura, Y., Ueno, Y., Hirooka, E., Ono, E.Y.S. (2006) Development of polyclonal antibodies against *Fusarium verticillioides* exo-antigens. *Food and Agricultural Immunology* **17**:69-77.

Blakeslee, G.M., Oak, S.W. (1979) Significant mortality associated with pitch canker infection of slash pine in Florida. *Plant Disease Reporter***63**:1023-1025.

Bloomberg, G.W. (1971) Diseases of Douglas-fir seedlings caused by *Fusarium oxysporum*. *Phytophathology* **61**:467-470.

Bloomberg, W.J. (1981) Disease caused by *Fusarium* in forest nurseries, in: P. E. Nelson, et al. (Eds.), *Fusarium*: Disease, Biology and Taxonomy. The Pennsylvania State University Press, London. pp. 178-187.

Bonello, D., Gordon, J.N., Storer, W. (2001) Systemic induced resistance in Monterey pine. *Forest Pathology* **31**:99-106.

Bossi, R., Dewey, F.M. (1992) Development of a monoclonal antibody-immunodetection assay for *Botrytis cinerea*. *Plant Pathology* **41**:472-482.

Brill, L.M., McClary, R.D., Sinclair, J.B. (1994) Analysis of two ELISA formats and antigen preparations using polyclonal antibodies against *Phomopsis longicolla*. *Phytopathology* **84**:173-179.

Britz, H., Coutinho, T.A., Gordon, T.R., Wingfield, M.J. (2001) Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Mexico. *South African Journal of Botany* **67**:609-614.

Brookhouser, L.W., Peterson, G.W. (1971) Infection of Austrian, Scot and Ponderosa pine by *Diplodia pinea*. *Phytopathology* **61**:409-414.

Budge, G.E., Shaw, M.W., Colyer, A., Pietravalle, S., Boonham, N. (2009) Molecular tools to investigate *Rhizoctonia solani* distribution in soil. *Plant Pathology* **58**:1071-1080.

Burgess, T., Wingfield, M.J. (2002) Quarantine is important in restricting the spread of exotic seedborne tree pathogens in the southern hemisphere. *International Forestry Reviews* **4**:56-65.

Cahill, D.M., Hardham, A.R. (1994) Exploitation of zoospore taxis in the development of a novel dipstick immunoassay for the specific detection of *Phytophthora cinnamomi*. *Phytopathology* **84**:193-200.

Calderon, C., Ward, E., . Freeman, J., McCartney, H.A. (2002a) Detection of airborne fungal spores sampled by rotating-arm and Hirst-type spore traps using polymerase chain reaction assays. *Journal of Aerosol Science* **33**:283-296.

Calderon, C., Ward, E., Freeman, J., McCartney, H.A. (2002b) Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. *Plant Pathology* **51**:303-310.

Carey, W.A., Kelley, W.D. (1994) First report of *Fusarium subglutinans* as a cause of late-season mortality in longleaf pine nurseries. *Plant Disease* **78**:754.

Carlucci, A., Colatruglio, L., Frisullo, S. (2007) First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Disease* **91**:1683.

Carver, T.L.W., Inger-Morris, S.M., Thomas, B.J. (1996) Early interactions during powdery mildew infection.*Plant Pathology* **73**:632-639.

Chen, F.S., Chen, J.W., Zhao, S., Gan, Z.B., Luo, X.C., Zhou, Q. (2000) Characterisation of chicken antisera raised against *Aspergillus spp*. by enzyme linked immunosorbent assay. *Nahrung* **44**:323-327.

Chou, C.K.S. (1978) Penetration of young stems of *Pinus radiata* by *Diplodia pinea*. *Physiological Plant Pathology* **13**:189-192.

Clark, M.F., Adam, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. *Journal of General Virology* **34**:475-483.

Clark, S.L., Gordon, T.L. (1998) Susceptibility of eleven California conifers to pitch canker, caused by *Fusarium subglutinans f. sp. pini*. *Phytopathology* **88**:17-22.

Clausen, C.A. (1997) Immunochemical detection of wood decay fungi- An overview of techniques developed from 1986 to the present. *International Biodeterioration and Biodegradation* **39**:133-143.

