Chapter 3: The Mycoflora Associated with Diseased Plants and Seeds of *Oryza sativa* (Rice)

Chapter 4: Pathogenicity of *Fusarium anthophilum* and *Fusarium fujikuroi* Associated with Bakanae Disease of Rice

Chapter 5: Responses of 54 Rice Cultivars and Lines to Bakanae Disease Caused by *Fusarium fujikuroi* and *Fusarium anthophilum*

Chapter 6: Comparative Effectiveness of Two Different Fungicides in Controlling Bakanae Disease of Rice

Student number: 4559-679-4

I declare that Studies of the impact of Mycoflora Associated with *Oryza sativa* (rice) in South Africa is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. Ethics clearance for this work was sought and approved by the Ethics Committee of University of South Africa (UNISA).

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SIGNATURE DATE

(MR MT HOSSAIN)

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I dedicate this thesis to Almighty God who gave me an opportunity to undertake this research for the doctoral thesis in Environmental Science. I dedicate this thesis in the sacred memory of my beloved late parents Moulavi A.H. Mohammed Makim Uddin and Shumarun Nesa and my wife's late parents Dr. M. Jamshed Uddin and Khodeza Khatun. In the same vein, this thesis is also dedicated to the late Mr. Nalini Mohan Das, Head Master and late Mr Matiur Rahman, teacher of Mathematics and Physics, Nandina M.H.K. Pilot High School, Jamalpur, Bangladesh, who encouraged me to study science, my late brother Mohammed Ashraf Hossain, Mohammed Mosharraf Hossain, Mohammed Muazzem Hossain and my late sister Firoza Hossain and Razia Hossain. All of them would be extremely happy to see this achievement, if they were alive.

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PUBLICATIONS

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- Hossain, M.T., Rong, I.H. and Modise, D.M. 2013**.** Studies on the pathogenicity of *Fusarium anthophilum* and *Fusarium fujikuroi* associated with bakanae disease of rice.

- Hossain, M.T., Rong, I.H. and Modise, D.M. 2013. Reaction of some rice cultivars and lines to bakanae disease caused by *Fusarium anthophilum* and *Fusarium fujikuroi.*

- Hossain, M.T., Rong, I.H. and Modise, D.M. 2013**.** Production of mycotoxins by strains of *Fusarium anthophilum* and *Fusarium fujikuroi* isolated from rice plants with bakanae disease.

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List of Symbols and Abbreviations

α	Alpha
%	Percentage
°C	Degree Celsius
mℓ	Millilitre
µg	Microgram
mg	Milligram
g	Gravity
g	Gram
kg	kilogram
µM	Micrometer
Lux	SI unit of illuminance
AAL	Toxin produced by fungus *Alternaria alternata*
AFLP	Amplified fragment length Polymorphism
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of Variance
BLAST	Basic local alignment search tool
Ca	Calcium
CO_2	Carbon dioxide
C_3 Crop-	Photo synthetically less efficient crop cultivar
C_4 Crop	Photo synthetically more efficient crop cultivar
CI	Consistency indices
DNA	Deoxyribonucleic acid
DEAE Sephadex	Used as a support for ion-exchange chromatography
DNTPS	Used for DNA amplification
EF 1	Elongation factor 1
EF 2	Elongation factor 2
ELEM	Leukoencephalomalacia
FAO	Food and Agricultural Organization of United Nations
FB_1	Mycotoxin fumonisin B_1
FB_2	Mycotoxin *f*umonisin B_2
FB_3	Mycotoxin fumonisin B_3
FIESC	*Fusarium incarnatum- equiseti* Species Complex
h	Hour
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ISTA	International Seed Test Association
ITS	Internal transcribed spacer
LSD	Least Significant Differences
MAFFT	Multiple Sequence Alignment Program
$Mgcℓ_2$	Magnesium chloride
MLST	Multilocus Sequence Typing
MPA	Mating Population A (*Fusarium verticillioides)*

MPC	Mating Population C (*Fusarium fujikuroi)*
MPD	Mating Population D (*Fusarium proliferatum)*
MRC	Medical Research Council, Tygerberg, Cape Town, South Africa
NaOCℓ	Sodium hypochlorite solution
NCBI	A Gene Bank
NERICA	New Rice for Africa
ND	Not detected
O_2	Oxygen
OPA	Ophthaldihyde
PAUP	Phylogenetic analysis using parsimony
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PIC	Paired Iron Chromatography
PPRI	National Collection Of Fungi Plant Protection Research Institute, Agriculture Research Council, Pretoria, South Africa
RAPD	Random Amplified Polymorphic DNA
RI	Retention indices
RNA	Ribosomal nucleic acid
SSA	Sub- Saharan Africa
TBR	Tree bisection reconnection
TEF	Translation Elongation Factor
T-2	MycotoxinTrichothecene 2
USA	United States of America
USDA	Department of Agriculture of United States of America
UK	United Kingdom
Voℓ	Volume

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SUMMARY

The objective of this research was to investigate the occurrence of mycoflora in rice plants and rice seeds in South Africa and their negative impact.

A total of six species of *Fusarium* were isolated from diseased rice plants and rice seeds and identified as *F. anthophilum, F. chlamydosporum, F. compactum, F. equiseti, F. fujikuroi* and *F. semitectum*. In the translation elongation factor data set, *Fusarium equiseti* isolates grouped together within the *F. incarnatum* - *equiseti* Species Complex (FIESC). The isolates from rice clustered together in a single clade with the *F. equiseti* and *F. incarnatum* isolates forming two separate sub-clades.The isolates of *F. equiseti* present a new phylogenetically distinct species in FIESC.

In the pathogenicity tests, isolates of both *F. anthophilum* and *F. fujikuroi* caused bakanae disease to rice plants. Fifty four rice cultivars and lines were tested by the standardized test tube inoculation method for resistance and susceptibility against bakanae isolate of *F. anthophilum* and the bakanae isolate *of F. fujikuroi.* None of the rice cultivars and lines was found to be resistant to bakanae isolates of *Fusarium* spp.

The fungicide, benomyl was found to be most effective as a seed treatment for controlling bakanae disease of rice due to isolates of both *F. anthophilum* and *F. fujikuroi*. Thiram was found to be the least effective fungicide for controlling bakanae disease of rice caused by isolates of both the *Fusarium* spp.

Apart from *Fusarium* species, other fungi that were also isolated from diseased rice plants and rice seeds were identified as *Alternaria alternata, Alternaria longipes, Cochliobolus miyabeanus, Nigrospora sphaerica, Phoma eupyrena, Phoma jolyana, Phoma sorghina* and *Pithomyces* sp.

In mycotoxin tests, the isolates of both *F. anthophilum* and *F. fujikuroi* produced moniliformin. None of the isolates of *F. anthophilum* and *F. fujikuroi* produced fumonisins.

This research is important as it identifies many fungal species in rice plants and seeds in South Africa for the first time. Currently, there is very little literature that makes reference to such findings under South African conditions. In addition, this investigation unravels previously unknown information on the resistance of rice to bakanese disease. Finally, information is provided on the effectiveness of commonly used fungicides (benomyl and thiram) to control rice diseases. This knowledge is crucial information that is useful to plant pathologists, the farming community and the scientists that are involved in strategies of fighting or reducing rice diseases so as to help contribute to food security.

Key terms:

Mycoflora; *Oryza sativa*; *Fusarium* species; Fungi; Translation Elongation Factor (TEF); Pathogenicity; Disease control; Mycotoxins; Impact

CHAPTER 1

GENERAL INTRODUCTION

1.1 Rice cultivation and consumption

Rice (*Oryza sativa* L.) is the world's most extensively cultivated cereal crop after wheat and it constitutes the staple diet of more than 50% of the world's population in terms of cultivation and consumption (Makun *et al*., 2007; FAO, 2012). It is grown in over one hundred countries on more than 150 million hectares of land and about 90% of the annual harvest is consumed in Asia and about 5-6% of rice produced, is traded on the world market (Bayer, 1997). While production and consumption are concentrated in Asia, rice is also grown in specific regions in North and South America, Africa, Australia and Europe (Grist, 1959; Sarla and Mallikarjuna Swamy, 2005; FAO, 2012). In the year 2011, about 721 million tonnes of paddy rice was produced in the world. China produced 28% of world production of rice, India 21%, Indonesia 9% and Bangladesh 7% (FAO, 2012).

China and India are the top two producers of rice in the world. These two countries consume the majority of rice produced domestically leaving little to be traded internationally. South Africa depends on imports to supply its domestic rice requirements (Wailes *et al*., 2000). In South Africa, per capita rice consumption was projected to range from 10.7 to 11.7 kg/annum over the period 1998-2010. Total consumption of rice was projected to grow from 450 000 tonnes in 1998 to 554 000 tonnes by 2010, an annual growth of 1.8%. Wailes *et al.* (2000) reported that until 1982, South Africa had to import 80-90% of its requirement of rice from United States of America (USA). Later on, the importance of the USA as a rice supplier to the country declined substantially. In the marketing years 2010/2011, South Africa imported 721 000 tonnes of rice and Thailand, with almost 80% of the market share, was South Africa's major importing country (USDA, 2012).

The production of rice in South Africa has a history of over 92 years. During 1920, rice was produced in the KwaZulu - Natal coastal area and also in the lowveld area

of Mpumalanga on a small scale for home consumption. Areas in North West and Northern Cape provinces are also suitable for rice production although most of the soil is currently under maize production.The Makhathini Flats of North Eastern part of KwaZulu - Natal is one of the most promising areas for cultivation of rice due to the warm climate and good rainfall (Dippenaar and Clarke, 1979; Dreyer, 2004). Rice has been grown intermittently on a commercial scale in South Africa since 1943 (Dippenaar and Clarke, 1979). However, cultivation of rice as a South African crop has not been particularly successful. Its cultivation was introduced to the former Bophuthatswana homeland, presently part of the North West, Free State and Northern Cape provinces of South Africa by Taiwanese rice experts in 1981 and produced about 253 tonnes of paddy rice during the 1986/87 crop season (Kung, unpublished). At the same time, cultivation was also introduced in KaNgwane, now Mpumalanga province, and in the Makhathini Flats in the KwaZulu - Natal province.

Research in rice production and especially the development of new cultivars may change the prospects for this crop in future in South Africa. The development of photosynthetically more efficient cultivars with a so called C4 cycle as opposed to the current C3 cycle, together with new innovations in breeding for nutritional value may change the future of this crop. In comparison to C3 crops such as rice, C4 crops have higher yields; reduced water loss and increased nitrogen use efficiency particularly when grown in hot and dry environments (Sage, 2004; Hibberd *et al*., 2008). The African rice species (*Oryza glaberrima* Steud) has great importance for cross breeding with the high yielding *Oryza sativa* (*O. sativa*) of South East Asia (Van der Walt, 2004).

Oryza glaberrima (*O. glaberrima*) has many important traits such as weed competitiveness, drought tolerance and ability to respond to low input conditions. It is also resistant to various pests and diseases of rice (Sarla and Mallikarjuna Swamy 2005). *Oryza glaberrima* has been grown in Africa for more than 3000 years and is well adapted to the African environment (Van der Walt, 2004).The cultivar “NERICA” (New Rice for Africa), a cross breed rice cultivar may offer a new dimension to rice production in South Africa through its drought tolerance and high yield potentials (Dreyer, 2004; Van der Walt, 2004). It is reported by the African Rice Centre (2009)

that some of the NERICA varieties have high yield advantage over *O. glaberrima* and *O. sativa* parents either through superior weed competitiveness, drought tolerance, and pest or disease resistance or simply through high yield potentials. Apart from that, the grain quality of some of the NERICAs is often better than that of their parents. For example, the protein content of some of the NERICAs is 25% higher than that of the Asian rice in the market (African Rice Centre, 2009). All these advantages combined can significantly contribute to food security and improved nutrition in Sub-Saharan Africa.

In the first 4-5 years of rice cultivation in the former Bophuthatswana homeland (now part of North West province, Free State province and Northern Cape province), no fungal diseases were reported by farmers. Later farmers complained about diseases of rice in the fields due to monoculture of the crop. Plant diseases caused by mycoflora (fungi) were reported to have reduced the quantity and quality of plant produce (Padmanabhan, 1973; Marin Sanchez and Jimenez Diaz, 1982; Copco and Karaca, 1983; Desjardins *et al*., 2000). Agrios (2005) also alluded to the fact that diseased plants may sometimes contain mycotoxins and render the produce unfit for consumption.

1.2 Fungi associated with rice

The fungus *Cochliobolus miybeanus* Ito and Kuribayashi Drechsler ex Dastur is known to cause the brown spot disease of rice (Padmanabhan, 1973). In 1942, an epidemic of the disease in Bengal (presently Bangladesh and West Bengal State of India) had caused yield losses of 40-90% and was largely responsible for the Bengal famine of 1943 (Padmanabhan, 1973; Webster and Gunnell, 1992; Agrios, 2005). Two million people died from starvation due to the famine. The fungus *Fusarium fujikuroi* Nirenberg has been reported as highly virulent and is the only *Fusarium* species involved in causing bakanae disease of rice in Malaysia and Indonesia (Zainudin *et al*., 2008; Heng *et al*., 2011), in Italy (Amatulli *et al*., 2010) and in Pakistan (Iqbal *et al*., 2011). It was reported by Ito and Kimura (1931) that bakanae disease caused a 20% loss in Hokkaido and losses were also reported to be as high as 40% in the Kinki-Chugoku Region of Japan (Anonymous 1975). The bakanae

disease has been reported as seed-borne disease and has become major limiting factor in rice production throughout the world (Ghazanfar *et al*., 2013).Therefore, in terms of rice production and diseases caused by fungi to rice have negative impact. Gorter (1977) reported some diseases of rice caused by fungi in South Africa but there was no information on the toxicity and their negative impact on human and animal health.

1.3 Mycotoxicosis

Fungal mycoflora not only cause diseases of plants, as well as they can produce mycotoxins (Desjardins *et al.,* 2000; Fandohan *et al*., 2003; Murray *et al*., 2005; Desjardins, 2006; Magan and Aldred, 2007; Nayaka *et al.,* 2011; Van Rensburg, 2012; Cao *et al*., 2013) that have been implicated in a variety of illnesses and clinical syndromes in humans and animals. Mycotoxins are secondary fungal metabolites that cause diseases known collectively as mycotoxicoses after ingestion, inhalation or through direct contact with the toxin and mycotoxicosis may manifest as acute or chronic disease ranging from rapid tumor formation to death (Peraica *et al.,* 1999; Murray *et al*., 2005).

Mycotoxicosis is caused or facilitated by one or a combination of genetic and environmental factors such as the consumption of plant material with sufficient quantities of toxin to cause disease but also by genetical as well as physiological susceptibility of the consumer (Marasas and Nelson, 1987).The toxins act as allergens or irritants and some might inhibit other micro-organisms with penicillin being a good example (Keller *et al*., 2005).The occurrence of mycotoxins in food is often caused by pre-harvest contamination of the material by toxigenic fungi that are plant pathogens.The mycotoxin moniliformin (sodium or potassium salt of 1-hydroxycyclo but-1-ene-3, 4-dione) is a highly toxic compound which was first isolated by Cole *et al*. (1973) from maize that had been inoculated with *Fusarium moniliforme* Sheldon (presently known as *F. verticillioides* Sacc. Nirenberg).The mycotoxin caused pathological lesions on heart tissue of experimental animals and ultimately caused the death of the animal (Burmeister *et al*., 1980; Allen *at al*., 1981; Zhao *at al*., 1993).The production of high levels of moniliformin (5000 to 7000µg /g)

by isolates of *F.* f*ujikuroi* and (200 to 5000µg/g) by isolates of *F. proliferatum* (Matsushima) Nirenberg was obtained from rice (Desjardins *et al.,* 1997). Fumonisins are mycotoxins with cancer promoting activity produced by *F. moniliforme*, was first reported in rats (Gelderblom *et al*., 1988) and there have been many publications dealing with the importance of fumonisins in maize in South Africa (Rheeder *et al.,* 2002). Fumonisin B_1 has been associated with a high incidence of oesophageal cancer in people living in China, Italy and South Africa (Murray *et al*., 2005).

1.4 Environmental factors in mycotoxin production

Hussein and Brasel (2001) postulated that the production of mycotoxins depend on the surrounding intrinsic and extrinsic environments and they vary greatly in their severity, depending on the organisms infected and their susceptibility, metabolism and defence mechanisms.The key environmental determinants pre- and post-harvest are water availability and temperature (Sinha, 1995; Magan *et al*., 2003; Magan and Olsen, 2004). Kungu (2004) reported that a few strains of mycoflora can nevertheless produce mycotoxins at some point during their growth even under sub-optimal growth conditions or limited nutrients.The conditions that favour production of one type of mycotoxin may not be favourable for production of another type. For example, aflatoxin production by *Aspergillus* is dependent on concentrations of oxygen, carbon dioxide, zinc and copper, as well as physical location, while ochratoxin production relates to air exhaustion (Kungu, 2004). Plant stress factors such as high temperatures, drought, poor fertilization and competition for nutrients are some of the aspects known to increase the mycotoxin production in the field (Rodrigues, 2008). Various environmental factors in mycotoxin production were categorized by D'Mello and MacDonald (1997) as physical factors such as temperature, relative humidity and insect infestation, chemical factors include the use of pesticides and or fertilizers and biological factors based on the interactions between the colonizing toxigenic fungal species and substrates. Marin *et al*. (1995) reported on the effect of different water activities (a_w) of 0.968, 0.956, 0.944 and 0.925 and temperature of 25^0C and 30^0C on colonization and production of fumonisin B_1 (FB_1) and B_2 (FB_2) by *F. proliferatum* and *F. moniliforme* on maize

grain. Isolates of both *F. proliferatum* and *F. moniliforme* grew faster with increasing water activities and best at 30^0C. Isolates of both *F*. *moniliforme* and *F*. *proliferatum* produced more FB_1 than FB_2 regardless of water activity or temperature. Very little FB_1 and FB_2 were produced at 0.925 a_w, with maximum produced at 0.956 and 0.968 a_w at both temperatures tested.The growth of *Fusarium* spp. and production of fumonisin result from the complex interaction of several factors such as biotic and /or abiotic. Water stress and temperature are the most relevant environmental factors that influence fungal growth and mycotoxin production (Charmley *et al*., 1994). It has been suggested that a differential regulation of fumonisin biosynthesis in the isolates of *F. verticillioides* and *F. proliferatum* might be related to their different host range and play an ecological role and additionally, environmental condition leading to water stress might result increased risk of fumonisin contamination of maize caused by *F. verticillioides* (Marin *et al*., 2010). The fungal infection and fuminisin accumulation during the development and drying of white maize kernels are related to environmental factors (Cao *et al*., 2013).

1.5 The research question and specific aims and objectives

The only information available on the mycoflora associated with rice in South Africa was given by Gorter (1977). However, he did not provide any recommendation on control measures.There was also no information on toxigenicity caused by fungi associated with rice in South Africa. The research question that was addressed in this research study was to gain an increased understanding of the mycoflora associated with rice plants and rice seeds in South Africa and their negative impact on the health of rice plants which has effect on rice production due to diseases caused by fungi and negative impact on human and animal health due to mycotoxins produced by fungi. Accurate species identification of plant pathogenic and toxigenic fungi is very important. Any error can have far reaching consquenecs impacting on biodiversity assessment, ecological studies and management decisions (Bortolus, 2008). It was important to devise methods to control or minimize their harmful effects or impacts on this economically important food crop. It was important to know whether the fungi could produce mycotoxins such as fumonisins and moniliformin. Production of mycotoxins by fungi has potential harmful effects on the plant health,

human health and animal health. It was also important to understand the identity of mycotoxins produced by the fungi associated with rice, a staple diet in many countries including South Africa. This knowledge would enable better strategies to combat the incidences of occurrence of these toxins and to able to ameliorate their harmful effects. In order to answer these questions a series of experiments were conducted and important informations were obtained on the mycoflora associated with rice, a staple diet in many countries including South Africa with available resources and fund.The specific objectives were:

- isolate and identify the mycoflora (fungi) associated with diseased rice plants and rice seeds of *Oryza sativa* (rice) that should be of concern because of their pathogenicity and toxigenic potential in South Africa.
- test the pathogenicity of some isolates of fungi associated with important diseases of rice in South Africa.
- screen the rice cultivars and lines for resistance to control important rice diseases of rice as part of economic and environmental friendly method of disease control.
- evaluate the effectiveness of seed treatment fungicides to control important diseases of rice.
- determine the potential for production of mycotoxins such as fumonisins and moniliformin by selected isolates of specific species of fungi with available resource and fund.
- assess the risks and potential harmful impact of mycoflora on the health of rice plants and risks on health of humans and animals due to mycotoxins.

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CHAPTER 2

LITERATURE REVIEW

2.1 Mycotoxins

Fungal mycoflora cause diseases to plants as well as they could affect negatively human and animal health with their mycotoxins (Marasas *et al*., 1984; Hussein and Brasel, 2001; Zaccardelli *et al.,* 2006; Glenn, 2007; Wulff *et al.,* 2010; Van Rensburg, 2012). Mycotoxins are secondary metabolites of fungi that cause diseases known collectively as mycotoxicoses. Mycotoxicosis is caused or facilitated by one or a combination of genetic and environmental factors such as the consumption of plant material with sufficient quantities of toxins to cause disease but also by genetical as well as physiological susceptibility of the consumer (Marasas and Nelson, 1987). Production of mycotoxins by fungi and their impacts on animal and human health have also been reported by other research scientists (Scott *et al.,* 1987; Desjardins *et al.,* 2000; Desjardins, 2006; Nayaka *et al*. 2011). Murray *et al.* (2005) reported that mycotoxins are involved in a variety of illnesses and clinical syndromes in humans and animals. Mycotoxicosis is caused after ingestion, inhalation or through direct contact with the toxin. Mycotoxicosis is manifested as acute or chronic disease ranging from rapid tumor formation to death (Murray *et al*., 2005). Mycotoxins act as allergens or irritants and some mycotoxins might inhibit other micro-organisms such as fungi or bacteria, penicillin being a good example (Keller *et al*., 2005). Mycotoxins can cause chronic toxicity in humans and animals that eat contaminated foods or crops depending on the quantities produced and consumed (Barrett, 2000; Lezar and Barros, 2010). Mycotoxins that adversely affect human and animal health are found in every variety of food and feed which supports fungal growth including pre and post harvest crop such as maize, rice, sorghum, wheat, oil seeds and edible nuts (Marasas *et al*., 1984; Marasas and Nelson, 1987; Sydenham and Thiel, 1996; Glenn, 2007; Magan and Aldred, 2007; Reddy *et al.,* 2008; Van Rensburg, 2012;

Cao *et al*., 2013). However, mycotoxin contamination is less reported for rice than many other cereal crops (Tanaka *et al*., 2007; Reddy *et al*., 2008). It has been reported that there are economic losses due to *Fusarium* mycotoxins contamination of grains, foods and feedstuffs (Charmley *et al*., 1994; Mellor, 2003). It is estimated that 25% of the world's food crops are affected by mycotoxins each year. Mycotoxins have very negative roles in contamination of grains, foods and feedstuffs and mycotoxin produced by fungi pose a continuous challenge to the safety and quality of food commodities in South Africa (Lezar and Barros, 2010).

Fungi can produce a wide range of mycotoxins such as aflatoxin, fumonisins, trichothecenes, ochratoxin,, zearalenone and moniliformin on food raw materials under environmental conditions which are conducive to growth and the key environmental determinants pre- and post-harvest are water availability (water activity, a_w) and temperature (Sinha, 1995; Peraica *et al*., 1999; Magan *et al*., 2003; Magan and Olsen, 2004; Glenn, 2007; Morgensen *et al*., 2010). Production of mycotoxins by *Fusarium* species have been reported in wheat, oats, barley, corn and rice as pre-harvest (Pitt, 1995; Amadi and Adeniyi, 2009). Aflatoxins occur in food raw materials such as nuts, maize, sorghum and rice and two major *Aspergillus* species such *A. flavus* and *A. parasiticus* produce aflatoxins (Peraica *et al*., 1999; Magan and Olsen, 2004).The aflatoxins are carcinogenic; therefore, contamination of food materials with the toxin has significant economic impact (Peraica *et al*., 1999; Magan and Olsen, 2004). Makun *et al*. (2007) showed rice samples contaminated with aflatoxin B_1 at cocentrations of between 20-1642µg/kg with a mean value of 200.19µg/kg. Fumonisins are produced by various *Fusarium* species including *F. anthophilum*, *F. fujikuroi*, *F. konzum, F. proliferatum* and *F. semitectum* (Desjardins, 2006; Lezar and Barros, 2010). *Aspergillus niger* aggregate strains isolated from harvested maize in Portugal have also been reported to produce FB_2 (Soares *et al*., 2013). Fumonisin producing *Fusarium* species can infect a wide variety of crop plants such as barley, maize, millet, rice, sorghum and wheat; thus, fumonisins could occur in a wide variety of foods and feeds (AOAC, 1994; Dutton, 1996; Desjardins *et al*., 1997; Rheeder *et al*., 2002; Desjardins, 2006). However, Maize is well known important component in the food and feed chain and fumonisins have been commonly found in a wide range of maize-based food in different parts of the world

(Doko and Visconti, 1994; Sanchis *et al*., 1994; Velluti *et al.,* 2001; Magan and Olsen, 2004). Trichothecenes are mycotoxins (carcinogenic type A and B) produced mostly by the members of the *Fusarium* genus and other genera (e.g.*Trichoderma, Trichothecium,* M*yrothecium* and *Stachybotry*s) can also produce these compounds (Peraica *et al*., 1999; Magan and Olsen, 2004). Contamination of cereal grains such as wheat, maize and rice with trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV) has been a particular problem for the flouring milling and baking industry and brewing industry (Magan and Olsen, 2004). Ochratoxins are produced by *Aspergillus* and *Penicillium* strains and found on a wide variety of foods including cereal products, nuts, spices, coffee, raisins and wine as well as on all kinds of food commodities of animal origin in many countries of the world (Speijers and Van Egmond, 1993, Peraica *et al.,* 1999; Magan and Olsen, 2004). Ochratoxin A (OTA) has received particular attention because of its association with cancer-promoting activity (IARC, 1993; Magan and Olsen, 2004). Zearalenone is produced by mainly by *F. graminearum* species complex in wheat, maize, sorghum, rice, barley and compounded feeds (Peraica *et al.,* 1999; Magan and Olsen, 2004; Makun *et al*., 2007). Zearalenone and its derivatives produce estrogenic effects such as infertility, vulval oedema, vaginal prolapse and mammary hypertrophy in females and feminization of males- atropy of testes and enlargement of mammary glands in various animal species (Peraica *et al.,* 1999). Moniliformin was initially isolated as a culture extract of *F. moniliforme;* hence the name moniliformin (Burmeister *et al.,* 1979; Glenn, 2007).There is international legislation for aflatoxin, fumonisins, trichothecene and ochratoxin. For moniliformin, there is no legislation at present. Important mycotoxins found in different food commodities and their negative impacts on human and animal health are shown in Table 2.1(adapted from Smith and Henderson, 1991; GASGA, 1997; Peraica *et al.,* 1999; Trucksess and Pohland, 2001; Magan and Olsen, 2004; Desjardins, 2006).

2.1.1 Moniliformin

Cole *et al*. (1973) first revealed the mycotoxin moniliformin (sodium or potassium salt of 1-hydroxycyclobut-1-ene-3, 4-Dione) a highly toxic compound was first isolated from maize that was inoculated with *Fusarium moniliforme* (*F. verticillioides*).The

production of moniliformin by strains of *Fusarium* species isolated from rice in Taiwan, Japan and Philippines with bakanae disease that resembled *Fusarium fujikuroi* (Nirenberg, 1976) was confirmed by Marasas *et al.* (1986). Marasas *et al.* (1986) also confirmed the production of moniliformin by strains of *F. anthophilum* (A. Braun) Wollenweber isolated from soil debris in New Guinea and from wheat in the USA.The production of moniliformin by various *Fusarium* species including *F. anthophilum*, *F. chlamydosporum* (Wollenweber and Reinking), *F. equiseti* (Corda) Sacc. Sensu Gordon and *F. semitectum* (Berkeley and Ravenel) has been reported by Farber *et al*. (1988).The production of moniliformin has been detected from various species of *Fusarium* (Chelkowski *et al.,* 1990), however, moniliformin was not detected from the isolates of *F. chlamydosporum*, *F. compactum* (Wollenweber) W.L. Gordon and *F. equiseti.* Marasas *et al*. (1991) reported the production of moniliformin from 19 isolates of *F. nygamai* (Burgess and Trimboli) from millet in Africa (4300- 18200μg/g) and 15 isolates from rice with bakanae disease (2300-19300μg/g) caused by uncertain taxa.The production of moniliformin (5000-7000μg/g) by isolates of *F. fujikuroi* and (200-5000μg/g) by isolates of *F. proliferatum* was reported from rice (Desjardins *et al*., 1997). These isolates of *F. fujikuroi* could not produce fumonisins (Desjardins *et al*., 1997).

Mycotoxin moniliformin caused death by developing pathological lesions on heart tissue of experimental animals (Kriek *et al*., 1977; Burmeister *et al*., 1980; Allen *et al.*, 1981). Zhao *et al*. (1993) confirmed that moniliformin might induce lesions of myocardium in rats and mice and could cause death of the experimental animals. However, Desjardins (2006) reported that although moniliformin had efficacy in experimental animals, the mycotoxin had not been associated with any chronic or fatal animal disease outbreak.

2.1.2 Fumonisins

Fumonisins are a group of mycotoxins produced by *F. verticillioides (F. moniliforme*) strain MRC 826 isolated from maize in the Transkei region of South Africa (Gelderblom *et al*., 1988). Gelderblom *et al*. (1988) first revealed the cancer promoting activity of the mycotoxin fumonisin in rats, produced by this strain of *F.*

verticillioides. Fumonisin has been reported as hepatoxic to ducklings and rats (Colvin and Harrison, 1992). Fumonisins B_1 and B_2 have toxicological significance, while the others such as B_3, B_4, A_1 and A_2 occur in very low concentrations (Peraica *et al*., 1999). Rheeder *et al*. (2002) explained the different aspects of novel, carcinogenic mycotoxin fumonisins. Fumonisin B_1 is responsible for leukoen cephalomalacia (ELEM) disease in horses (Kellerman *et al*., 1990; Wilson *et al*., 1990).Thiel *et al*. (1991) and Gelderblom *et al.* (1992) confirmed that fumonisin could cause ELEM disease in horses. Harrison *et al.* (1990) revealed that Fumonisin B_1 is responsible for pulmonary edema in pigs and other studies (Ross *et al*., 1990; Gelderblom *et al*., 1992; Nelson *et al*., 1994; Sala *et al*., 1994; Moheshwar *et al*., 2009) also showed fumonisin B_1 is responsible for pulmonary edema in pigs. Sydenham *et al.* (1991) and Thiel *et al*., (1992) suggested that fumonisins could be associated with oesophageal cancer in humans. Murray *et al*. (2005) also reported that fumonisins were associated with a high incidence of oesophageal cancer in people living in South Africa, China and Italy. Domijan *et al.* (2005) reported that International Agency for Research on Cancer (IARC) evaluated FB_1 as a possible carcinogen (Group 2B) to humans. Recenty, production of fumonisin B_1 has been shown by13 of the 32 strains of *Fusarium fujikuroi* isolated from rice in the Philippines (Cruz *et al.,* 2013). Based on phylogenetic studies, these scientists showed that the strains of *F. fujikuroi* are distinct from *F. proliferatum* strains. However, they could not find any relationship between fumonisin production and pathogenicity.

2.2 Mycoflora (fungi) in rice plants and rice seeds

Different species of fungi such as *Fusarium* species and other fungi have been reported from diseased rice plants and rice seeds and from other crop plants (Table 2.2) and these fungi negatively affect human and animal health (Ou, 1985; Desjardins *et al*., 2000; Hussein and Brasel, 2001; Murray *et al*., 2005; Desjardins *et al*., 2006; Makun *et al*., 2007; Nayaka *et al*., 2011).Therefore, accurate species identification of plant pathogenic and toxigenic fungi in association with rice plants and rice seeds is very important. Any error can have far reaching consequences

impacting biodiversity assessment, ecological studies and management decisions (Bortolus, 2008).

2.2.1 *Fusarium* spp.

Fusarium species have been reported from different geographical regions of the world to cause diseases of rice and they produce mycotoxins that negatively affect human and animal health (Marin-Sanchez and Jimenez –Diaz, 1982; Desjardins *et al*., 1997; Desjardins *et al*., 2000; Desjardins, 2006; Zainudin *et al*., 2008; Wulff *et al*., 2010; Heng *et al*., 2011 Van Rensburg; 2012; Latiffah *et al.,* 2013).

2.2.1.1 *Fusarium anthophilum*

Gordon (1956) first reported the isolation of *F. anthophilum* from copra in Trinidad without any information on pathogenicity and toxigenicity of the fungus. However, Gordon (1960) revealed that *F. anthophilum* has the ability to produce substances with gibberellin like biological properties and this was confirmed by Marasas *et al*. (1984).

Abdel-Hafej *et al*. (1987) reported the isolation of *F. anthophilum* from paddy grains in Egypt but no information on pathogenicity and toxigenicity of the fungus. Nelson *et al*. (1992) confirmed the isolation of *F. anthophilum* from rice grains in Australia and from pearl millet in Zambia but without any information on pathogenicity and toxigenicity. Nyvall *et al*. (1999) also confirmed the isolation of *F. anthophilum* from both cultivated rice (*Oryza sativa*) and wild rice (*Zizania palustris*) with head blight symptoms in Minnesota, USA but pathogenicity was demonstrated. Mantecon *et al*. (1984) reported the isolation of *F. anthophilum* from wheat in Argentina and demonstrated that the fungus could reduce the height of wheat seedlings. This was the first report on the pathogenicity of the fungus *F. anthophilum*. Mansuetus *et al*. (1997) and Sreenivasa *et al.* (2008) confirmed the isolation of *F. anthophilum* from sorghum in Tanzania and from sorghum in India respectively. However, they could not provide any information on the pathogenicity of the fungus. Sala *et al*. (1994) confirmed the isolation of *F. anthophilum* from maize in Spain. Montazeri and

Mojaradi (2008) isolated *F. anthophilum* from barnyard grass (*Echinochloa crus-galli*) in Guilan, the North of Iran. Several other studies suggest the association of *F. anthophilum* with other crop plants and a negative impact on plant health (Sharfun and Mustaq, 2006; Moghaddam and Taherzadeh, 2007). Mustaq and Sharfun (2006) found *F. anthophilum* to be the causal agent of wilting and damping off of sunflower in Pakistan. Moghaddam and Taherzadeh (2007) confirmed *F. anthophilum* as the causal pathogen of crown and root rots of hazelnut in Iran.

Marasas *et al*. (1984) revealed that *F. anthophilum* could cause toxicity to rabbit skin and Desjardins (2006) also suggested that the fungus could cause toxicity to rabbit skin. Summerbell *et al*. (1988) reported the isolation of *F. anthophilum* along with *F. solani* (Mart.) Sacc. from disseminated cutaneous *Fusarium* infection in a leumic patient in Japan but unfortunately they could not establish the degree to which each of the two *Fusarium* species contributed to the infection. The production of beauvericin, fumonisins and moniliformin by *F. anthophilum* has been reported (Marasas *et al*., 1986; Desjardins, 2006).

2.2.1.2 *Fusarium chlamydosporum*

Nath *et al*. (1970) first isolated *F. chlamydosporum* from rice seeds in India. Desjardins *et al*. (2000) and Broggi and Molto (2001) isolated *F. chlamydosporum* from rice seeds in Nepal and Argentina respectively.They suggested that *F. chlamydosporum* is important as a seed-borne pathogen of rice. Marasas *et al.* (1987) also confirmed the isolation of *F. chlamydosporum* from other cereal crop plants such as maize and sorghum but without any information on pathogenicity of the fungus. However, Sharfun and Mushtaq (2006) suggested that *F. chlamydosporum* is also an important pathogen of other crops than cereals. Sharfun and Mushtaq (2006) reported *F. chlamydosporum* as the causal pathogen of collar rot, wilting and stunting of sunflower in Pakistan. Chaudhury *et al.* (2009) isolated of *F. chlamydosporum* from lentils and suggested that the fungus can cause root rot and wilting at different growth stages of lentil. Palmero *et al*. (2009) reported *F. chlamydosporum* as the causal pathogen of pre-and post-emergence damping-off on melon and tomato. These were confirmed by pathogenicity tests.

Segal *et al*. (1998) reported the isolation of *F. chlamydosporum* from an infected patient with plastic anemia. Naiker and Odhav (2004) and Desjardins (2006) confirmed the association of *F. chlamydosporum* in human infection. Rabie *et al*. (1978) and Marasas *et al*. (1984) isolated *F*. *chlamydosporum* from millet linked to onyalai disease, hemorrhagic syndrome in Namibia. Cultures of *F. chlamydosporum* caused toxicity to ducklings and rats in experiments but these cultures of *F. chlamydosporum* could not produce the characteristics symptoms of onyalai disease.