Conradie, E., Swart, W.J., Wingfield, M.J. (1990) Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**:43-49.

Consolandi, C., Castiglioni, B., Bordoni, R., Busti, E., Battaglia, C., De Bellis, G. (2001) Development of oligonucleotide arrays to detect mutations and polymorphisms. *Minerva Biotechnological* **13**:261-268.

Correll, J.C., Gordon, T.R., McCain, A.H., Fox, J.W., Koehler, C.S., Wood, D.L., Schultz, M.E. (1991) Pitch canker disease in California: pathogenicity, distribution, and canker development on Monterey pine (*Pinus radiata*).*Plant Disease* **75**:676-682.

Coutinho, T.A., Steenkamp, E.T., Mongwaketsi, K., Wilmot, M., Wingfield, M.J. (2007) First outbreak of pitch canker in a South African pine plantation. *Australasian Plant Pathology* **36**:256-261.

Crous, J.W. (2005) Post establishment survival of *Pinus patula* in Mpumalanga, one year after planting. *Southern African Forestry Journal* **205**:3 - 8.

da Silva, D.W., Tambourgi, D.V. (2010) IgY: A promising antibody for use in immunodiagnostic and in immunotherapy. *Veterinary Immunology and Immunopathology* **135**:173-180.

de Saeger, S., van Peteghem, C. (1996) Dipstick enzyme immunoassay to detect *Fusarium* T-2 toxin in wheat. *Applied and Environmental Microbiology* **62**:1880-1884.

de Wet, J., Burges, T., Slippers, B., Preisig, O., Wingfield, B.D., Wingfield, M.J. (2003) Multiple gene genealogies and microsatellite markers reflect relationship between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. *Mycological Research* **107**:557-566.

de Wet, J., Wingfield, M.J., Coutinho, T.A. (2000) Characterisation of *Sphaeropsis sapinea* isolates from South Africa, Mexico, and Indonesia. *Plant Disease* **84**:151-156.

De-Bernardis, F., Molinari, A., Boccanera, M., Stringaro, A., Robert, R., Senet, J.M., Arancia, G. (1994) Modulation of cell surface-associated mannoprotein antigen expression in experimental *candidal vaginitis*. *Infection immunology* **62**:509-519.

Dewey, F.M., Grose, M.J., Twiddy, D.R., Phillips, S.I., Wareing, P.W. (1992) Development of a quantitative monoclonal antibody-based immunoassay for *Humicola lanuginosa* and comparison with conventional assays. *Food and Agriculture Immunology* **4**:153-168.

Dewey, F.M., MacDonald, M.M., Phillip, S.I., Priestley, R.A. (1990) Development of monoclonal-antibody-DIP-STICK immunoassays for *Penicillium islandicum* in rice grains. *Journal of Genetics and Microbiology* **136**:753-760.

Dewey, F.M., Meyer, U. (2004) Rapid, quantitative tube immunoassays for on-site detection of *Botrytis, Aspergillus* and *Penicillium*antigens in grape juice. *Analytical Chimical Acta* **513**:11-19.

Dick, M.A., Dobbie, K. (2002) Species of *Fusarium* on *Pinus radiata* in New Zealand. *New Zealand Plant Protection* **55**:58-62

Dwinell, D.L., Barrows-Broaddus, J., Kuhlman, E.G. (1985) Pitch canker: A disease complex of southern pines. *Plant Disease* **69**:270-276.

Dwinell, L.D. (1978) Susceptibility of southern pines to infection by *Fusarium moniliforme var. subglutinans*. *Plant Diseases Reports* **62**:108-111.

Dwinell, L.D., Fraedrich, S.W. (1998) Contamination of Pine Seeds by the Pitch Canker Fungus, National proceedings of the forest and conservation nursery associations,general technical report, USDA, Forest Service, Southern Research Station. pp. 41-42.

Edel, V., Steinberg, C., Gautheron, N., Alabouvette, C. (2000) Ribosomal DNA-targeted oligonucleotide probe and PCR assay specific for *Fusarium oxysporum*. *Mycological Research* **104**:518-526.