Rabie *et al*. (1978) reported the production of moniliformin, a mycotoxin from *F. chlamydosporum* (previously known as *F. fusarioides*). Dejardins (2006) confirmed that *F. chlamydosporum* can produce moniliformin. Savard *et al*. (1990) revealed the production of chlamydosporal, a new mycotoxin with relatively low toxicity from *F. chlamydosporum*. Solfrizzo *et al*. (1994) and Desjardins (2006) confirmed the production of chlamydosporal from *F. chlamydosporum*.

2.2.1.3 *Fusarium compactum*

Marasas and Van Rensburg (1986) and Desjardins (2006) isolated *F. compactum* from maize in South Africa. Schj (2002) was also able to isolate this species from maize in Zambia. These studies provided no information on relative pathogenicity and toxigenicity stratus of this fungus. Van Wyk *et al*. (1987) reported the isolation of *F. compactum* from wheat in South Africa and suggested that the fungus could cause root and crown rot disease. Rochacic and Hudec (2005) confirmed the isolation of *F. compactum* from wheat kernels in the Slovak Republic. Gonzalez and Trevathan (2000) in the USA showed that *F. compactum* was responsible for root and crown rot of wheat grown on the upper coastal plains of the Mississipi. Sarbjeet *et al*. (2000) reported that the fungus had caused head-blight of wheat. Onyike and Nelson (1992) were also able to isolate *F. compactum* from sorghum grains in Lesotho and Nigeria. Somorin and Bankole (2010) reported the isolation *F. compactum* from rice seeds in Nigeria. This was the first report of *F*. *compactum* in association with rice. However, Wang *et al*. (2004) suggested that the fungus not only caused disease to cereal crops but it could also cause wilting disease of *Gossypium* in Australia.

Marasas and van Rensburg (1986) investigated mycotoxicological aspects on maize and groundnuts from the endemic areas of Mseleni joint disease in the KwaZulu-Natal area of South Africa. They found that strains of *F. compactum* isolated from maize were toxic to ducklings but they did not produce the characteristics symptoms of Mseleni disease. Desjardins (2006) suggested that the fungus *F. compactum* may not be responsible for Mseleni disease. Cole *et al*. (1988) confirmed the isolation of trichothecenes from *F. compactum* that was suspected in the aetiology of a major intoxication of sandhill cranes in Western Texas, USA.These scientists further reported that trichothecenes of *F. compactum* were the cause of fatal toxicoses and death of 9500 wild birds in the USA. Nelson *et al*. (1990) confirmed that trichothecenes of *F. compactum* isolated from waste peanuts was the cause of fatal toxicoses of sandhill cranes. Desjardins (2006) also suggested that trichothecenes of *F. compactum* was the cause of this environmental disaster. Lezar and Barros (2010) also suggested that *F. compactum* has the ability to produce fumonisins.

2.2.1.4 *Fusarium equiseti*

Joffe and Palti (1967) isolated *F. equiseti* from soils and a wide range of host plants in Israel but no information on pathogenicity and toxigenicity of the fungus was reported. Marin-Sanchez and Jimenez-Diaz (1982) isolated *F. equiseti* from mature rice plants in Spain and showed that fungus can cause discoloration in vascular tissues of the plants. Singh and Khare (1983) reported *F. equiseti* as a seed-borne pathogen of rice in India. Kang and Rattan (1983) isolated *F. equiseti* from rice plants with sheath rot symptoms in India. In a pathogenicity test, Kang and Rattan (1983) found that the fungus really caused sheath rot symptoms on rice plants. Reckhause and Adamon (1986) also reported that *F. equiseti* can cause disease on rice plants. Van Wyk *et al*. (1987) isolated *F. equiseti* from wheat plants in South Africa. Marasas *et al*. (1987) confirmed the isolation of *F. equiseti* from maize, wheat, sorghum and other crop plants such as pasture and from soils in South Africa. Onyike and Nelson (1992) also isolated *F. equiseti* from sorghum grains in Lesotho, Nigeria and Zimbabwe. Sriovasta *et al*. (2008) suggested that *F. equiseti* not only causes disease on cereal crops, it can also cause disease on other crop such as wilting disease of guava seedligs.

Fusarium equiseti was found in association with myelomonocytic leukemia (Wray *et al*., 1979; Marasas, *et al*., 1984). Marasas *et al*. (1984) reported that *F. equiseti* from mouldy rice straw caused Degnala disease of water buffaloes in India. *Fusarium semitectum* was also present in the mouldy straw and therefore the roles of these two *Fusarium* species in Degnala disease remained unclear. However, Hokonohara *et al*. (2003) and Desjardins (2006) suggested that *F. equiseti* is not responsible for Degnala disease because it was alleviated by tetracycline treatment of affected water buffaloes and that suggested that the disease is not primarily a mycotoxicosis.

O'Donnell *et al.* (2009) reported that *F. equiseti* is a member of the *Fusarium incarnatum-equiseti* species complex (FIESC) and this comprises at least 20 mycoses among 28 reciprocally monophyletic lineages resolved by multilocus molecular phylogenetics.

Hossein *et al.* (1991) and Desjardins (2006) reported that *F*. *equiseti* has the ability to produce the mycotoxins trichothecenes, zearalenone and moniliformin.

2.2.1.5 *Fusarium fujikuroi*

Ito and Kimura (1931) revealed that *Gibberella fujikuroi* (Sawada) Ito (anamorph: *Fusarium moniliforme*) was the causal agent of bakanae disease of rice in Japan. Thomas (1931) isolated *G. fujikuroi* from diseased rice plants in India and confirmed that the fungus could cause bakanae disease of rice plants. Reyes (1939) isolated *G. fujikuroi* in the Philippines and confirmed the fungus as the causal organism of bakanae disease of rice. *Gibberella fujikuroi* was found to cause bakanae disease in India and Thailand (Pavgi and Singh, 1964; Kanjanasoon, 1965). Fifty four glutinous and 78 non-glutinous varieties of rice were examined to find possible resistant varieties for controlling bakanae disease of rice in Thailand. He found only 2 varieties moderately resistant and both were non-glutinous. Booth (1971) considered the causal organism of bakanae disease as *Fusarium moniliforme* (presently known as *F. verticillioides*). However, Nirenberg (1976) differentiated it as a separate species, *Fusarium fujikuroi*. Nelson *et al.* (1983) did not accept *F. fujikuroi* as a separate species but included it in *F. moniliforme* as the "short-chained" type of *F.*

moniliforme. Mia and Zaman (1973) isolated *G. fujikuroi* from rice plants with bakanae symptoms in Bangladesh and confirmed the fungus as causal organism of bakanae disease of rice. Ou (1985) also described *G. fujikuroi* as the causal pathogen of bakanae disease of rice. The most common symptom caused by *G. fujikuroi* is the elongation of the stems of diseased plants. Bakanae symptoms may be observed in the seedbeds as well as in the field. Infected seedlings are taller than normal seedlings and are thin and pale yellow-green in colour. Diseased seedlings usually die after transplanting although healthy seedlings may be infected in the fields after transplanting. Bakanae disease has been reported from other rice producing countries such as Philippine (Lee *et al*., 1989), Malaysia and Indonesia (Zainudin *et al*., 2008) and Italy (Amatulli *et al*., 2010). Desjardins *et al*. (1997) confirmed the isolations of *F. verticillioides* and *F. proliferatum* from bakanae infected rice seedlings from various geographic areas. Desjardins *et al.* (2000) suggested that bakanae disease of rice is caused by one or more *Fusarium* species and is a complex of disease symptoms including root rot and crown rot, abnormal elongation of stems, wilting, stunting and formation of adventitious roots at nodes on the lower portions of stems. Several studies (Yamanaka and Honkura, 1978; Sun and Snyder, 1981; Ou, 1985; Webster and Gunnell, 1992) confirmed that these symptoms are characteristic of bakanae disease of rice due to *G. fujikuroi*. Carter *et al.* (2008) showed *F. fujikuroi* as the causal pathogen of bakanae disease of rice (*Oryza sativa* L.), water grass (*Echinochloa oryzoidis*) and barnyard grass (*Echinochloa crus-galli*) in California, USA. Amatulli *et al.* (2010) isolated several *Fusarium* species associated with rice plants with bakanae symptoms in Italy. However, in pathogenicity tests, they found only *F. fujikuroi* could cause bakanae disease of rice plants. Wulff *et al.* (2010) found that *F. fujikuroi*, *F. proliferatum and F. verticillioides* could cause bakanae disease of rice plants. Heng *et al.* (2011) suggested that both *F. fujikuroi and F. proliferatum* have the ability to cause bakanae disease of rice plants.

Realms *et al.* (1997) reported that *F. fujikuroi* with a known concentration of moniliformin caused sudden death syndrome in chicks and turkey poults. *Fusarium fujikuroi* culture material containing moniliformin could cause toxicity to turkey poults (Bermudez *et al*., 1997; Bermudez and Ledoux, 1997; Kubena *et al*., 1997).

Desjardins *et al*. (2000) and Desjardins (2006) confirmed that *F. fujikuroi* could produce moniliformin and fusaric acid. Proctor *et al*. (2004) found that only one of six strains of *F. fujikuroi* was able to produce fumonisins. Webster and Gunnell (1992) and Desjadins (2006) also reported that *F. fujkuroi* could produce gibberellic acid. A summary on *F. fujikuroi* has been shown in Table 2.3.

2.2.1.6 *Fusarium semitectum*

Fusarium semitectum has been isolated from different crop plants such as mung beans, soya beans, paddy rice, sorghum grains and different types of beans, sesame seeds and cassava in Thailand (Pitt *et al*., 1994), from redfleshed dragon fruit (*Hylocereus polyrhizus*) and from several vegetable fruits in Malaysia (Masratul Hawa *et al.,* 2010; Latiffah *et al*., 2013) and from rice in Argentina and Paraguay (Sergio *et al.,* 1997). Broggi and Molto (2001) confirmed that *F. semitectum* was a seed borne pathogen of rice in Argentina. Butt *et al*. (2011) isolated *F. semitectum* from stored rice grains in Pakistan and it was a seed borne pathogen. Madbouly *et al*. (2012) isolated *F. semitectum* from rice and maize seeds in Egypt and showed the fungus as a seed-borne. Little information is available on the pathogenicity of *F. semitectum.* However, previously Jain *et al*. (1979) reported that *F. semitectum* could cause stalk rot and ear rot of maize. Francis and Burgess (1975) isolated *F. semitectum* from maize with stalk rot symptoms in Eastern Australia.

Marasas *et al.* (1984) reported an association of strains of *F. semitectum* with moldy rice straw with Degnala disease of water buffaloes. However, Hokonohara *et al*. (2003) and Desjardins (2006) found that Degnala disease was alleviated by tetracycline treatment of affected water buffaloes, suggesting that the disease is not due to mycotoxicosis. Lezar and Barros (2010) demonstrated the ability of *F. semitectum* to produce fumonisins.

2.2.2 Other fungi

Not only *Fusarium* species, but other fungi have also been reported from rice plants and rice seeds and some are plant pathogenic and toxic to animals and humans (Ou, 1985; Rheeder *et al*., 2002; Reddy *et al*., 2008).

2.2.2.1 *Alternaria* species

Copcu and Karaca (1983) reported *Alternaria* species as plant pathogenic. Mirocha *et al.* (1996) and Rheeder *et al*. (2002) demonstrated that *Alternaria* species could be toxigenic.

2.2.2.1.1 *Alternaria alternata* (Fr.) Keissler

Siddiqi (1980) isolated *A. alternata* from rice and suggested that it caused glume spoting in Malawi. Nesterov (1981) found that *A. alternata* was a pathogen causing root rot of wheat in Kazakhstan. Copcu and Karaca (1983) also isolated *A. alternata* from rice plants in Turkey and demonostrated it as the causal pathogen of minute leaf spot of rice. Koroleva *et al*. (1984) isolated *A. alternata* from rice in the Ukraine. Ou (1985) suggested that *A. alternata* could cause leaf spot of rice. In the same year, Saini (1985) reported that *A. alternata* causes discoloration of rice grain in India. Lee *et al*. (1986) revealed *A. alternata* as the causal pathogen of dirty panicle disease of rice in Philippines. Broggi and Molto (2001) confirmed *A. alternata* as a seed borne pathogen of rice in Argentina. Butt *et al*. (2011) reported A. *alternata* as a seed borne pathogen of rice in Pakistan. Pose *et al*. (2009) suggested that *A. alternata* is responsible fungus for black mould of ripe tomato fruits in Argentina. Taba *et al*. (2009) suggested *A. alternata* as pathogen of leaf spot of basil in Japan. Thomidis *et al.* (2009) also confirmed *A. alternata* as a pathogen of fruit rot of peaches in Greece.

Rabie and Thiel (1985) reported the isolation of strains of *A. alternata* from sorghum malt which was found to be toxic to ducklings. Gruber-Schley and Thalmann (1988) suspected animal illness due to *A. alternata* toxin in animal feeds. Ahn *et al.* (2009) reported that *A. alternata* induced rhinosinusities. Alqurashi (2009) confirmed the

isolation of *A. alternata* in eastern Saudi Arabia and suggested that the fungus has a role in causing ophthalmic infection.

Production of mycotoxins such as alternariol, alternariol methyl ether, altenuene and and a derivative of tetramic acid and tenuazonic acid by *A. alternata* has been reported (Meronuck *et al*., 1972; Pero *et al*., 1973; Harvan and Pero, 1976; Wei and Swartz, 1985; Gruber-Schley and Thalmann, 1988). Production of these mycotoxins by *Alternaria* species has been shown on wheat (Magan and Lucey, 1985), tomato (Harwig *et al.,* 1979), sorghum (Sauer *et al*., 1978; Magan and Baxter, 1994), pecans (Schroeder and Cole, 1977) and on cotton (Young *et al.,* 1980). A culture of *A. alternata* on corn flower has been found to be carcinogenic in rats and culture extracts were mutagenic in various microbial and cell systems (Dong *et al*., 1987; Zhen *et al*., 1991). It has been reported that *A. alternata* might be one of the etiological factors for human oesophageal cancer in Linxian, China (Dong *et al.,* 1987; Trucksess and Pohland, 2001). Chen *et al*. (1992) revealed that *A. alternata* can produce mycotoxin fumonisin B_1 and AAL-toxin in culture. Abbas and Riley (1996) and Mirocha *et al*. (1996) confirmed the production of fumonisins FB_2, FB_3 and AAL- toxin by *A. alternata*. Rheeder *et al.* (2002) alluded to the fact that *A. alternata* does not belong to the genus *Fusarium*, but has the ability to produce fumonisins (FB_1, FB_2 and FB_3). Frisvad *et al*. (2007) also found that fungus *Aspergillus niger* that does not belong to the genus *Fusarium* could also produce fumonisin B_2. Later, Noonim *et al*. (2009) showed production of fumonisin B_2 by *A. niger* on coffee. Morgensen *et al*., (2010) detected fumonisin B_2 and B_4 by *A. niger* on grapes and raisins. Recently Soares *et al*. (2013) confirmed the production of fumonisin B_2 by *A. niger* aggregate strains isolated from harvested maize in three Portuguese regions. A summary on *A. alternata* has been shown in Table 2.4.

2.2.2.1.2 *Alternaria longipes* (Ellis & Everth) Mason

Giri and Murugesan (1996) reported that *A. longipes* causes small, chlorotic, water soaked lesions on groundnut leaves in India. The fungus has been reported to cause leaf blight of *Medicago sativa* in India (Maiti *et al*., 2007). Vintal *et al.* (2002) isolated *A. longipes* from carrots in Israel and confirmed the fungus as the causal pathogen

of leaf blight of carrot. Yiyong *et al*. (2009) confirmed *A. longipes* as the causal pathogen of brown spot of tobacco in China. Long *et al*. (2009) also confirmed *A. longipes* as the causal pathogen of leaf spot of smilax in China. Pose *et al.* (2004) reported the production of several toxins such as alternariol, alternariol monomethyl ether and tenuazonic acid. However, the fungus has not been reported to cause any human or animal disease.

2.2.2.2 *Cochliobolus miyabeanus*

Padmanabhan (1973) confirmed *C. miyabeanus* as the causal pathogen of brown spot disease of rice and largely responsible for the Bengal (presently Bangladesh and West Bengal of India) famine of 1943. In 1942, the fungus caused yield losses of 40 to 90% in this region. Two million people died from starvation due to famine (Webster and Gunnell, 1992; Agrios, 2005). Reddy and Khare (1978) and Singh and Khare (1983) isolated the fungus from rice seeds in India. Zainun and Nik (1977) and Mujim *et al*. (1983) also isolated the fungus from rice seeds in Malaysia and Indonesia respectively. Kim *et al.* (1981) reported the isolation of *C. miyabeanus* from rice seeds in Vietnam. In Africa, Gorter (1977) isolated *C. miyabeanus* from rice plants with brown spot disease in South Africa. Imolehin (1983) also confirmed the isolation of the fungus from rice seeds in Nigeria.

Ishlah and Gendeh (2005) reported that *C. miyabeanus* has a role in the pathogenesis of allergic diseases of rhinitis patients in the Klang Valley in Malaysia.

2.2.2.3 *Nigrospora sphaerica* (Sacc.)

Nigrospora sphaerica has been reported to cause disease on turf grasses in China (Liu and Pu, 2004), foliar dieback on carpet grass (*Axonopus compresses*) in Argentina (Palmucci and Arcidiacono, 2003), leaf spot on *Nyctanthese arbortistis* (Panday and Roy, 2008), leaf spot, twig and shoot blight on blueberry (Wright *et al*., 2008), leaf spot on *Glycyrrhiza glabra* (Verma and Gupta, 2008) and on *Aloe barbadensis* Mill (Sharma and Samota, 2007) in India. Luan *et al.* (2004) confirmed the isolation of *N. sphaerica* from wheat seeds with black embryo disease in China.

Isolation of *N*. *sphaerica* from various plants in Argentina has also been reported (Piontell *et al*., 2005) but without any information on the pathogenicity of the fungus. Wright *et al.* (2008) showed that *N. sphaerica* causes leaf spot, twig and shoot blight on blueberry in Argentina. Lucca *et al*. (2008) reported the isolation of *N. sphaerica* from diseased grape berries and stems but no report on the pathogenicity of the fungus.

Nigrospora sphaerica has been reported to produce a compound (mycotoxin) called nigrosporolide which inhibited growth in etiolated wheat coloeptiles (Harwooda *et al*., 1995). However, *N. sphaerica* has not been reported to cause any disease to humans or animals.

2.2.2.4 *Phoma* species

Phoma species are widely distributed in warm regions of the world (tropical, subtropical and continental climate) and they have been reported from plants, soil, human beings, animal and air (Dorenbosch, 1970; Boerema *et al*., 1971; Boerema *et al*., 1973b; Ou, 1985). Some species of *Phoma* such as *P. sorghina* secrete phytotoxins and anthraquinone pigments as secondary metabolites which have potential for biological control of weeds. Rai (2009) reported that secondary metabolites secreted by some species of *Phoma* are antitumor, antimicrobial and anti-HIV. Equisetin and phomasetin obtained from species of *Phoma* are useful against AIDS (Rai, 2009). Ou (1985) also reported the isolation of *Phoma* species from discolored rice grains.

2.2.2.4.1 *Phoma eupyrena* (Sacc.)

Malcolmson (1958) first found *P. eupyrena* as a secondary harmless fungus on potato tubers. Dorenbosch (1970) isolated *P. eupyrena* from potato tubers and from underground parts of all kinds of other plants but was not pathogenic. The fungus has not been reported from any country of the world as rice mycoflora. The fungus has not been reported to cause disease in humans and animals and has not been reported to produce any toxins.

2.2.2.4.2 *Phoma jolyana* Pirozynski and Morgan Jones

Boerema *et al*. (1971) reported the isolation of *P. jolyana* from all kinds of plants including rice. Boerema *et al*. (1973b) confirmed that the fungus acts as a secondary invader of dead or weakend plant material. *Phoma jolyana* has not been reported to cause any disease in humans or animals. The fungus has not been reported to produce any toxins.

2.2.2.4.3 *Phoma sorghina* (Sacc.) Boerema, Dorenb.and van Kest

Chantarasnit (1971) and Zainun and Parbery (1974) reported the isolation of *P. sorghina* from various crop plants and showed that the fungus caused considerable reduction in germination and post-emergence death of seedlings of various crops. Boerema *et al*. (1973a) confirmed *P. sorghina* as a pathogen of maize, millet, rice, sorghum, sugarcane and wheat. Gorter (1977) reported *P. sorghina* as the causal agent of glume blight of rice in South Africa. Marley and Ajay (1999) also showed *P. sorghina* to be the causal pathogen of sorghum in West and Central Africa. Amaral *et al.* (2004) found *P. sorghina* caused leaf spot of maize in Brazil. Ram *et al.* (2005) confirmed *P. sorghina* as a pathogen of sheath rot of rice in Haryana, India. Raj *et al.* (2007) found *P. sorghina* to be a seed borne pathogen of paddy, sorghum, sunflower and cowpea seeds in Karnataka State, India. Velez-Rodriques and Rivera-Vargas (2007) confirmed that *P. sorghina* is pathogenic to young roots and bulbs of onions.

Boerema *et al*. (1973b) reported that *P. sorghina* produces a metabolite which is acutely toxic to rats and chickens and other mammals and birds. The following anthraquinones have been reported to be produced by *P. sorghina* (Borges and Pupo, 2006):1,7-dihydrox-3-methyl-9,10-anthraquinone,1,6-dihydroxy-3-methyl-9,10-anthraquinone and 1-hydroxy-3-methyl-9, 10-anthraquinone, one new anthraquinone (1,7- dihydroxy-3-hydroxy methyl-9, 10 - anthraquinone) and two new hexahydro anthraquinone derivatives, dendryols E and F, have been isolated from culture of the endophytic fungus *P. sorghina* and the fungus was found in association with *Tothonia diversifolia* (*Asteraceae*).

2.2.2.5 *Pithomyces* species

Liu and Zhang (2007) reported the isolation of *Pithomyces* species from soil in the warm temperate zone of Eastern China. Manoch *et al*. (2007) isolated *Pithomyces* species from various substrates such as soil, water, plants, food stuffs and paper in Thailand. Jeamjitt *et al*. (2006) isolated *Pithomyces* species from dung in Thailand. Green *et al*. (2006) reported *Pithomyces* species from North New South Wales, Australia. Hilda *et al*. (2003) reported *P. chartarum* from rice seeds in Cuba. Toth *et al.* (2007) confirmed that fungus *P. chartarum* causes leaf damage to wheat in Europe.

Brewer *et al*. (1988) reported the production of sporidesmin and sporidesmolides by *P. chartarum.* Aas and Ulvund (1989) found that *P. chartarum* caused hepatic damage when ingested by grazing lambs in Norway and that the fungus could produce the toxin sporidesmin. Waghorn *et al.* (2002) reported that *P. chartarum* causes facial eczema in cattle and sheep. Dodd and Stewart (2003) confirmed these findings. Hum (2005) reported that *P. chartarum* caused chronic hepatotoxicity in a 2-year old, male Eastern grey kangaroo in Australia and killed the animal with exposure to the mycotoxin sporidesmin.

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Table 2.1 Mycotoxins in food commodity

Mycotxin	Food commodity	Effects of ingestion	Countries of outbreaks of mycotoxicosis
1. Aflatoxin B_1, B_2, G_1, G_2	Rice, maize, peanuts, oats, barley, sorghum, wheat	Aflatoxin B_1 and naturally occurring mixtures of aflatoxins are identified as potent human carcinogens by the International at Agency for Research on Cancer (IARC). They have adverse effects in various animals, especially chicken	Ghana, India, Kenya, Liberia, Nigeria, South Africa, Sudan, USA and Zimbabwe
2. Fumonisins	Rice, maize, barley, wheat	Suspected by IARC as human carcinogen. Toxic to pigs and poultry. It causes equine Leucoencephalomalacia (ELEM), a fatal disease of horses	China, India, Italy and South Africa
3. Trichothecene (deoxynivalenol/ nivalenol)	Wheat, maize, barley, oats, rye, sorghum	Human toxicoses reported from India, China, Japan and Korea. Toxic to animals especially pigs	China, India, Japan and Russia
4. Ochratoxin A	Rice, wheat, oats, rye, barley	Suspected by IARC as human carcinogen. Carcinogenic in laboratory animals and pigs	Bosnia, Bulgaria, Croatia Herzegovina, Romania and Yugoslavia
5. Zearalenone	Rice, maize, barley, sorghum	Zearalenone is identified by IARC as a possible human carcinogen. Affects reproductive system in pigs	China and Puerto Rico
6. Moniliformin	Rice, maize, wheat, rye, triticale, oats	Moniliformin is a potent cardiotoxic mycotoxin. Broiler chickens (3-wk-old) injected with moniliformin (1mg/Kg body wg) died of cardiac failure	Moniliformin has not been associated with any chronic or fatal animal disease outbreak. Moniliformin proposed as a candidate causal agent of Keshan disease, a heart disease endemic some regions of China, but a role for moniliformin has not established. Very frequent contamination of commercial maize with moniliformin in South Africa.

- Adapted from Smith and Henderson, 1991; GASGA, 1997; Peraica *et al*., 1999; Trucksess and Pohland, 2001; Magan and Olsen, 2004; Desjardins, 2006; Reddy *et al*., 2008.

Table 2.2 Fungi in association with diseases of rice and other plants

Fungus	Disease Name	References
Fusarium anthophilum	• Associated with head blight of rice • Stunting of wheat seedlings • Wilting and damping off on sunflower • Crown and root rots of hazelnut • Fruit rots of paw paws	• Nyvall *et al*., 1999. • Mantecon *et al*., 1984. • Sharfun and Mushtaq, 2006. • Moghaddam and Taherzadeh, 2007. • Muniz et *al*., 2003.
Fusarium chlamydosporum	• Seed-borne pathogen of rice • Wilting and root rot of lentil • Pre and post emergence damping off on melon and tomato • Collar rot, wilting and stunting of sunflower	• Nath *et al.,* 1970; Desjardins *et al*., 2000; Broggi and Molto, 2001. • Chaudhury *et al*., 2009. • Palmero *et al*., 2009. • Sharfun and Mushtaq, 2006.
Fusarium compactum	• Seed-borne pathogen of rice • Crown rot and root rot of wheat • Wilting of cotton • Head- blight of wheat	• Somorin and Bankole, 2010 • Van Wyk *et al*., 1987; Gonzalez and Trevathan, 2000. • Wang *et al*., 2004. • Sarbjeet *et al*., 2000.
Fusarium equiseti	• Sheath rot of rice • Adult rice plants show discolouration in vascular tissues of the culm • Seed borne pathogen of rice • Wilting of guava seedlings	• Kang and Rattan, 1983. • Marin- Sanchez and Jimenez-Diaz, 1982. • Singh and Khare, 1983; Reckhause and Adamon, 1986. • Sriovasta *et al*., 2008.

Fusarium fujikuroi	• Bakanae disease of rice, water grass and barnyard grass	• Kanjanasoon, 1965;Yamanaka and Honkura, 1978; Ou, 1985; Desjardins *et al*., 2000; Carter *et al*., 2008; Zainudin *et al*., 2008; Amatulli *et al*., 2010; Wulff *et al*., 2010; Heng *et al*., 2011; Iqbal *et al*., 2011.
Fusarium semitectum	• Seed borne pathogen of rice, discoloration of rice grain, seed borne pathogen of maize and sorghum • Stalk rot and ear rot of maize • Wilting of alfalfa • Fruit rot of long bean, okra, bitter gourd, cucumber and green chilli	• Nath *et al*., 1970; Siddiqi, 1980; Singh and Khare, 1983; Saini, 1985; Broggi and Molto, 2001. • Francis and Burges, 1975; Jain *et al*., 1979. • Zaccardelli *et al*., 2006. • Latiffah *et al*., 2013.
Alternaria alternata	• Leaf spot of rice • Glume spotting of rice • Dirty panicle disease of rice • Seed borne pathogen of rice, seed discoloration • Root rot of wheat • Black point of wheat • Seed borne pathogen of wheat, corn and sorghum • Black mold of ripe tomato fruits • Leaf spot of basil • Fruit rot of peaches	• Copcu and Karaca, 1983; Ou, 1985. • Siddiqi, 1980. • Lee *et al*., 1986. • Broggi and Molto, 2001; Koroleva *et al*., 1984; Saini, 1985; Nesterov, 1981. • Nesterov, 1981. • Adlakha and Joshi, 1974; Agrawal *et al.,* 1987; Patil *et al*., 1981. • Moubasher *et al.,* 1972. • Pose *et al*., 2009. • Taba *et al.,* 2009. • Thomidis *et al*., 2009.
Alternaria longipes	• Leaf blight of carrot • Chlorotic water soaked lesion on ground- nut leaves • Brown spot of tobacco	• Vintal *et al.,* 2002. • Giri and Murugesan, 1996 • Yiyong *et al.,* 2009.

	• Leaf spot of Smilax	• Long *et al*., 2009.
Cochliobolus miyabeanus	• Brown spot and seedling blight of rice • Sheath rot of rice	• Padmanabhan, 1973; Ou. 1985; Webster and Gunnel, 1992; Agrios, 2005. • Singh *et al*., 2005.
Nigrospora sphaerica	• Black embryo disease of wheat seeds • Foliar dieback of carpet grass • Leaf spot of *Nyctanthese arbortistis* • Wilting of turf grass • Leaf spot twig and shoot blight on blue berry • Leaf spot on *Glycyrrhiza glabra* • Black end and squirter disease in bananas • Leaf spot of *Aloe barbadensis* Mill • Lesion on grapes and stems • Lesion on leaves of *Rhododendron* L.	• Liu and Pu, 2004. • Palmucci and Arcidiacono, 2003. • Panday and Roy, 2008. • Liu and Pu, 2004. • Wright *et al*., 2008. • Verma and Gupta, 2008. • Allen, 1970. • Sharma and Samota, 2007. • Lucca *et al*., 2008. • Kowalik, 2008.
Phoma eupyrena	• There is no report on pathogenecity • Isolated as secondary unharmful on potato tuber	• Kowalik, 2008. • Malcomson, 1958; Dorenbosch, 1970.
Phoma jolyana	• Reported as secondary invader of dead and weakened plant material • Isolated from all kinds of plants including rice	• Boerema *et al*., 1971. • Boerema *et al.,* 1973b.
Phoma sorghina	• Spots on leaves and stems of rice, sorghum, sugarcane, wheat, maize and millet • Post emergence death	• Boerema *et al*., 1973a • Chantarasnit, 1971; Zainun and Parbery,

	of seedlings • Sheath rot of rice • Glume blight of rice • Seed borne pathogen of paddy, sorghum, sunflower, cowpea • Leaf spot of aromatic *Poceae*, wheat leaves and maize leaves • Leaf spot on *Trifolium competre* • Leaf spot and podblight of pasture legume • Seed borne pathogen of Macroptilium and *stylosanthes* species • Root rot and bulb rot of onion	1974. • Ram *et al*., 2005 • Gorter, 1977; Prabhu and Bedendo, 1988. • Marley and Ajay, 1999; Navi, 2006; Raj *et al.,* 2007. • Madia and Gaetan, 2004; Perello Amaral *et al*., 2004. • Sert and Sumbul, 2005. • Nik and Parbery, 1977. • Zainun and Parbery, 1974. • Velez- Rodrigues and Rivera- Vargas, 2007.
Pithomyces sp.	• Seed borne pathogen of rice • Leaf spot of wheat	• Hilda *et al*., 2003 • Toth *et al.,* 2007.

Table 2.3 Summary on *Fusarium fujikuroi*

Geographical distribution	Host	Disease	Mycotoxin production
Bangladesh, India, Indonesia, Italy, Japan, Malayasia, Thailand and California of USA	Rice (Oryza *sativa.)* Water grass (*Echinochloa oryzoidis*) and barnyard grass (Echinochloa *crus-galli)*	The fungus causes bakanae disease. Which is a complex of disease symptoms including root rot and crown rot, abnormal elongation of stems, wilting, stunting and formation of adventitious roots at nodes on the lower portions of stems	Fumonisins moniliformin and fusaric acid

- Adapted from: Ito and Kimura, 1931; Thomas, 1931; Reyes, 1939; Pavgi and Singh, 1964; Both, 1971; Mia and Zaman, 1973; Nirenberg, 1976; Nelson *et al*; 1983; Ou, 1985; Lee *et al.,* 1989; Desjardins *et al.,* 1997; Desjardins, 2000; Webster and Gunnel, 1992; Zainuddin and Carter *et al.,* 2008; Carter *et al.,*2008; Amattulli *et al.,* 2010

Table 2.4 Summary on *Alternaria alternata*

Geographical distribution	Host	Diseases	Mycotoxin production
Argentina, India, Greece, Malawi, Pakistan, Philippines, Turkey and Ukraine	Rice, wheat, basil, peaches and tomatoes	Leaf spot of rice, dirty paniicle of rice, discolouration of rice grains, root rot of wheat, leaf spot of basil, fruit rot of peaches and black mould of ripe tomato fruit	Alternariol, altherneriol methyl ether, altenuene derivative of tetramic acid and tenuazonic acid, fumonisim $FB_{1,}$ $FB_{2,}$ FB_3 and AAL toxin.

CHAPTER 3

THE MYCOFLORA ASSOCIATED WITH DISEASED PLANTS AND SEEDS OF *ORYZA SATIVA* (RICE)

3.1 Abstract

Various mycoflora (fungi) were isolated from diseased rice plants and rice seeds from rice growing regions of South Africa.The isolates of various fungi were initially identified on the basis of their morphological characteristics and the identification of the representative isolates of *Fusarium* spp. were confirmed based on the DNA sequence of the translation elongation factor 1-α (TEF-1-α) gene. A total of six species of *Fusarium* were identified namely, *F. anthophilum*, *F. chlamydosporum*, *F. compactum*, *F. equiseti*, *F. fujikuroi and F. semitectum.*This is the first report regarding the *Fusarium* species from rice in South Africa. *Fusarium anthophilum* has not been found associated with bakanae disease of rice from any country of the world before. Fungi other than *Fusarium* spp. were also isolated and identified only on the basis of their morphological characteristics. A total of eight other species of fungi were identified namely, *Alternaria alternata*, *Alternaria longipes*, *Cochliobolus miyabeanus*, *Nigrospora sphaerica*, *Phoma eupyrena*, *Phoma jolyana*, *Phoma sorghina* and *Pithomyces* sp.This is the first report regarding *A. alternata, A. longipes, N. sphaerica, P. eupyrena, P. jolyana* and *Pithomyces* sp. from rice in South Africa. To the best of my knowledge, *A. longipes* and *P. eupyrena* have not been reported from rice before from any country in the world.

3.2 Introduction

Rice belongs to the family of Gramineae and the genus *Oryza*. *Oryza sativa* is native to tropical and subtropical southern Asia, while the African rice, *Oryza glaberrima* is native to West Africa (Habib *et al*., 2012). Improved varieties of rice *(Oryza sativa*)

were introduced in the regions of the North West province, Free State province, Northern Cape province, Mpumalanga province and KwaZulu-Natal province of South Africa by Taiwanees experts towards 1980. Yields of up to 8-12 tonnes/ha paddy rice were obtained from some areas (Billette, 1986; Dreyer, 2004). Bakanae disease symptoms were observed in the rice fields in the North West province and Kuroman area of Northern Cape province during the 1988-89 crop seasons. The disease symptoms observed were yellow and abnormal elongation of infected rice seedlings due to gibberellic acid production by the bakanae causal agent (Copco and Karaca, 1983; Ou, 1985; Amatulli *et al*., 2010; Iqbal *et al*., 2011). In various rice growing countries, losses by the disease could be 70% (Ito and Kimura, 1931; Ou, 1985; Iqbal *et al.,* 2011). The disease causes both quantitative and qualitative losses with severe losses under field conditions and the disease is able to attack the rice plant from pre-emergence to flowering (Ou, 1985; Iqbal *et al.,* 2011). The bakanae disease has become major limiting factor in rice production throughout the world (Ghazanfar *et al*., 2013).

It is very important to identify pathogenic and toxigenic fungi correctly for effective disease control management, quarantine purposes and as a basis for making decisions to protect agricultural crops as well as other natural resources (Rossman and Palm-Hernandez, 2008; Heng *et al*., 2011).

The objective of this investigation was to isolate and identify various pathogenic and toxigenic fungi associated with the different plant parts of diseased rice plants in the fields of North West province, Free State province and Northern Cape province, during three crop seasons (1988/89, 1989/90 and 1990/91) and from rice seeds from Mpumalanga Province during the crop seasons 1995/96 with the support of the established protocols by the Department of Agriculture in the region. The investigator did not intend to isolate all possible micro-organisms that can occur on rice such as the welknown toxin producing *Aspergillus* and *Penicillium* species. Since many of these fungi are saprophytic and it can be expected that vast numbers of the species would be found. These organisms are not known to be pathogens of rice.