EI-Nashaar, H.M., Moore, L.W., George, R.A. (1986) Enzyme-linked immunosorbent assay quantification of initial infection of wheat by *Gaeumannomyces graminis var. tritici* as moderated by biocontrol agents *Phytopathology* **76**:1319-1322.

Enebak, S.A., Stanosz, R. (2003) Responses of conifer species of Great Lakes region of North America to inoculation with the pitch canker pathogen *Fusarium circinatum*. *Forest Pathology* **33**:333-338.

Faria, C.B., Abe, C.A.L., Novais da Silva, C.N., Tessmann, D.J., Barbosa-Tessmann, I.P. (2012) New PCR assays for the identification of *Fusarium verticillioides, Fusarium*

subglutinans, and other species of the *Gibberella fujikuroi* complex. *International Journal of Molecular Sciences* **13**:115-132.

Fraaije, B., Lovell, D.J., Rohel, E.A., Hollomon, D.W. (1999) Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction. *Jonournal of Applied Microbiology* **86**:701-708.

Gan, Z., Marquardt, R.R., Abramson, D., Clear, R.M. (1997) The characterisation of chicken antibodies raised against *Fusarium spp*. by enzyme-linked immunosorbent assay and immunoblotting. *International Journal of Food Microbiology* **30**:191-200.

Garrido, C., Carbú, M., Fernández-Acero, F.J., Boonham, N., Colyer, A., Cantoral, J.M., G., B. (2009) Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using real-time PCR. *Plant Pathology* **58**:43–51.

Gerik, J.S., Lommel, S.A., Huisman, O.C. (1987) A specific serological staining procedure for *Verticillium dahliae* in cotton root tissue. *Phytopathology* **77**:261-265.

Glancy, H., Palfreyman, J.W., Button, D., Bruce, A., King, B. (1990) An immunological method for the detection of *Lentinus lepideus* in distribution poles. *Journal of the Institute of Wood Science* **12**:59-64.

Godoy-Lutz, G., Kuninaga, S., Steadman, J.R., Powers, K. (2008) Phylogenetic analysis of *Rhizoctonia solani* subgroups associated with web blight symptoms on common bean based on ITS-5.8S rDNA. *Journal of General Plant Pathology* **74**:32-40.

Gordon, T.R., Okamoto, D., Storer, A.J., Wood, D.L. (1998) Susceptibility of five landscape pines to pitch canker, caused by *Fusarium subglutanans f. sp. pini*. *HortScience* **33**:866-871.

Gordon, T.R., Storer, A.J., Wood, D.L. (2001) The pitch canker epidemic in California. *Plant Disease* **85**:1128-1139.

Grimm, C., Geisen, R. (1998) A PCR-ELISA for the detection of potential fumonisin producing *Fusarium species*. *The Society for Applied Microbiology* **26**:456-462.

Hahn, R., Werres, S. (1997) Development of a dot immunobinding assay to detect *Phytophthoru spp*. in naturally dark-rooted woody plants. *Annals of Applied Biology***130**:453-466.

Hayashi, Y., Arie, T., Yoneyama, K., Yamaguchi, I. (1998) Characterization of the antigenic determinant on *Fusarium oxysporum* recognized by a genus-specific monoclonal antibody. *The Journal of General and Applied Microbiology* **44**:43-47.

Hepting, G.H., Roth, E.R. (1953) Host relations and spread of pitch canker disease. *Phytopathology* **43**:475.

Hitchcock, P., Gray, T.R.G., Frankland, J.C. (1997) Production of a monoclonal antibody specific to *Mycena galopus* mycelium. *Mycological Research* **101**:1051-1059.

Holtz, B.A., Karu, A.E., Weinhold, A.R. (1994) Enzyme-linked immunosorbent assay for detection of *Thielaviopsis basicola*. *Disease Detection and losses* **84**:977-983.

Homechin, M., Pizzinatto, M.A., Menten, J.O.M. (1986) Health of seeds of *Pinus elliottii* var. *elliottii* and *Pinus taeda* and pathogenicity of *Fusarium oxysporum* on *Pinus elliottii* var. *elliottii* seedlings. *Plant Pathology* **12**:102-112.