3.3 Materials and Methods

3.3.1 Field surveys

Disease surveys were conducted to determine the fungal species associated with various plant parts of diseased rice plants in the field. The surveys were repeated at three growth stages of rice plants, namely as seedlings-tillering, elongation-booting and ripening in the same fields. Disease infected rice plant samples were collected during three growing seasons (1988/89, 1989/90, 1990/91). Samples were collected from rice fields at six rice projects situated in the regions of North West, Free State and Northern Cape provinces respectively. During each survey forty rice fields were sampled in each growing season. Five sample areas were randomly selected in each field by throwing a wire counting square 1 meter in diameter. Samples were collected from the fields and isolation made within shortest possible time from each symptom type recorded.

3.3.2 Isolation of fungal species from diseased rice plants

Isolations of fungal species were done from diseased plant parts by direct plating of plant tissues. The affected tissues namely roots, stems and leaves were washed under running tap water and surface sterilised with 1% sodium hypochlorite solution (NaOCℓ) for 1 minute and rinsed twice in sterilised water. Small pieces of affected tissues were placed in a 9 cm Petri dish containing potato dextrose agar (PDA) and incubated at 25±1°C in darkness for up to 10 days or until sufficient growth or spores enabled isolation. Single spore pure cultures were obtained and stored on PDA slants for identification (Marin-Sanchez and Jimenez Diaz, 1982; Copcu and Karaca, 1983; Nelson *et al*., 1983; Leslie and Summerel, 2006).

3.3.3 Isolation of fungal species from rice seeds

Seed samples of six lowland rice cultivars/lines (TK5, TK6, TK7, TK9, TC10, and USA 201) were obtained from a rice project at Bushbuckridge in Mpumalanga province to determine fungi associated with rice seeds in the areas. The seeds were the harvest of 1995/96 crop season. The researcher could not visit the rice project to collect disease infected rice plants during the growing seasons. Therefore, rice

samples were obtained to determine the fungi associated with rice seeds in the areas. A total of 400 seeds were selected at random and used for each seed sample. Fungi associated with rice seeds were isolated using two methods namely the blotter method and the potato dextrose agar (PDA) method (ISTA, 1985; Mew and Misra, 1994). The blotter method involved placing 20 non-sterilised seeds on three layers of Whatman's 9 cm blotter (Whatman No.1) in a 9 cm Petri dish moistened with sterile water.The plates were incubated at 25±1ºC for 8 days in an alternating cycle of 12 hours light and 12 hours darkness (ISTA, 1985; Mew and Misra, 1994). The PDA method involved plating of 20 surfaced sterilized seeds from each sampled cultivar/line on to Petridishes and subsequent incubation at 25±1°C for 8 days in an alternation of cycles of 12 hours light and 12 hours darkness. Single spore cultures were obtained and stored on PDA slants for later identification.

3.3.4 Identification of fungal isolates based on morphological characteristics

Fusarium strains isolated from diseased rice plants and rice seeds were initially identified on the basis of the morphological species concepts of Gerlach and Nirenberg (1982) and Nelson *et al*. (1983). Morphological species concepts are based on similarity of observable morphological characters e.g. size of spore and shape of spore (Summerell *et al.,* 2003). Cultural characters of fungal species were assessed morphologically through examination using a stereomicroscope and a compound microscope with reference to Booth (1971, 1977), Gerlach and Nirenberg (1982) and Nelson *et al*. (1983).The identification of *Fusarium* spp., based on morphological features, were further correlated with the descriptions of Leslie and Summerell (2006). Cultural characters of fungi other than *Fusarium* were assessed morphologically by examination using a stereomicroscope and a compound microscope with reference to Ellis (1971), Carmichael *et al*. (1980), Sutton (1980), Ellis and Ellis (1985) and Barnett and Hunter (1998) for morphological identification.

3.3.5 Identification of *Fusarium* isolates on the basis of molecular characters

DNA Extraction and Amplification

Isolates were grown on PDA at 25°C for 7 days. Deoxyribonucleic acid (DNA) was isolated using the DNA easy plant mini extraction kit (Qiagen, Valencia, CA) by following the manufacturer's protocol after mycelium placed in Eppendorf tubes and ground with ca. 10µg sterile, chemically treated sand.

The extracted DNA was used as a template in the polymerase chain reactions (PCR).The Part of the TEF gene was amplified using the primer set EF1 '(5'-CGAATCTTTGAACGCACATTG-3)'EF2(5'-CCGTGTTTCAAGACGGG-3') (O'Donnell *et al*., 1998).The PCR reaction consisted of 1 x Dream Taq reaction buffer with $MgC\ell_2$, dNTPs (250µM each), primers (O.2µM each), and template DNA 9 (25ng) and Dream Taq polymerase (0.5U).The PCR reaction conditions, for the TEF gene region was amplified by initial denaturation at 94°C for 2 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and then elongation at 72°C for 1 min and elongation step at 72°C for 5 min. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany).

DNA Sequencing and Sequence Comparisons

DNA sequences were determined from PCR amplicons using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq ® DNA Polymerase (Applied Biosystems, Warrington, UK) using the primers EF1 and EF2. Sequences generated in this study were deposited in GenBank.

The partial sequence data for translation elongation factor 1-α gene was compared against both the NCBI (GeneBank) database and the *Fusarium* Database (Geiser *et al*., 2004). Reference sequences were selected on the bases of the BLAST (Basic Local Alignment Search Tool) results and previously published phylogenetic relationships within the *Fusarium incarnatum-equiseti* species complex (FIESC). DNA sequences were aligned using a multiple sequence alignment programme, MAFFT (Katoh *et al*., 2002). MAFFT is a novel method for rapid multiple sequence alignment based on Fast Fourier Transform (FFT). Gaps were treated as missing data in the subsequent analysis. Phylogenetic analysis was based on parsimony

using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods Version 4; Swofford, 2002). Heuristic searches were done with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPARS effective and MaxTrees set to auto increase. Phylogenetic signal in the data sets (g1) was assessed by evaluating tree length distributions over 100 randomly generated trees (Hillis and Huelsenbeck, 1992).The consistency (C1) and retention (R1) indices were determined for the TEF data set. Phylogenetic trees were rooted with *Fusarium oxysporum* Schlechtend. Emend.Snyder & Hansen as monophyletic sister outgroup to the rest of the taxa. Bootstrap analyses were performed to determine branching point confidence intervals (replicates) for the most parsimonious trees generated for the TEF data set.

3.4 Results

3.4.1 Field surveys

Bakanae disease

Diseased rice plants showed symptoms of abnormal elongation of stems and were several centimeters taller than healthy plants in the fields.The diseased plants were thin, pale and yellowish-green.The diseased rice plants developed adventitious roots from the lower nodes.The crown tissues were so rotten that the root system could be separated easily from the culms. The diseased rice plants died at the booting stage, showing dark brown to black roots and erect panicles with no kernels.These symptoms were similar to bakanae disease of rice. Bakanae disease symptoms were found in the rice fields at Manyeding near to kuroman in the Northern Cape province, Bodibe near to Itsoseng, Dinokana near to Zeerust, Moiletsoane near to Odi and Taung in the North West province (Table 3.1).

Sheath rot

Diseased rice plants showed sheath rot symptoms in the rice fields. The symptoms were usually found at late booting stage. The upper most part of leaf sheaths were

found affected. Early symptoms were oblong to irregular brown to black spots. The centre of the spot became greyish white with brown margin. Sheath rot lesions enlarged and affected the entire leaf sheath. Brown to black discolouration was seen occasionally on the rice culms. In case of severe infection, panicles were found partially exserted. Partially emerged panicles produced poorly filled grains. Sheath rot symptoms were found in the rice fields at Manyeding near to Kuroman in the Northern Cape province, Bodibe near to Itsoseng, Dinokana near to Zeerust, and Moiletsoane near to Odi in the North West province and Woodbridge near to ThabaNchu in the Free State province (Table 3.1).

Brown spot

Brown spot symptoms were found on both surfaces of leaves of rice plants. Symptoms of brown spots were also found on the leaf sheaths and stems. The spots were oval in shape and relatively uniform and fairly evenly distributed over leaf surface. The spots were found as brown with grey or whitish centres when fully developed. The centre of the lesion became straw coloured but the margin remained dark brown. Brown spot symptoms were found on leaves of both young and adult plants. The brown spot disease symptoms were found in the rice fields at Dinokana near to Zeerust in the North West province and Manyeding near to Kuroman in the Northern Cape province (Table 3.1).

Minute leaf spot

Rice plants showed characteristics symptoms of numerous minute dark brown or black lesions on the leaves. Rice plants also showed necrotic symptoms on the leaves. Minute leaf spot symptoms were found in the rice fields at Manyeding near to Kuroman in the Northern Cape province, Dinokana near to Zeerust and Bodibe near to Itsoseng in the North West province (Table 3.1).

3.4.2 Isolation and identification of different species of fungi

The different species of fungi were isolated and identified from diseased rice plants in the fields and rice seeds in South Africa. Representative cultures of the various species were deposited in the culture collection of PROMEC, Medical Research Council (MRC), P O Box 19070, Tygerberg, 7505, South Africa and the duplicate cultures were later also deposited in the National Collection of Fungi, Culture Collection of the Plant Protection Research Institute (PPRI), Agricultural Research Council, South Africa for long time storage and for future use.

3.4.2.1Isolation and identification of *Fusarium* isolates based on morphological characteristics

A total of 6 morphologically known *Fusarium* species such as *F. anthophilum*, *F. chlamydosporum*, *F*. *compactum*, *F. equiseti*, *F. fujikuroi* and *F. semitectum* were isolated from diseased rice plants and rice seeds (Table 3.2).

Fusarium anthophilum (MRC 5519, MRC 5520 and MRC 5806) was isolated from diseased rice plants with bakanae symptoms from the rice fields in the North West and Northern Cape provinces. Diseased rice plants showed abnormal elongation of stems and the plants were thin, pale and yellowish green. The diseased rice plants developed adventitious roots from the lower nodes. The crown tissues were found seriously rotten. The diseased rice plants died at the booting stage and showed dark brown to black roots and erect panicles with no kernels (Table 3.1). This *Fusarium* species produced dense aerial mycelium with abundant microconidia of oval globose and pear shaped on potato dextrose agar medium. Microconidia were borne on polyphialides. Chlamydospores were absent.

Fusarium chlamydosporum (MRC 7368) was isolated from a seed sample from the rice cultivar TK9 from the Lowveld area of Mpumalanga province.The fungus produced characterized abundant aerial mycelium from white to pink to brown in colour. The fungus produced spindle shaped microconidia, which were formed on polyphialides.

Fusarium compactum (MRC 7369, MRC 7370) was isolated from a seed sample from the rice cultivar TC10 from the Lowveld area of Mpumalanga province. *Fusarium compactum* produced mycelium with white to grayish rose in colour on potato dextrose agar medium. The fungus produced thick walled, shorter, fatter and compact macroconidia on potato dextrose agar medium and produced abundant chlamydospores. The fungus did not produce microconidia.

Fusarium equiseti (MRC 5817, MRC 5818 and MRC 5819) was isolated from diseased rice plants with symptoms of sheath rot from rice fields in the North West, Free State and Northern Cape provinces. The diseased rice plants showed sheath rot symptoms at booting stage. The upper most part of leaf sheaths were found affected. The early symptoms were oblong to irregular brown to black spots. The centre of the spot became greyish white with brown margin. Sheath rot lesions enlarged and affected the entire leaf sheath. The rice culms showed brown to black discolouration. The panicles of the diseased rice plants were found partially exserted and partially emerged panicles produced poorly filled grains (Table 3.1).The fungus *Fusarium equiseti* produced abundant aerial mycelium of white to brown in colour on PDA. Chlamydospores were abundant. Macroconidia were variable in size and shape and strongly curved or bent with elongated apical cells.

Fusarium fujikuroi (MRC 5807, MRC 5808 and MRC 5809) was isolated from the diseased rice plants with bakanae symptoms from the rice fields in the North West and Northern Cape provinces. Diseased rice plants showed symptoms of abnormal elongation of stems and the plants were found thin, pale and yellowish-green. The diseased rice plants developed adventitious roots from the lower nodes. The crown tissues were found seriously rotten.The diseased rice plants died at the booting stage and showed dark brown to black roots and erect panicles with no kernels (Table 3.1). *Fusarium fujikuroi* was characterized by dense white mycelium with abundant ovoid to clavate shaped microconidia in chains and in false heads on monophialides and polyphialides on potato dextrose agar medium.The fungus did not produce chlamydospores.

Fusarium semitectum (MRC 7363, MRC 7364, MRC 7365, MRC 7366, and MRC 7367) was isolated from seed samples from the rice cultivars TK5, TK7 and TK9 from the lowveld area in Mpumalanga province. *Fusarium semitectum* produced abundant uniform mycelium that varied from white to pink in colour on PDA medium.The microconidia were not produced. Macroconidia were produced in sporodochia and were strongly curved with an elongated apical cell.

3.4.2.2 Identification of *Fusarium* isolates based on molecular characteristics

DNA Extraction and Amplification

Aplicons of the TEF gene region were 640 bp in size.

DNA Sequencing and Sequence Comparisons

The partial sequence data for the translation elongation factor 1-α (TEF) was compared against the NCBI (Gene/Bank) database, the *Fusarium* database (Geiser *et al*., 2004) and *Fusarium* MLST database (O'Donnell *et al*., 2009) to confirm the identity of isolates of *Fusarium* species isolated from diseased rice plants and rice seeds. All the strains of *Fusarium* species isolated from diseased rice plants and rice seeds were confirmed as *Fusarium* spp. by comparing three *Fusarium* identification databases.The results of these independent analyses are summarized in Table 3.3.

Parsimony analysis of the TEF gene region was done to determine the phylogenetic placement of the *Fusarium equiseti* rice isolates (Figure 3.1).The TEF data set by inserting gaps resulted in a total of 587 characters used in the comparisons of the different species. All the parsimony uninformative and constant characters were excluded, resulting in 185 parsimony informative characters. Heuristic searches on the data set generated one hundred most parsimonious trees. In the TEF data set, the rice isolates of *F. equiseti* grouped together within the *Fusarium incarnatum-equiseti* species complex (FIESC).The South African isolates from rice were clustered together in a single clade with the *F. equiseti* and *F. incarnatum* isolates

forming two separate sub-clades (Figure 3.1). The South African isolates clustered with none of the 28 phylogenetic lineages in the FIESC (O'Donnell *et al*., 2009). The South African *F. equiseti* rice isolates proved to present a new phylogenetically distinct species in this complex.

3.4.2.3 Isolation and identification of other fungi

Fungi other than *Fusarium* species were isolated from diseased rice plants and rice seeds and identified only on the basis of their morphological characters.The species identified were, *Alternaria alternata*, *Alternaria longipes*, *Cochliobolus miyabeanus*, *Nigrospora sphaerica*, *Phoma eupyrena*, *Phoma jolyana*, *Phoma sorghina* and a species of *Pithomyces* (Table 3.2).

Alternaria alternata was isolated from diseased rice leaves showing characteristics symptoms of numerous minute dark brown or black lesions and it was isolated from rice projects of North West and Northern Cape provinces (Table 3.1).The fungus was also isolated from a seed sample from the rice cultivar TK5 of the Lowveld area of Mpumalanga province. The fungus produced mycelium that was usually black or grey in colour.The conidiophores were borne singly or in small groups, simple or branched and could be straight or flexuous. Conidia were formed in long, branched chains and obclavate, obpyriform, oboid or ellipsoidal in shape with short conical or cylindrical beak.

Alternaria longipes was isolated from a seed sample from the rice cultivar TK5 of the Lowveld area of the Mpumalanga province. The fungus produced amphigenous type of mycelium. Conidiophores were borne singly or in groups, erect or ascending simple or loosely branched. Conidia were solitary and were in chains obclavate, rostrate, pale to mid pale brown.

Cochliobolus miyabeanus was isolated from diseased rice plants showing brown spot symptoms. Dark brown spots were found on both leaf surfaces.The brown spots were also found on leaf sheath and stems. It was isolated from the rice projects of North West and Northern Cape provinces (Table 3.1). The fungus was also isolated

from the rice seed samples from the rice cultivar TK7 and USA 201 of the Lowveld area of Mpumalanga province. The conidiophores of the fungus were solitary or in small groups, might be straight, flexuous or geniculate and were pale to mid brown or olivaceous brown. Conidia were curved, navicular, fusiform or obclavate and cylindrical in shape.

Nigrospora sphaerica was isolated from a seed sample of the rice cultivar TK6 of the Lowveld area of Mpumalanga province.The fungus produced shining white mycelium colonies. Hyphae were creeping, short and vaguely branched and produced brown to black conidia. Conidia were borne on apices of small branches, perfectly globose in shape.

Phoma eupyrena was isolated from a seed samples from the rice cultivar USA 201 of the Lowveld area of Mpumalanga province. The fungus produced aerial mycelium that is dark brown to olivaceous green to brown grey in colour. The fungus produced chlamydospores with pale to brown colour.Conidia were found cylindrical or ellipsoid, straight or slightly curved in shape, biguttulate and aseptate.

Phoma jolyana was isolated from seed samples of the rice cultivars TK5, TK7, TK9 and TC10 of the Lowveld area of Mpumalanga province.The fungus produced abundant fruit bodies on hyphae (pycnidia).Conidia were ellipsoid or slightly irregular in shape and biguttulate.The chlamydospores were terminal and lateral.

Phoma sorghina was isolated from diseased rice plants with sheath rot symptoms. Short linear and brownish lesions were found on the leaves and leaf sheaths of diseased rice plants.The upper most part of leaf sheaths were found affected. Sheath rot lesion enlarged and affected the entire leaf sheath (Table 3.1).It was isolated from rice projects of North West and Northern Cape provinces.The fungus was also isolated from seed samples of rice cultivars TK5, TK7, TK9 and TC10 of the Lowveld area of Mpumalanga province.The fungus produced fluffy to dense aerial mycelium which was grey-green to olivaceous or darker, but with characteristic white to saloman pink tinges. Conidia were found ellipsoid in shape and eguttulate. Chlamydospores were singled celled.

***Pithomyces* sp.** was isolated from seed samples of the rice cultivar TK7 of the Lowveld area of Mpumalanga province.The fungus produced black mycelium colonies. The fungus produced conidia wth mid to dark brown, echinulate or verruculose, mostly with 3 trasverse and1 or 2 longitudinal septa, each with a protruding fractured denticle at its base.

3.5 Discussion and conclusion

In this study, different species of fungi such as *Fusarium* and other fungi were isolated from diseased rice plants showing various diseases symptoms in the rice fields of North West, Northern Cape and Free State provinces and isolated from rice seed samples from Mpumalanga province, South Africa.

Fusarium species

Six species of *Fusarium* were isolated from diseased rice plants and rice seeds and identified as *F. anthophilum*, *F. chlamydosporum*, *F*. *compactum*, *F. equiseti*, *F. Fujikuroi* and *F. semitectum* on the basis of morphological species concepts of Gerlach and Nirenberg (1982) and Nelson *et al.* 1983). Morphological identification of *Fusarium* species provides a great deal of information but the system may not suffice for a complete identification (Summerell *et al*., 2003). Morphological identification of representative strains of *Fusarium* species isolated from diseased rice plants and rice seeds in this study, were therefore, confirmed with the DNA sequences of the translation elongation factor 1-α (TEF-1-α) gene (O'Donnell *et al.,* 1998).The partial sequence data for the translation elongation factor 1-α (TEF) was compared against the NCBI database (GenBank), the *Fusarium* database (Geiser *et al*., 2004) and *Fusarium* MLST database (O'Donnell *et al*., 2009).The identification of strains of *Fusarium* species based on *Fusarium* ID and MLST database largely correlated with morphological identification, whereas Gene/Bank database did not provide significant insight to the identification of the strains but at least confirmed all the strains as *Fusarium* spp. MLST database and phylogenetic analyses confirmed the placement of strains of *Fusarium* spp. in the *F. incarnatum-equiseti* species complex and *Gibberella fujikuroi* species complex.The identification of all strains of

Fusarium species isolated and identified morphologically from diseased rice plants and rice seeds were confirmed as *Fusarium* species by comparing three *Fusarium* identification databases.

In parsimony analysis of the TEF gene, the isolates of *F. equiseti* from rice grouped together within the *Fusarium incarnatum-equiseti* species complex (FIESC).The South African isolates of *F. equiseti* from rice were clustered together in a single clade with the *F. equiseti* and *F. incarnatum* isolates forming two separate sub-clades.The South African rice isolates of *F. equiseti* clustered with none of the 28 phylogenetic lineages in the FIESC (O'Donnell *et al*., 2009).The South African *F. equiseti* rice isolates proved to present a new phylogenetically distinct species in this complex.

Fusarium anthophilum and F. fujikuroi

Fusarium anthophilum and F. fujikuroi were isolated from the diseased rice plants showing bakanae symptoms in the rice seedbeds and in the main rice fields of North West and Northern Cape provinces. *Fusarium fujikuroi* is well known fungus isolated from rice plants in association with bakanae disease of rice (Kanjanasoon, 1965; Ou, 1985; Desjardins *et al.,* 2000; Carter *et al*., 2008; Zainudin *et al*., 2008; Amatulli *et al*., 2010; Wulff *et al*., 2010; Heng *et al*., 2011).This is the first report of bakanae disease of rice in South Africa and both *F. anthophilum* and *F. fujikuroi* have been isolated for the first time from rice plants in association with bakanae disease of rice in South Africa. However, Gorter (1977) reported "*F. moniliforme*/*G. fujikuroi*" to be associated with foot rot of rice in South Africa. *Fusarium anthophilum* has been isolated from cultivated and wild rice in association with headblight of rice in Minnesota, USA (Nyvall *et al*., 1999).To the best of my knowledge, *F. anthophilum* has not been reported before from any country in the world in association with bakanae disease of rice. The association of two *Fusarium* spp. such *F. anthophilum* and *F. fujikuroi* with bakanae disease of rice in South Africa will complicate the control measures of the disease. Moreover both *F. anthophilum* and *F. fujikuroi* produce multiple mycotoxins (Desjardins, 2006) and would increase the risks of

contamination of rice grains with mycotoxins in the fields and in the stores of South Africa.

Fusarium chlamydosporum and F. compactum

In this study, *Fusarium chlamydosporum and F. compactum* have been isolated from a rice seed sample from the rice cultivarTK9 and TK10 respectively from the Lowveld area in the Mpumalanga province of South Africa. *Fusarium chlamydosporum* has also been isolated from rice seeds in India (Nath *et al*., 1970), in Nepal (Desjardins, *et al*., 2000) and in Argentina (Broggi and Molto, 2001). Previously, Marasas *et al*. (1987) confirmed the isolation of *F. chlamydosporum* from various crop plants such as maize, sorghum and pasture plants in South Africa. Because of its association with major food grains including rice and because of its ability to produce toxins, Desjardins (2006) suggested to do risk assessment for *F. chlamydosporum*.This is the first report of isolation of *F. chlamydosporum* from rice in South Afric. *Fusarium compactum* has also been isolated from rice in Nigeria (Somorin Bankole, 2010), from maize in South Africa (Marasas and Van Rensburg, 1986; Desjardins, 2006). The fungus has been isolated from wheat in South Africa (Van Wyk *et al*., 1987) and from wheat kernels in the Slovak Republic (Rochacik and Hudec, 2005).The fungus has been reported to produce trichothecenes and fumonisins (Cole *et al.,* 1988; Desjrdins, 2006; Lezar and Barros, 2010).Therefore, there are risks of contamination of rice grains with mycotoxins produced by the fungus in the stores of South Africa. This is the first report of isolation of *F. compactum* from rice in South Africa.

Fusarium equiseti and F. semitectum

In this study, *F. equiseti* has been isolated from diseased rice plants showing sheath rot symptoms in the fields of warm climates in the North West and Northern Cape provinces and in the fields of Free State province, where climate is relatively cool. *Fusarium equiseti* has been isolated from the diseased rice plants with sheath rot symptoms in India (Kang and Rattan, 1983). *Fusarium equiseti* has been isolated from the adult rice plants showing a discoloration in the vascular tissues of the culm (Marin-Sanchez and Jimenez-diaz, 1982).The fungus has been reported as a seed

borne pathogen of rice in India (Singh and Khare, 1983) and in Nigeria (Reckhause and Adamon, 1986).The fungus has been reported to produce trichothecene (T2), zearalenone and moniliformin (Hussein, 1991).There are risks of contamination of rice grains in the stores of South Africa. This is the first report of isolation of *F. equiseti* from rice in South Africa. In this investigation, *F. semitectum* was isolated from rice seeds from the region of warm climate in the Mpumalanga province, South Africa. *Fusarium semitectum* has been isolated from rice seeds in India (Nath *et al*., 1970; Singh and Khare, 1983; Saini, 1985), from rice seeds in Malawi (Siddiqi, 1980), from rice seeds in Thailand (1994) and from rice seeds in Paraguay and Argentina (Sergio *et al*., 1997; Broggi and Molto, 2001).The fungus has been isolated from rice seeds and maize seeds in Egypt (Madbouly *et al*., 2012).The fungus has been reported to produce fumonisins, moniliformin, trichothecene (T2) and zearalenone (Desjardins, 2006; Zaccardelli *et al*., 2006; Lezar and Barros, 2010).There are risks of contamination of rice grains with mycotoxins of *F. semitectum* in the stores of South Africa. This is the first report of isolation of *F. semitectum* from rice in South Africa.

Other fungi

Apart from *Fusarium* species, other fungi such as *Alternaria alternata, Alternaria longipes, Cochliobolus miyabeanus, Nigrospora sphaerica, Phoma eupyrena, Phoma jolyana, Phoma sorghina* and *Pithomyces* species were found in association with diseased rice plants and rice seeds in South Africa.

Alternaria alternata and A. longipes

Alternaria alternata was isolated from diseased rice plants showing characteristics symptoms of numerous minute dark brown or black spots on the leaves of rice plants in the fields of warm region in the North West and Northern Cape provinces.The fungus was also isolated from a rice seed sample from the warm region in the Mpumalanga province,South Africa. *Alternaria alternata* has been reported to cause minute leaf spot of rice in Turkey (Copcu and Karaca, 1983) and glume spoting of rice in Malawi (Siddiqi, 1980).The fungus has also been reported as seed-borne

pathogen of rice (Moubasher *et al*., 1972; Koroleva *et al*., 1984; Ou, 1985; Saini, 1985; Lee *et al*., 1986; Broggi and Molto, 2001; *Butt et al*., 20011). *Alternaria alternata* is not just a weak parasite, but was found as pathogen of several crop plants in the various countries of the world.The fungus has been reported to produce various mycotoxons such as fumonisins (FB_1, FB_2, FB_3), a family of food borne carcinogenic mycotoxin in culture (Chen *et al*., 1992; Abbas and Riley, 1996; Mirocha *et al*., 1996; Rheeder *et al.,* 2002). Production of other mycotoxins such as alternariol, alternariol monomethyl ether, alternuene and tenuazonic acid have also been reported (Meronuck *et al*., 1972; Pero *et al*., 1973; Wei and Swartz, 1985; Pose *et al*., 2004). It has been reported that *A. alternata* might be one of the etiological factors for human oesophageal cancer in Linxian, China (Dong *et al.,* 1987; Trucksess and Pohland, 2001). *Alternaria* toxins have been demonstrated to be produced by *Alternaria* species on wheat (Magan and Lacey, 1985) and on sorghum (Sauer *et al*., 1978; Magan and Baxter, 1994).Therefore, there are risks of contamination of rice and other cereal grains with mycotoxins in the stores of South Africa.This is the first report of isolation of *A. alternata* from rice in South Africa. In this investigation, *A. longipes* was isolated from a rice seed sample from the warm region in the Mpumalanga province of South Africa.The fungus has been reported to produce mycotoxins such as alternariol, alternariol monomethyl ether and tenuazonic acid (Pose *et al*., 2004).This is the first report of isolation of *A. longipes* from rice in South Africa. To the best of my knowledge, the fungus has not been reported from rice before from any country in the world.

Cochliobolus miyabeanus and N. sphaerica

Cochlioblus miyabeanus was isolated from diseased rice plants showing symptoms of dark brown spots on both leaf surfaces in the fields of North West and Northern Cape provinces, during this investigation.The brown spot symptoms were found on leaves of both young and adult plants. It was also isolated from a rice seed sample from Mpumalanga province. The fungus is well known as causal pathogen of brown spot of rice in various countries of the world (Padmanabhan, 1973,; Webster and Gunnell, 1992; Agrios, 2005).The fungus has been isolated from sheath of rice in India (Singh *et al*., 2005).The brown spot of rice caused by *C. miyabeanus* has also

been reported before from rice in South Africa (Gorter, 1977). In this investigation, *N. sphaerica* has been isolated from a rice seed sample from Mpumalanga province, South Africa.This is the first report of isolation of *N. sphaerica* from rice in South Africa.

Phoma eupyrena, P. jolyana and P. sorghina

Phoma eupyrena has been isolated from a rice seed sample from Mpumalanga province of South Africa during this study. *Phoma eupyrena* is known as secondary unharmful organisms on potato tuber (Malcolmson, 1958; Dorenbosch, 1970). There is no pathogenicity report of *P. eupyrena*. This is the first report of *P. eupyrena* from rice in South Africa.To the best of my knowledge, *P. eupyrena* has not been reported from rice before from any country in the world. In this study, *P. jolyana* has been isolated from a rice seed sample from Mpumalanga province.The fungus has been reported from different kinds of plants including rice (Boerema *et al*., 1971) from different regions of the world.This is the first report of *P. jolyana* from rice in South Africa. *Phoma sorghina* has been isolated from diseased rice plants showing sheath rot symptoms in the fields of North West and Northern Cape provinces of South Africa.The fungus was also isolated from a rice seed sample from Mpumalanga province, South Africa. The fungus has been reported as pathogen of sheath rot of rice in India (Ram *et al.,* 2005). *Phoma sorghina* was reported as the causal agent of glume blight of rice in South Africa (Gorter, 1977). *Phoma sorghina* has been reported from rice seeds in Brazil (Malavolta *et al*., 2007), from sorghum grains originating from South Africa and Texas, USA and from pearl millets in Namibia (Pazoutova, 2009).

***Pithomyces* species**

In this study, *Pithomyces* species have been isolated from a rice seed sample from Mpumalanga province of South Africa. *Pithomyces chartarum* has been isolated from rice seeds in Cuba (Hilda *et al*., 2003).The fungus has been reported to cause leaf damage to wheat in Europe (Toth *et al.,* 2007).This is the first report of *Pithomyces* species from rice in South Africa.

Various mycoflora have been isolated from diseased rice plants and rice seeds in South Africa.There are risks of infection of rice grains with various fungi resulting in contamination of rice grains with multiple mycotoxins produced particularly by the species of *Fusarium* and *Alternaria* in the fields as pre-harvest and in the stores as post harvest in South Africa. Mycoflora have negative impacts on plant health and human and animal health (Marasas *et al*., 1984; Peraica *et al*., 1999; Desjardins *et al*., 2000; Desjardins, 2006; Van Rensburg, 2012; Latiffah *et al.,* 2013).The co-occurrence of mycoflora, aflatoxins and fumonisins with high concentration in rice and maize seeds have been reported from markets of various districts in Cairo, Egypt (Madbouly *et al*., 2012).They detected total aflatoxins and fumonisins in rice averaged 5.15 and 1014µg/kg respectively.Total aflatoxins and fumonisins were detectected in maize averaged 9.75 and 33µg/kg respectively. Accurate identification of fungi such as *Fusarium* species and fungi other than *Fusarium* spp. in association with rice plants and rice seeds in South Africa is important because they cause diseases to plants and produce mycotoxins and cause mycotoxicosis in humans as well as in animals (Marin *et al*., 1999; Peraica *et al.,* 1999; Peraica and Domijan, 2001; Magan and Olsen, 2004; Marin *et al*., 2004; Frisvad *et al.,* 2007; Reddy *et al*., 2008; Das *et al*., 2010; Morgensen *et al*., 2010; Nayaka *et al*., 2011; Latiffah *et al*., 2013).Therefore, it is important to obtain information on the mycoflora associated with rice plants and rice seeds in South Africa, their phytopathogenicity, control, mycotoxicosis in humans and animals and production of mycotoxins to assess risks with food products.

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Table 3.1 Rice diseases found in the fields of South Africa

Name of disease	Symptoms of the disease	Geographical region of occurrence
Bakanae Disease	• Diseased rice plants showed abnormal elongation of stems • Plants were thin pale and yellowish- green • The diseased rice plants developed adventitious roots from lower nodes • The crown tissues were found seriously rotten • The diseased rice plants died at the booting stage and showed dark brown to black root and erect panicles with no kennels	Northern Cape province, North West province,South Africa
Sheath rot	• Diseased rice plants showed sheath rot symptoms at booting stage • The upper most part of sheaths were found affected • Early symptoms were oblong to irregular brown to black spot • The centre of the spot became greyish white with brown margin • Sheath rots lesions enlarged and affected entire leaf sheath • Brown to black discolouration was found on the rice culms • Panicles were found partially exserted • Partially emerged panicles produced poorly filled grains	Northern Cape province, North West province and Free State province, South Africa
Brown Spot	• Diseased rice plants showed brown spot symptoms on both surfaces of leaves • Brown spots were also found on the leaf sheaths and stems • The spots were oval in shape and relatively uniform and fairly evenly distributed over leaf surface • The spots were found as with grey whitish centres when fully developed • The centre of the lesion became straw coloured but the margin remained dark brown • Brown spot were found on leaves of both young and adult plants	North West province and Northern Cape province, South Africa
Minute leaf spot	• Diseased rice plants showed characteristics symptoms of numerous minute dark brown or black lesions on the leaves • Rice plants also showed neurotic symptoms	Northern Cape province and North West province, South Africa

Table 3.2 Isolation of fungi from diseased rice plant tissues and rice seeds

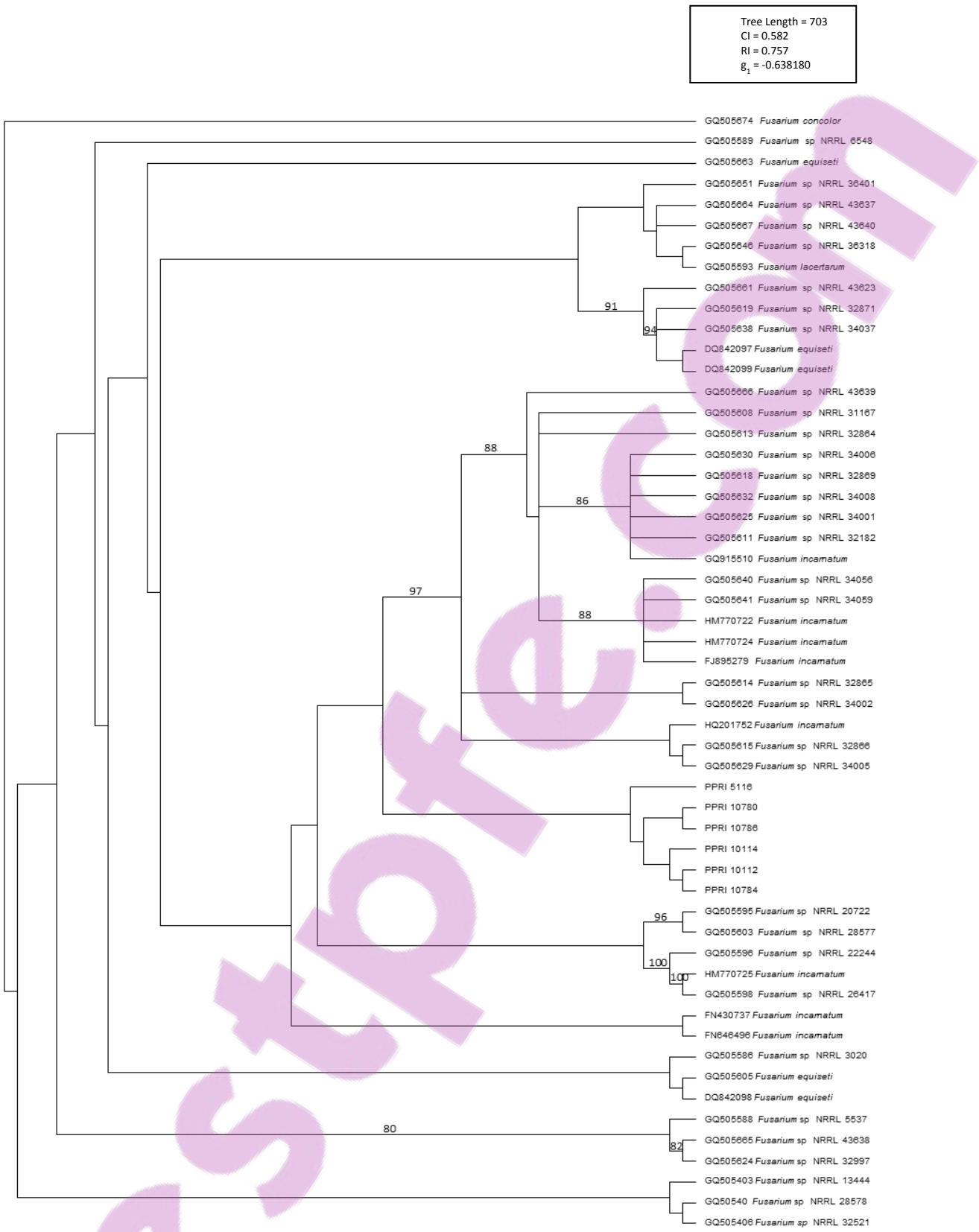
Fungal species	Plant tissues	Seeds
Fusarium anthophilum	Root, crown root and stems	
Fusarium chlamydosporum		seeds
Fusarium compactum		seeds
Fusarium equiseti	Leaf sheaths	
Fusarium fujikuroi	Root, crown roots and stems	
Fusarium semitectum		seeds
Alternaria alternata	Leaves	seeds
Alternaria longipes		seeds
Cochliobolus miyabeanus	Leaf, leaf sheaths and stems	seeds
Nigrospora sphaerica		seeds
Phoma eupyrena		seeds
Phoma jolyana		seeds
Phoma sorghina	Leaf, leaf sheaths	seeds
Pithomyces sp.		seeds