Hudson, L., Hay, F.C. (1980) Molecular weights and special properties of immunoglobulins and antigens of immunological interest Practical Immunology Blackwell Scientific Publications, London. pp. 347.

Inami, K., Yoshioka, C., Hirano, Y., Kawabe, M., Tsushima, S., Teraoka, T., Arie, T. (2010) Real-time PCR for differential determination of the tomato wilt fungus, *Fusarium oxysporum f.sp lycopersici*, and its races. *Journal of General Plant Pathology* **76**:116-121.

Iyer, M.S., Cousin, M.A. (2003) Immunochemical detection of *Fusarium* species in cornmeal. *Journal of Food Protection* **66**:451-456.

Johnson, R.D., Johnson, L., Kohomoto, K., Otani, H., Lane, C.R., Kodama, M. (2000) A polymerase chain-reaction based method to specifically detect *Alternaria alternata apple pathotype (A. mali)*, the causal agent of Alternaria blotch of apple. *Phytopathology* **90**:973-976.

Juhasova, G., Adamcikova, K., Kobza, M. (2006) *Sphaeropsis* tip blight disease of Austrian pine in urban greenery. *Horticultural Science* **33**:11-15.

Karpovich-tate, N., Dewey, F.M. (2001) Quantification of *Ulocladium atrum* in necrotic plant tissues by monoclonal antibody-based enzyme-linked immunosorbent assay. *Mycological Research* **5**:567-574.

Kaur, J., Robinboro, K.V., Thampi, K.R., Varsheney, M.G., Ramansuri, C. (2007) Immunochromatographic dipstick assay format using gold nanoparticles labeled protein-hapten conjugate for the detection of atrazine. *Environmental Science and Technology* **41**:5028-5036.

Kennedy, R., Wakeham, A.J., Cullington, J.E. (1999) Production and immunodetection of ascospores of *Mycosphaerella brassicicola*: ringspot of vegetable crucifers. *Plant Pathology* **48**:297-307.

Kimishima, E., Kobayashi, Y. (1990) Use of dot immunobinding assay DIBA and immunoenzymatic staining method IESM for rapid detection of *Phytophthora-spp*. *Research Bulletin of the Plant Protection Service of Japan* **26**:7-14.

Kitagawa, T., Sakamoto, Y., Furumi, K., Ogura, H. (1989) Novel enzyme immunoassays for specific detection of *Fusarium oxysporum f. sp. cucumerinum* and for general detection of various *Fusarium* pecies. *Phytopathology* **79**:162-165.

Kong, P., Hong, C.X., Tooley, P.W., Ivors, K., Garbelotto, M., Richardso, P.A. (2004) Rapid identification of *Phytophthora ramorum* using PCR-SSCP analysis of ribosomal DNA ITS-1. *Letters in Applied Microbiology* **38**:433-439.

Kuhlman, E.G., Dianis, S.D.a., Smith, T.K. (1982) Epidemiology of pitch canker disease in a loblolly pine seed orchard in North Carolina. *Phytopathology* **72**:1212-1216.

Kuo, K.C., Hoch, H.C. (1996) Germination of *Phyllosticta anpelicida* pycnidiospores: prerequisite of adhesion to the substratum and the relation of substratum wettability.*Fungal Genetics and Biology* **20**:18-29.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**:680-685.

Landeras, E., Garcia, P., Fernandez, Y., Braña, M., Fernandez-Alonso, O., Mendez-Lodos, S., Perez-Sierra, A., Leon, M., Abad-Campos, P., Berbegal, M., Beltran, R., Garcia-Jimenez, J., Armengol, J. (2005) Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus spp*. in Northern Spain. *Plant Disease* **89**:1015.

Lievens, B., Rep, M., Thomma, B.P.H.J. (2008) Recent developments in the molecular discrimination of formae speciales of *Fusarium oxysporum*. *Pest Management* **64**:781-788.

Lievens, B., Thomma, B.P.H.J. (2005) Recent developments in pathogen detection arrays: implementations for fungal plant pathogens and use in practice. *Phytopathology* **95**:1374-1380.

Livak, K.J. (1999) Allelic discrimination using fluorogenic probes and the 5′ nuclease assay. *Genetic Analysis* **14**:43-149.

Loos, R., Fourrier, C., Iancu, G., Gordon, T. (2009) Sensitive detection of *Fusarium circinatum* in pine seed by combining an enrichment procedure with real-time polymerase chain reaction using dual-labeled probe chemistry. *Phytophathology* **99**:582-590.

Luchi, N., Capretti, P., Maresi, G., Fedicci, M. (2007) Detection of *Diplodia pinea* in asymptomatic pine shoots. *Forest Pathology* **70**:111-114.

Lunchi, N., Prates, N., Simi, L., Pazzagli, M., Capretti, P., Scala, A., Slippers, B., Pinzani, P. (2011) High-Resolution Melting Analysis: a new molecular approach for the early detection of *Diplodia pinea* in Austrian pine. *Fungal Biology* **115**:715-723.

Maresi, G., Lunchi, N., Pinzani, P., Pazzagli, M., Capretti, P. (2007) Detection of *Diplodia pinea* in asymptomatic pine shoots and its relation to normalized insolation index. *Forest Pathology* **37**:272-280.

McCain, A.H., Koehler, C.S., Tjosvold, S.A. (1987) Pitch canker threatens California pines. *California Agriculture* **41**:22-23.

McCartney, H.A., Foster, S.J., Fraaije, B.A., Ward, E. (2003) Molecular diagnostics for fungal plant pathogens. *Pest Management Science* **59**:129-142.

Meyer, U., Dewey, F.M. (2000) Efficacy of different immunogens for raising monoclonal antibodies to *Botrytis cinerea*. *Mycological Research* **104**:979-988.

Michael, A., Meenatchisundaram, S., Parameswari, G., Subbraj, T., Selvakumaran, R., Ramalingam, S. (2010) Chicken egg yolk antibodies (IgY) as an alternative to mammalian antibodies. *Indian Journal of Science and Technology* **3**:468-474.

Mitchell, R., Wingfield, M., Hodge, G., Steenkamp, E., Coutinho, T. (2012) Selection of *Pinus spp*. in South Africa for tolerance to infection by the pitch canker fungus. *New Forests* **43**:473-489.

Mitchell, R.G., Hongwane, P., Olivier, R. (2009) Pitch canker outbreak on mature *Pinus patula* at Roburnia, Sabie: Komatiland Forests, Unpublished internal research report 4/2009.

Mitchell, R.G., Steenkamp, E.T., Coutinho, T.A., Wingfield, M.J. (2011) The pitch canker fungus, *Fusarium circinatum*: implications for South African forestry. *Journal of Forest Science* **73**:1-13.

Mojca, N. (2003) Production of antibodies in chickens. *Food Technology and Biotechnology* **41**:259-267.

Muramoto, M., Dwinell, L.D. (1990) Pitch canker of *Pinus luchuensis* in Japan. *Plant Disease* **74**:530.

Murray, M.G., Thompson, W.F. (1980) Rapid isolation of high molecular weight plantDNA. *Nucleic Acid Research***8**: 4321-4326.

Nazar, R.N., Hu, X., Schmid, J., Culham, D., Robb, J. (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiological and Molecular Plant Pathology* **31**:1-11.

Nelson, P.E. (1981) Life cycle and epidemiology of *Fusarium oxysporum*, in: M. E. Mace, et al. (Eds.), Fungal Wilt Diseases of Plants. Academic Press, New York. pp. 51-88.

Nicholson, R.L., Kunoh, H. (1995) Early interaction, adhision and establishment of the infection court by *Erysiphe graminis*. *Phytopathology* **73**:609-615.

Notermans, S., Soentoro, P.S.S. (1986) Immunochemical relationship of extra-cellular polysaccharide antigens produced by different mould species. *Antonie van Leeuwenhoek* **52**:393-401.

O'Donnell, K., Kistler, H.C., Cigelnik, E., Ploetz, R.C. (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Applied Biological Sciences* **95**:2044-2049.