Table 3.3 Identification of *Fusarium* isolates from diseased rice plants and rice seeds based on *Fusarium* MLST, *Fusarium* ID and NCBI (GenBank) databases

PPRI number	**MLST**	**GenBank**	***Fusarium* ID**	**Morphological identification**
4580	*F. brachygibbosum*	*Fusarium sp.*	*Fusarium sp.*	*Fusarium chlamydosporum*
5116	*F. incarnatum-equiseti* species complex (MLST type: 22-a)	*Fusarium equiseti* ➢ *KF539764*	*Fusarium pallidoroseum*	*Fusarium chlamydosporum*
10112	*F. incarnatum-equiseti* species complex (MLST type: 4-a)	*Fusarium lacertarum* ➢ *KF539766*	*Fusarium sp.*	*Fusarium equiseti*
10114	*F. incarnatum-equiseti* species complex (MLST type: 20-b)	*Fusarium sp.*	*Fusarium sp.*	*Fusarium semitectum*
10779	*Gibberella fujikuroi* species complex	*Fusarium sp.*	*Gibberella fujikuroi* species complex	*Fusarium anthophilum*
10780	*F. incarnatum-equiseti* species complex (MLST type: 5-d)	*Fusarium equiseti* ➢ *KF539765*	*Fusarium sp.*	*Fusarium equiseti*
10783	*Gibberella fujikuroi* species complex	*Fusarium sp.*	*Gibberella fujikuroi* species complex	*Fusarium anthophilum*
10784	*F. incarnatum-equiseti* species	*Fusarium sp.* ➢ *KF539767*	*Fusarium sp.*	*Fusarium equiseti*

	complex (MLST type: 5-f)			
10785	*F. chlamydosporum* species complex (MLST type: 2-a)	*Fusarium sp.*	*Fusarium nelsonii*	*Fusarium chlamydosporum*
10786	*F. incarnatum-equiseti* species complex (MLST type: 24-b)	*Fusarium incarnatum*	*Fusarium sp.*	*Fusaium semitectum*
10787	*Gibberella fujikuroi* species complex	*Gibberella fujikuroi* species complex	*Gibberella fujikuroi* species complex	*F. fujikuroi*
10788	*Gibberella fujikuroi* species complex	*Gibberella fujikuroi* species complex	*Gibberella fujikuroi* species complex	*F. fujikuroi*
10879	*Gibberella fujikuroi* species complex	*Gibberella fujikuroi* species complex	*Gibberella fujikuroi* species complex	*Fusarium fujikuroi*
10880	*Gibberella fujikuroi* species complex	*Fusarium sp.*	*Gibberella fujikuroi* species complex	*Fusarium semitectum*

- GenBank accession number



Legends:

Figure 3.1: Phylogenetic tree produced using parsimony of the translation elongation factor-1α gene with *Fusarium concolor* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated above the branches of the tree

Appendix 3.1 *Fusarium* strains isolated and identified morphologically from diseased rice plants and rice seeds

Name	PPRI No.	MRC NO.	GenBank submission group number 4166318
Fusarium chlamydosporum	4580		
Fusarium chlamydosporum	5116		
Fusarium fujikuroi	7751	MRC 8534	
Fusarium equiseti	10112		
Fusarium semitectum	10114		
Fusarium anthophilum	10783	MRC 5806	
Fusarium equiseti	10784	MRC 5818	
Fusarium chlamydosporum	10785	MRC 7368	
Fusarium semitectum	10786	MRC 7364	
Fusarium fujikuroi	10787	MRC 5809	
Fusarium fujikuroi	10788	MRC 5807	
Fusarium semitectum	10880		
Fusarium fujikuroi	10879		
Fusarium anthophilum	10779	MRC 5519	
Fusarium equiseti	10780	MRC 5817	

Appendix 3.2 *Fusarium* isolates in the phylogenetic study

NRRL culture number*	Phylogenetic species	Isolate source	Geographical origin	GenBank accession no.	Reference
3020	FIESC 10	unknown	unknown	GQ505586	O'Donnell *et al.*, 2009
5537	FIESC 8	Fescue hay	Missouri	GQ505588	O'Donnell *et al.*, 2009
6548	FIESC 12	wheat	Germany	GQ505589	O'Donnell *et al.*, 2009
13459	*Fusarium concolor*			GQ505674	O'Donnell *et al.*, 2009
20423	FIESC 4	lizard skin	India	GQ505593	O'Donnell *et al.*, 2009
20722	FIESC 27	*Chrysanthemum* sp.	Kenya	GQ505595	O'Donnell *et al.*, 2009
22244	FIESC 25	rice	China	GQ505596	O'Donnell *et al.*, 2009
26417	FIESC 26	leaf litter	Cuba	GQ505598	O'Donnell *et al.*, 2009
28577	FIESC 28	grave stone	Romania	GQ505603	O'Donnell *et al.*, 2009
29134	FIESC 9	pasture soil	Australia	GQ505605	O'Donnell *et al.*, 2009
31167	FIESC 18	human sputum	USA	GQ505608	O'Donnell *et al.*, 2009
32182	FIESC 15	human blood	USA	GQ505611	O'Donnell *et al.*, 2009
32864	FIESC 17	human	USA	GQ505613	O'Donnell *et al.*, 2009
32865	FIESC 21	human endocarditis	Brazil	GQ505614	O'Donnell *et al.*, 2009
32866	FIESC 23	human cancer patient	USA	GQ505615	O'Donnell *et al.*, 2009

32869	FIESC 15	human cancer patient	USA	GQ505618	O'Donnell *et al.*, 2009
32871	FIESC 5	human abscess	USA	GQ505619	O'Donnell *et al.*, 2009
32997	FIESC 7	human toenail	USA	GQ505624	O'Donnell *et al.*, 2009
34001	FIESC 15	human foot wound	USA	GQ505625	O'Donnell *et al.*, 2009
34002	FIESC 22	human etmoid sinus	USA	GQ505626	O'Donnell *et al.*, 2009
34005	FIESC 24	human intravitreal fluid	USA	GQ505629	O'Donnell *et al.*, 2009
34006	FIESC 15	human eye	USA	GQ505630	O'Donnell *et al.*, 2009
34008	FIESC 15	human lung	USA	GQ505632	O'Donnell *et al.*, 2009
34037	FIESC 5	human abscess	USA	GQ505638	O'Donnell *et al.*, 2009
34056	FIESC 16	human bronchial wash	USA	GQ505640	O'Donnell *et al.*, 2009
34059	FIESC 16	human blood	USA	GQ505641	O'Donnell *et al.*, 2009
36318	FIESC 3	unknown	unknown	GQ505646	O'Donnell *et al.*, 2009
36401	FIESC 2	cotton	Mozambique	GQ505651	O'Donnell *et al.*, 2009
43623	FIESC 5	human maxillary sinus	USA	GQ505661	O'Donnell *et al.*, 2009
43635	FIESC 13	horse	USA		O'Donnell *et al.*, 2009
43636	FIESC 14	Dog	USA	GQ505663	O'Donnell *et al.*, 2009
43637	FIESC 1	Dog	USA	GQ505664	O'Donnell *et al.*, 2009

43638	FIESC 6	Manatee	USA	GQ505665	O'Donnell *et al.*, 2009
43639	FIESC 19	Manatee	USA	GQ505666	O'Donnell *et al.*, 2009
43640	FIESC 1	Dog	USA	GQ505667	O'Donnell *et al.*, 2009

FIESC = *Fusarium incarnatum* / *Fusarium equiseti* species complex

PPRI = PPRI culture collection of the National Collections of Fungi, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council (ARC-PPRI), Pretoria, South Africa.

CHAPTER 4

PATHOGENICITY OF *FUSARIUM ANTHOPHILUM AND FUSARIUM FUJIKUROI ASSOCIATED* WITH BAKANAE DISEASE OF RICE

4.1Abstract

Pathogenicity of three representative isolates of each of *Fusarium anthophilum* (MRC 5519, MRC 5520, and MRC 5806) and *Fusarium fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) obtained from naturally infected rice plants in the fields with bakanae symptoms was tested. All isolates of both *F. anthophilum* and *F. fujikuroi* were able to cause bakanae symptoms on seedlings of four different rice cultivars and lines. This is the first report of the occurrence of bakanae disease of rice in South Africa and both *F. anthophilum* and *F. fujikuroi* are new records as pathogens of rice in South Africa. *Fusarium anthophilum* has not been reported before from any country in the world as a pathogen of rice (*Oryza sativa*) and as a causative fungus of bakanae disease.

4.2 Introduction

In the first 4-5 years of rice growing in the North Western areas of South Africa, no fungal diseases were reported. Due to on-going cultivation of rice without crop rotation, disease started to develop and farmers complained about disease problems with rice.There was an outbreak of bakanae disease in the fields of North West province and Kuruman area of Northern Cape province during the 1988-89 crop seasons.The most evident symptom of this disease is yellowing and abnormal elongation of infected rice seedlings due to gibberellic acid production by bakanae causal agent and this led to the Japanese name bakanae, meaning 'foolish seedlings' (Ou, 1985; Amatulli *et al.,* 2010).The diseased rice plants developed adventitious roots from the lower nodes. Bakanae caused by *F. fujikuroi*

(teleomorph: *Gibberella fujikuroi*) is well known as important disease of rice, especially at the seedling stage. It has been reported from most rice producing countries such as Japan (Ito and Kimura, 1931), India (Thomas, 1931), Philippines (Reyes, 1939), Thailand (Kanjanasoon, 1965), Bangladesh (Mia and Zaman, 1973), Spain (Marin-Sanchez and Jimenez-Diaz, 1982), Turkey (Copcu and Karaca, 1983), China (Yang *et al*., 2003), Italy (Amatulli *et al*., 2010) and Pakistan (Butt *et al.,* 2011; Ghazanfar *et al*., 2013). It was reported by Ito and Kimura (1931) that the disease caused a 20% yield loss in Hokkaido. Yield losses were reported as high as 40-50% in Kinki-Chugoku Region of Japan (Anonymous, 1975). Pavgi and Singh (1964) reported losses of up to 15% due to bakanae disease in the eastern districts of the State of Uttar Pradesh in India. Grain yield losses of up to 40% due to bakanae disease have been reported by others (Maragos *et al*., 1997; Desjardins *et al*., 2000). In various rice growing countries, losses by bakanae disease could be higher than 70% (Iqbal *et al*., 2011).

Desjardins *et al*. (2000) reported that bakanae disease of rice caused by one or two *Fusarium* species. *Fusarium fujikuroi, F. proliferatum and F. verticillioides* (*F. moniliforme*) have been reported to cause bakanae disease of rice (Wulff *et al.,* 2010; Heng *et al*., 2011). Bakanae disease caused by *F. moniliforme* (*F. verticillioides*) has become major limiting factor in rice production throughout the world (Ghazanfar *et al.,* 2013). *Fusarium anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) have been isolated from naturally infected rice plants showing bakanae symptoms in the fields of North West and Northern Cape provinces in South Africa. There could be multiple causes of the bakanae disease since different species of *Fusarium* are known to cause the disease. The objectives of this research study were to establish the pathogenic nature of isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) in four different rice cultivars and lines.

4.3 Materials and Methods

4.3.1 Collection of rice seeds and inoculation procedures

Rice seeds of Hsinchu 56, Chainan 8, Lemont and IRRI 52287-15 were used and collected from the Provincial Department of Agriculture and Rural Development, North West province and from Grain Crops Institute of Agricultural Research Council, Potchefstroom, South Africa.

Inoculum for the pathogenicity trial was obtained by washing conidia from 10 days old cultures on PDA plates of the test isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809).The resultant conidial suspensions were concentrated by centrifugation at 4000 rpm for 20 minutes and re-suspending the pellets in sterilised water so that each suspension contains $125x10^3$ spores/ml (Ahmed *et al*., 1986). Pathogenicity of isolates was tested on seedlings of the cultivars/lines Hsinchu 56, Chainan 8, Lemont and IRRI 52287-15. Rice seeds were pre-soaked in cold water for four hours and soaked again in hot water at 54°C for 15 minutes to eliminate external micro-organisms.The hot water treated seeds were then dried at room temperature for future use.

The hot water treated seeds were soaked for 48 hours. Total 40 sprouted seeds of each cultivar/line were inoculated with each test isolate of *F. anthophilum* (MRC5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 MRC 5809) by immersing them in 1ml inoculum for 5 minutes in a test tube. Ten inoculated seeds were individually planted in a plastic pot of 12.5 cm diameter containing sterilised sand. For each isolate, 16 potted seedlings were grown for treatment and 16 potted seedlings were similarly grown but not inoculated and used as control. The seedlings were grown in a growth chamber with 14 hours per day photo period of 15,000 ℓux fluorescent light. Temperature and relative humidity ranged from 20°C to 25°C and 70 to 90% respectively in the growth chamber (Marin-Sanchez and Jimenez-Diaz, 1982). A half strength Hoagland nutrients solution (Dhingra and Sinclair, 1995) was used to provide nutrients for growth of rice seedlings. The pots were arranged in a randomized block design with four replications (Mead and Curnow, 1987; Box *et al*., 2005). Final counts of diseased and healthy seedlings were made when they were 28 days old.

4.3.2 Statistical analysis

The pathogenicity tests of three isolates of each of *F. anthophilum* (MRC 5519, MRC 5520, and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) on seedlings of four rice cultivars/lines Hsinchu 56, Chainan 8, Lemont and IRRI 52287-15 were measured and statistically analysed by using the Least Significant Difference (LSD) as an Analysis of Variance (ANOVA), as well as cluster analyses. The LSD was calculated at $p<0.05$. Data analysis was performed using STATISTICA software (Statsoft Inc., version 17.0). The raw data sets were expressed as a percentage of diseased plants. This percentage was used for the rest of the analyses. For each isolate, the final disease index was calculated as the mean of the four pots in each of the four repetitions. Cluster analysis was performed to assign treatment combinations similar to each other into groups.

4.4 Results

In pathogenicity test, all the representative isolates of both *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) were able to cause bakanae symptoms on seedlings of four different rice cultivars and lines.The diseased seedlings showed abnormal growth of stems and development of adventitious roots from lower nodes.The diseased rice plants showed dark brown to black roots.Typical bakanae symptoms appeared,14 days after seeds sowing only in seedlings of four test rice cultivars inoculated with all the isolates of both *F. anthophilum* and *F. fujikuroi*. The Bakanae symptoms were not seen on any of the control rice plants. All the pathogenic isolates of *F. anthophilum* and *F. fujikuroi* were re-isolated from diseased seedlings showing bakanae symptoms. All the four test rice cultivars were susceptible to the isolates of *F. anthophilum* and *F. fujikuroi*.The disease incidence caused by isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) varied from 70 to 95% and the disease incidence caused by *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) varied from 78 to 100% on a 0-100 disease index scale (Table 4.1).The isolates of *F. anthophilum* such as MRC 5519 caused disease incidence from 80 to 83%, MRC 5520 caused disease incidence from 70 to 85% and isolate MRC 5806 caused disease incidence from 80 to 95%

on a o-100 disease index scale. The isolates of *F. fujikuroi* such as MRC 5807 caused disease incidence from 98 to 100%, MRC 5808 caused disease incidence 78 to 93% and MRC 5809 caused disease incidence from 85 to 88% on a 0-100 disease index scale. In this study, all isolates of *F. anthophilum* (MRC 55519, MRC 5520 and MRC 5806) and *F. fujikuoi* (MRC 5807, MRC 5808 and MRC 5809) were found as causal pathogen of bakanae disease of rice. None of the test cultivars/lines was found resistant to the disease caused by *F. anthophilum* and *F. fujikuroi*.The cluster analyses were performed to assign treatment combinations similar to each other into groups and analyses clearly showed treatment combinations similar to each other into few groups (Figure 4.1, Figure 4.2).

4.5 Discussion and conclusion

Fusarium anthophilum and *F. fujikuroi* were isolated from diseased rice plants showing bakanae symptoms in the fields of North West and Northern Cape provinces, South Africa. All the isolates of *F. anthophilum* and *F. fujikuroi* caused bakanae disease in four different rice cultivars and lines in artificial inoculation of pathogenicity tests. None of the test rice cultivars was found resistant to the disease caused by isolates of *F. anthophilum* and *F. fujikuroi*.The isolates of *F. fujikuroi* were ranked based on their pathogenicity and rated between 78 to 100% on the 0-100 disease index scale.The isolates of *F. anthophilum* were ranked on the basis of their pathogenicity and rated between 70 to 95% on the same scale. Cluster analyses on different rice cultivars and lines clearly showed the treatments similar to each other into groups. These groupings were the indication of intrinsic genetic differences in rice cultivars and lines. The environment had little influence on the variables, because the experiments were conducted under controlled environment.

The Bakanae disease showed complex of disease symptoms include root and crown rot, abnormal elongation of stems, wilting, stunting and the formation of adventitious roots at nodes on the lower portions of stems (Yamanaka and Honkura, 1978; Sun and Snyder, 1981; Ou, 1985; Webster and Gunnell, 1992).The Bakanae disease was discovered 100 years ago in Japan but it seems the species causing diseases are known but not exhaustive yet (Amatulli *et al*., 2010).