Paoletti, E., Danti, R., Strati, S. (2001) Pre- and post-inoculation water stress affects *Sphaeropsis sapinea canker* length in *Pinus halepensis* seedlings. *Forest Pathology* **31**:209-218.

Papp, A.C., Pinsonneault, J.K., Cooke, G., Sadee, W. (2003) Single nucleotide polymorphism genotyping using allele-specific PCR and fluorescence melting curves. *Biological Techniques* **34**:1068-1072.

Parent, J.G., Belanger, F., Desjardins, S., Brisson, J.D. (1985) Dot-immunobinding for detection of tomato mosaic virus and potato virus X infecting greenhouse tomatoes. *Phytoprotection* **66**:53-57.

Pascholati, S.F., Yoshioka, H., Kunoh, H., Nicholson, R.L. (2002) Preparation of the infection by *Erysiphe graminis f. sp. hordei* cutinase is a component of the conidial exudate.*Physiological and Molecular Plant Pathology* **41**:53-59.

Pauly, D., Chacana, P.A., Calzado, E.G., Brembs, B., Schade, R. (2011) IgY technology: extraction of chicken antibodies from egg yolk by polyethylene glycol (PEG) precipitation.*Journal of Visualized Experiments* **51**:1-6.

Peltonen, S. (1995) Comparison of xylanase production by fungal pathogens of barley with special reference to *Bipolaris sorokinia*. *Mycological Research* **99**:717-723.

Perez-Sierra, A., Landeras, E., Leon, M., Berbegal, M., Garcia-Jimenez, J., Armengol, J. (2007) Characterisation of *Fusarium circinatum* from *Pinus spp*. in northern Spain. *Mycological Research* **3**:832-839.

Petrini, O., Fisher, P.J. (1988) A comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. *Mycological Society* **91**:233-238.

Polson, A., Coetzer, T.H.T., Kruger, J., von Maltzahn, E., van der Merwe, K.J. (1985) Improvements in the isolation of IgY from the yolks of eggs laid by immunised hens. *Immunological Investigations***14**: 323-327

Polson, A., von Wechmar, M.B., van Regenmortel, M.H. (1980) Isolation of viral IgY antibodies from yolks of immunised hens. *Immunology Communications* **9**:475.

Porter, B., Wingfield, M.J., Coutinho, T.A. (2009) Susceptibility of South African native conifers to the pitch canker pathogen, *Fusarium circinatum*. *South African Journal of Botany* **75**:380-382.

Recorbet, G., Steinberg, C., Olivain, C., Edel, V., Trouvelot, S., Dumas-Gaudot, E., Gianinazzi, S., Alabouvette, C. (2003) Wanted: Pathogenesis-related marker molecules for *Fusarium oxysporum*. *Phytopathology* **159**:73-92.

Ricker, R.W., Marois, J.J., Dlott, R.M. (1991) Immunodetection and quantification of *Botrytis cinerea* on harvested wine grapes. *Phytopathology* **81**:404-411.

Runion, G.B., Cade, S.C., Bruck, R.I. (1993) Effects of carbofuran and thiabendazole on incidence of pitch canker of loblolly pine. *Plant Disease* **77**:166 -169.

Sakamoto, J.M., Gordon, T.R. (2006) Factors influencing infection of mechanical wounds by *Fusarium circinatum* on Monterey pines (*Pinus radiata*). *Plant Pathology* **55**:130-136.

Schade, R., Erhard, M. (2001) Short introduction to hens' humoral immune system in: R. Schade, et al. (Eds.), Chicken Egg Yolk Antibodies, Production and Application. IgY-technology Springery, New York. pp. 1-8.

Schilling, A.G., Moller, E.M., Geiger, H. (1996) Polymerase chain reaction-based assay for species-specific detection of *Fusarium culmorum, Fusarium graminearum*, and *Fusarium avanaceum*. *Molecular Plant Pathology* **86**:515-522.

Schmechel, D., Simpson, J.P., Lewis, D.M. (2005) The production and characterisation of monoclonal antibodies to the fungus *Aspergillus versicolor*. *Indoor Air* **15**:11-19.

Schmechel, D., Simpson, J.P., Reponen, T., Grinshpun, S.A., Lewis, D.M. (2003) Limitations of monoclonal antibodies for monitoring of fungal aerosols using *Penicillium brevicompactum* as a model fungus. *Journal of Immunological Methods* **283**:235-245.

Schweigkofler, W., O'Donnell, K., Garbelotto, M. (2004) Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Applied and Environmental Microbiology* **70**:3512-3520.

Skottrup, P.D., Nicolaisen, M., Justesen, A.F. (2008) Towards an on-site pathogen detection using antibody-based sensors. *Biosensors and Bioelectronics* **24**:339-348.

Skottrup, S., Frokiaer, S., Hearty, S., O'kennedy, R., Hejgaard, J., Nicolaisen, M., Justesen, A.F. (2007) Monoclonal antibodies for the detection of *Puccinia striiformis* urediniospores. *Mycological research* **3**:332-338.

Smith, H., Wingfield, P.W., Coutinho, T.A. (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic *Pinus spp*. and *Eucalyptus spp*. in South Africa. *South African Journal of Botany* **62**:86-88.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**:76-85.

Smith, S.N., Snyder, W.C. (1975) Persistence of *Fusarium oxysporum f.sp. vasinfectum* in fields in the absence of cotton. *Phytopathology* **65**:190-196.

Stanosz, G.R., Blodgett, J.T., Smith, D.R., Kruger, E.L. (2001) Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phythologist* **149**:531-538.

Stanosz, G.R., Smith, D.R., Gutmiller, M.A. (1996) Characterisation of *Sphaeropsis sapinea* from the west central United States by means of random amplified polymorphic DNA marker analysis. *Plant Disease* **80**:1175-1178.

Stanosz, G.R., Smith, D.R., Leisso, R. (2007) *Diplodia* shoot blight and asymptomatic persistence of *Diplodia pinea* on or in stems of jack pine nursery seedlings. *Forestry Pathology* **37**:145-154.

Stewart, J.E., Abdo, Z., Dumroese, R.K., Klopfenstein, N.B., Kim, M.S. (2012) Virulence of *Fusarium oxysporum* and *F. commune* to Douglas-fir (*Pseudotsuga menziesii*) seedlings. *Forest Pathology***42**:220-228.

Storer, A.J., Gordon, T.R., Clark, S.L. (1998) Association of pitch pine canker fungus, *Fusarium subglutinans f. sp. pini* with Monterey pine seeds, and seedlings in California. *Plant Pathology* **47**:649-654.

Storer, A.J., Wood, D.L., Gordon, T.R. (2002) The epidemiology of pitch canker of Monterey pine in California. *Forest Science* **48**:646-700.

Swart, W.J., Wingfield, M.J. (1991) Biology and control of *Sphaeropsis sapine*a on *Pinus species* in South Africa. *Plant Disease* **75**:761-766.

Thornton, C.R., Gilligan, C.A. (1999) Quantification of the effect of the hyperparasite *Trichoderma harzianum* on the saprotrophic growth dynamics of *Rhizoctonia solani* in compost using a monoclonal antibody-based ELISA. *Mycological Research* **103**:443-448.

Thornton, C.R., Pitt, D., Wakley, G.E., Talbot, N.J. (2002) Production of a monoclonal antibody specific to the genus *Trichoderma* and closely related fungi, and its use to detect *Trichoderma spp.* in naturally infested composts. *Microbiology* **148**:1263-1279.

Toussoun, T.A. (1981) Prologue., in: P. E. Nelson, et al. (Eds.), *Fusarium*: Disease, Biology and Taxonomy. Pennsylvania State University Press, University Park, U.S. pp. 3-8.

Towbin, N., Staehlin, T., Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.*Proceedings of the National Academy of Science of the United States of AmericaUSA***76**: 4350-4354.

Validov, S.Z., Kamilova, F.D., Lugtenberg, B.J.J. (2011) Monitoring of pathogenic and nonpathogenic *Fusarium oxysporum* strains during tomato plant infection. *Microbial Biotechnology* **1**:82-88.

Vidhyasekaran, P. (2008) Fungal pathogenesis in plants and crops: Molecular biology and host defense mechanisms CRC Press, London. pp. 45**.**

Viljoen, A., Wingfield, M.J., Kemp, G.H.J., Marasas, W.F.O. (1995) Susceptibility of pines in South Africa to the pitch canker fungus *Fusarium subglutinans f. sp. pini*. *Plant Pathology* **44**:877-882.

Viljoen, A., Wingfield, M.J., Marasas, W.F.O. (1994) First report of *Fusarium subglutinans f. sp. pin*i on pine seedlings in South Africa. *Plant Disease* **78**:309-312.

Vivas, M., Zas, R., Solla, A. (2012) Screening of maritime pine (*Pinus pinaster*) for resistance to *Fusarium circinatum*, the causal agent of Pitch Canker disease. *Forestry Oxford* **85**:185-192.

Ward, E., Foster, S.J., Fraaije, B.A., McCartney, H.A. (2004) Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology* **145**:1-16.

Waterman, A.M. (1943) *Diplodia pinea*, the cause of disease for hard pines. *Phytopathology***33**:1018-1031.

Weber, K., Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Jonournal of Biochemistry* **244**:4406-4412.

Weiland, J.J., Sundsbak, J.L. (2000) Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rRNA gene. *Plant Disease* **84**:475-482.

Wingfield, M.J. (1980) Association of *Diplodia pinea* with a root disease of pines in South Africa. *Plant Disease* **64**:221-223.

Wingfield, M.J., Coutinho, T.A., Roux, J., Wingfield, B.D. (2002a) The future of exotic plantation forestry in the tropics and southern hemisphere: lessons from pitch canker. *Southern African Forestry Journal* **195**:79-82.

Wingfield, M.J., Jacobs, A., Coutinho, T.A., Ahumada, R., Wingfield, B.D. (2002b) First report of the pitch canker fungus, *Fusarium circinatum*, on pines in Chile. *New Disease Reports* **4**:16.

Wingfield, M.J., Hammerbacher, A., Ganley, R.J., SteenkampA, E.T., Gordon, T.R., Wingfield, B.D., Coutinho, T.A. (2008) Pitch canker caused by *Fusarium circinatum* - a growing threat to pine plantations and forests worldwide. *Australasian Plant Pathology Society* **37**:319-334.

Wingfield, M.J., Knox-Davies, P.S. (1980) Association of *D. pinea* with root disease of pines in South Africa. *Plant Disease* **64**:221-223.

Wingfield, M.J., Slippers, B., Roux, J., Wingfield, B.D. (2001) Worldwide movement of exotic forest fungi, especially in the tropics and the Southern Hemisphere. *BioScience* **51**:134-140.

Wright, S.F., Morton, J.B., Sworobuk, J.E. (1987) Identification of a vesicular-arbuscular mycorrhizal fungus by using monoclonal antibodies in an enzyme-linked immunosorbent assay. *Applied and Environmental Microbiology* **53**:2222-2225.

Wycoff, K.L., Ayers, A.R. (1990) Monoclonal antibodies to surface and extracellular antigens of a fungal plant pathogen, *Phytophthora* megasperma f.sp. glycinea, recognise specific carbohydrate epitopes. *Physiological and Molecular Plant Pathology* **37**:55-79.

Wycoff, K.L., Jellison, J., Ayers, A.R. (1987) Monoclonal antibodies to glycoprotein antigens of a fungal plant pathogen, *Phytophthora megasperma f.sp. glycinea*. *Plant Physiology* **85**:508-515.

Xue, B., Goodwin, P.H., Annis, S.L. (1992) Phytotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. *Physiological and Molecular Plant Pathology* **41**:179-188.

Zhang, H., Wang, S. (2008) Development of flow-through and dip-stick immunoassays for screening of sulfonamide residues. *Journal of Immunochemical Methods* **337**:1-6.

Zwolinski, B., Swart, W.J., Wingfield, M.J. (1990) Economic impact of a post-hail outbreak of dieback induced by *Sphaeropsis sapinea*. *European Journal of Pathology* **20**:405-411.