The causal organism of bakanae disease was described in Japan as *Gibberella fujikuroi* (Sawada) Ito (Ito and Kimura, 1931).The anarmorph was considered to be *Fusarium moniliforme* (*F. verticillioides*) (Saccardo) Nirenberg by Booth (1971), but Nirenberg (Nirenberg,1976) differentiated it as a separate species, *Fusarium fujikuroi* Nirenberg. Nelson *et al*. (1983) did not accept *F. fujikuroi* as a separate speices but included it in *F. moniliforme* as the "short-chained" type of *F. moniliforme*. Marasas *et al*. (1986) reported the presence of polyphialides in *Fusarium* isolates from rice with bakanae disease ("bakanae strains") and excluded them from *F. moniliforme*. These authors concluded, however, that the use of the name *F. fujikuroi* sensu Nirenberg (Nirenberg, 1976) for these cultures would create the problem of separating this sp. from *F. proliferatum* (Matsushima) Nirenberg only on the basis of the host plant (= rice). On the basis of studies on the ultastructure of collarette formation, Tiedt and Jooste (1988) concluded that *F. fujikuroi* could be clearly differentiated from *F. moniliforme*, but not from *F. proliferatum.* Prof W.F.O. Marasas who confirmed the identity of two *Fusarium* spp., expressed concern that these three isolates identified as *F. fujikuroi* could not be differentiated morphologically from *F. proliferatum*. Thus these three isolates could either be called "*F. fujikuroi*" (because they were isolated from rice with bakanae disease) or short-chained strains of *F. proliferatum.* In this study and according to current understanding of the taxonomy of this fungus, two *Fusarium* species *F. anthophilum* and *F. fujikuroi* caused bakanae disease in South Africa. Desjardins *et al*. (1997) reported the isolation of some strains of *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* from bakanae infected rice seedlings from various geographic areas. Bakanae disease of rice is caused by one or more *Fusarium* species (Desjardins *et al*., 2000).The results of this study on the pathogenicity of *F. anthophilum* and *F. fujikuroi* isolated from rice plants showing bakanae symptoms in the fields of North West and Northern Cape province, South Africa corroborate the conclusion of Desjardins *et al.* (2000). *Fusarium fujikuroi, F. proliferatum* and *F. vertcillioides* have been reported to cause bakanae disease of rice (Wulff *et al*., 2010 and Heng *et al.,* 2011). Amatulli *et al.,* (2010) reported the isolation of few *Fusarium* species such as *F. fujikuroi, F. proliferatum, F. verticillioides*, and *F. equiseti* from bakanae diseased rice plants and seeds in Italy. In pathogenicity test only *F. fujikuroi* isolates caused bakanae disease. Carter *et al*. (2008) reported *Fusarium fujikuroi* as the causal pathogen of bakanae disease of rice (*Oryza*

sativa), water grass (*Echinochloa oryzoidis* O. Bolos *et* Masclans) and barnyard grass (*Echinochloa crus-galli* L. Beauv.) in Carlifornia of USA. Zainudin *et al.* (2008) reported the isolation of *F. fujikuroi, F. proliferatum, F. verticillioides, F. sacchari* (E.J Butler and Hafiz Kahn) W. Gams and *F. subglutinans* (Wollenw. and Reinking) P.E. Nelson, Toussoun and Marasas from bakanae diseased rice plants in Malaysia and Indonesia. However, in pathogenicity test, only the isolates of *F. fujikuroi* caused bakanae disease.

This is the first report of the occurrence of bakanae disease of rice in South Africa. Both *F. anthophilum and F. fujikuroi* are new records as pathogens of rice in South Africa, although Gorter (1977) reported "*Fusarium moniliforme* / *Gibberella fujikuroi*" to be associated with foot rot of rice in South Africa. Marasas *et al*. (1987) did not report *F. anthophilum* in South Africa but later the fungus was isolated from oats in South Africa (Thiel *et al*., 1991). *Fusarium anthophilum* has been isolated from Copra in Trinidad (Gordon, 1956). *Fusarium anthophilum* (as *moniliforme* var. *anthophilum*) has been reported to produce substances with gibberellin-like biological properties (Gordon, 1960; Marasas *et al*., 1984). *Fusarium anthophilum* has been reported to reduce the height of wheat seedlings (Mantecon *et al*., 1984) in Argentina. To the best of my knowledge, *F. anthophilum* has not been reported before from any country in the world as pathogen of rice (*Oryza sativa*) or as causative fungus of bakanae disease.

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Table 4.1 Pathogenicity of *Fusarium anthophilum* and *Fusarium fujikuroi* isolates to seedlings of four rice cultivars/lines

FUNGAL ISOLATES	RICE CULTIVARS																					
	Hsinchu 56						Chainan 8						Lemont						IRRI 52287-15					
	Diseased seedlings Replicates						Diseased seedlings Replicates						Diseased seedlings Replicates						Diseased seedlings Replicates					
F. anthophilum	**1**	**2**	**3**	**4**	**Mean**	**%**	**1**	**2**	**3**	**4**	**Mean**	**%**	**1**	**2**	**3**	**4**	**Mean**	**%**	**1**	**2**	**3**	**4**	**Mean**	**%**
MRC 5519	7	8	7	10	8	80^{a}		9	9	7	8.25	83^{ab}	8	9	6	9	8	80^{ab}	7	8	8	9	8	80^{a}
MRC 5520	9	9	7	9	8.5	85^{ab}	6	8	9	7	7.5	75^{b}	8	6	9	5	7	70^{a}	9	7	8	8	8	80^{a}
MRC 5806	7	10	10	10	9.25	93^{ab}	9	10	10	9	9.5	95^{a}	10	9	8	5	8	80^{ab}	8	9	8	8	8.25	83^{a}
F. fujikuroi																								
MRC 5807	10	10	10	10	10	100^{b}	10	10	10	10	10	100^{a}	10	9	10	10	9.75	98^{b}	10	10	10	9	9.75	98^{c}
MRC 5808	8	10	9	10	9.25	93^{ab}	8	8	8	10	8.5	85^{ab}	8	8	7	8	7.5	78^{a}	9	10	9	9	9.25	93^{bc}
MRC 5809	8	9	8	10	8.75	88^{ab}	8	9	8	9	8.5	85^{ab}	8	9	8	9	8.5	85^{ab}	8	9	9	8	8.5	85^{ab}

*LSD (ANOVA at p<0.05) of 4 rice cultivars and lines disease index after inoculation for pathogenicity tests of three isolates of each of *Fusarium anthophilum* (MRC 5519, MRC 5520, MRC 5806) and *Fusarium fujikuroi* (MRC 5807, MRC 5808, MRC 5809). The values with similar superscript are not significantly different at P< 0.05 (T-test), **Note**: No disease symptoms were seen on any of the rice control plants (control: 0)

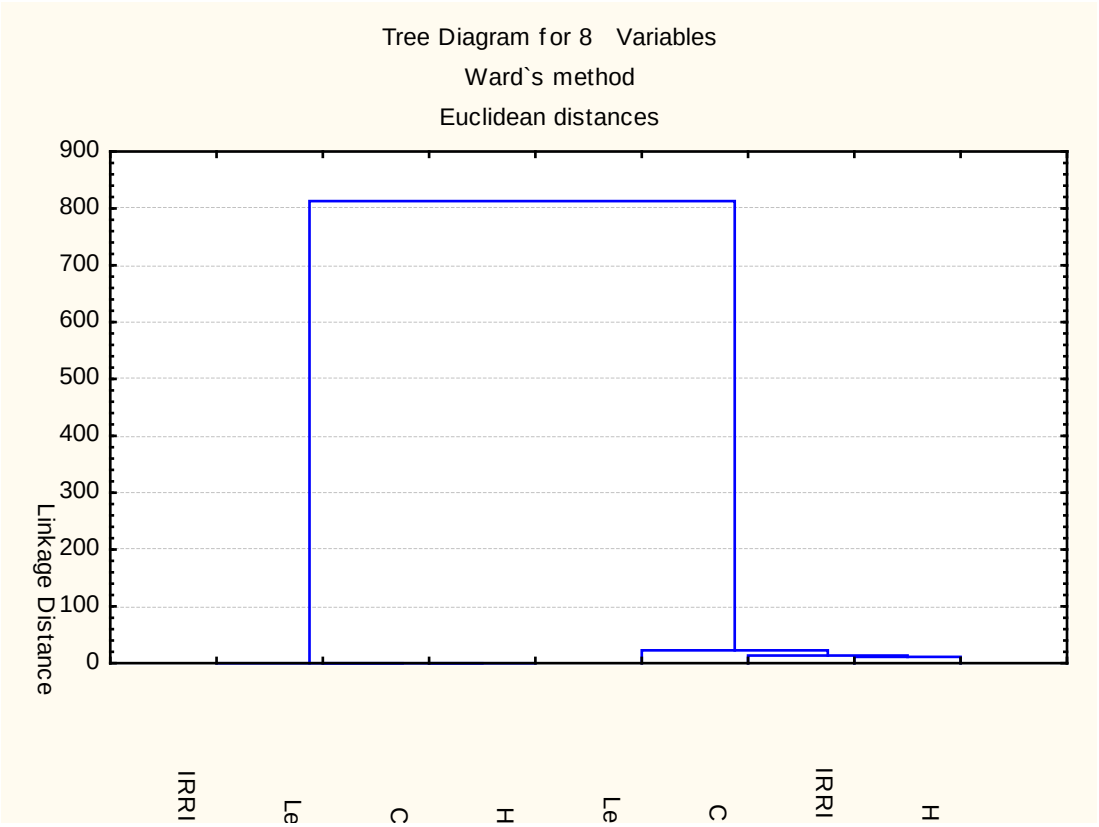

Figure 4.1 Cluster analyses of four rice cultivars and lines for pathogenicity test

Hsinchu = Hsinchu 56 C= Rice cultivar control

Chainan = Chainan 8 T= Rice cultivar treatment

Lemont = Lemont

IRRI = IRRI 52287-15

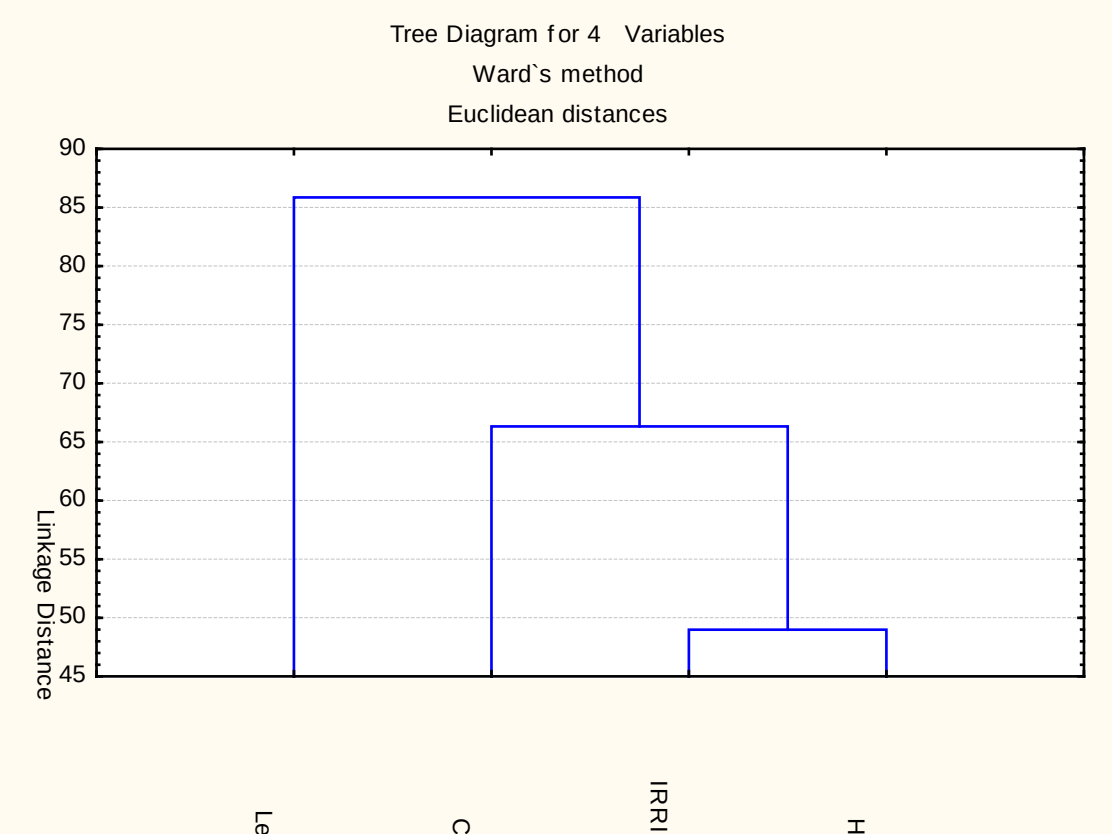

Figure 4.2 Cluster analyses of four rice cultivars for pathogenicity test

Hsinchu = Hsinchu 56 T= Rice cultivar treatment

Chainan = Chainan 8

Lemont = Lemont

IRRI = IRRI 52287-15

CHAPTER 5

RESPONSES OF 54 RICE CULTIVARS AND LINES TO BAKANAE DISEASE CAUSED BY *FUSARIUM FUJIKUROI* AND *FUSARIUM ANTHOPHILUM*

5.1 Abstract

Fifty four rice cultivars and lines were tested by the standardized test tube inoculation method for their inherent properties of resistance and susceptibility to bakanae isolate MRC 5807 of *Fusarium fujikuroi* and bakanae isolate MRC 5806 of *Fusarium anthophilum.* Cultivar E 7034 and rice line RP 2199-16-2-2-1 were found moderately resistant to bakanae disease caused by one of the most virulent isolates of *Fusarium fujikuroi* (MRC 5807). Fifty two cultivars of rice and lines were found moderately susceptible to susceptible to bakanae isolate MRC 5807 of *Fusarium fujikuroi*.Only cultivar E 7034 was found to be moderately resistant to bakanae disease caused by one of the most virulent isolates of *Fusarium anthophilum* (MRC 5806). Fifty three cultivars and lines were found moderately susceptible to susceptible to bakanae isolate (MRC 5806) of *Fusarium anthophilum*. No rice cultivars and rice lines were found resistant to *F. fujikuroi* (MRC 5807) and *F. anthophilum* (MRC 5806) isolates.

5.2 Introduction

Fusarium fujikuroi (teleomorph: *Gibberella fujikuri*) is the causal organism of bakanae disease of rice (Nirenberg, 1976). Bakanae is known to be an important fungal disease of rice (*Oryza sativa*) particularly at the seedling stage of rice plants in China, India, Iran, Japan, Pakistan, Philippines, Taiwan, and USA (Thomas, 1933; Reyes, 1939; Sun,

1975; Ou, 1985; Carter *et al*., 2008; Saremi *et al*., 2008; Ghazanfar *et al*., 2013).The disease has been reported in Nepal (Desjardins *et al.,* 2000) and in Italy (Amatulli *et al.*, 2010).Ito and Kimura (1931) reported that the disease caused a 20% yield loss in Hokkaido of Japan. Losses of up to 15% due to bakanae disease of rice were reported by Pavgi and Singh (1964) in the eastern districts of the State of Uttar Pradesh in India. Desjardins *et al*. (2000) reported the loss of grain yield of rice of up to 40%, due to bakanae disease. The bakanae disease, also known as root rot disease in Iran caused up to 75% yield losses of rice in Northwest region of Iran (Saremi *et al.,* 2008). Bakanae disease of rice has been reported as a major factor limiting rice production in Pakistan and other parts of the world (Junaid *et al*., 2000; Iqbal *et al*., 2011; Ghazanfar *et al*., 2013).

In South Africa, there was an outbreak of bakanae disease of rice in the fields of North West province and Northern Cape province during the 1988-1989 crop seasons. *Fusarium fujikuroi* and *F. anthophilum* were found associated with the disease. In pathogenicity tests, isolates of both *F. fujikuroi* and *F. anthophilum* caused bakanae disease to four different rice cultivars.

The ideal method for controlling bakanae disease is the cultivation of resistant varieties because the method is economic and environmentally friendly disease control method (Ou, 1985; Li *et al.,* 1993; Lv, 1994; Khokhar and Jaffrey, 2002; Iqbal *et al*., 2011; Ghazanfar *et al*., 2013). However these studies found that only a few accessions were reported to have high resistance to bakanae disease. Ito and Kimura (1931) tested some rice cultivars for resistance against bakanae disease in Japan.Three cultivars were found as resistant and 2 cultivars were found as susceptible.Thomas (1931) had tested 41 rice cultivars in India and found 3 cultivars as resistant and 3 cultivars as susceptible. Other cultivars were intermediate in response. Reyes (1939) reported in the Philippines that 4 rice varieties posses some degree of resistance against bakanae disease while an other four varieties were susceptible. Hashioka (1952) tested 200 rice cultivars and found that cultivars from the temperate regions were less affected than those from the tropics. Two cultivars of rice have been reported as resistant against

bakanae disease in Italy (Anonymous, 1959). Rajagopalan (1961) reported 6 resistant cultivars among the 20 cultivars which he tested in India.Tweenty rice varieties/lines were screened in a greenhouse during the 1990-1992 to overcome bakanae disease problems caused by *F. verticillioides* (*F. moniliforme*) in Pakistan and only 5 varieties and 2 lines were found as highly resistant against bakanae disease (Gill *et al*., 1993). In field survey, the cultivar Binam was found as the main resistant cultivar against bakanae disease and had the most yield production in the field of Iran (Saremi *et al*., 2008).

Kanjanasoon (1965) tested 78 non glutinous varieties and 54 glutinous varieties of rice to find possible resistant varieties for the control of bakanae disease caused by *G. fujikuroi* in Thailand and found two varieties to be moderately resistant. Haque *et al.* (1979) tested several methods of artificial inoculation for screening rice varieties against bakanae disease in Bangladesh. Among these, a method of mixing the fungal spore suspension to sprouted seeds proved suitable to obtain good infection. They also found that in vitro incubation of inoculated sprouted seeds for seven to ten days was advantageous to field sowing.They screened 21 rice varieties and lines by this method and six were found to be comparatively resistant to bakanae disease. Ahmed *et al.* (1986) recommended standardized test tube inoculation for rapidly screening rice varieties for resistance to bakanae disease.To screen rice varieties for resistance to bakanae disease, they recommend soaking the seeds for 48 hours and to inoculate them with 125 X 10^3 spores/ ml. Iqbal *et al.* (2011) screened rice germplasm consisting of total 9 cultivars for resistance against bakanae disease in Pakistan.Two cultivars were found as resistant, 3 cultivars as moderately resistant and other cultivars as susceptible. Ghazanfar *et al*. (2013) screened rice germplasm consisting of 3 medium coarse grain varieties and 6 fine grain varieties and revealed that coarse grain varieties are more resistant than fine grain varieties.

This research study was therefore undertaken to screen some rice cultivars and lines to find possible resistant cultivars and lines, so as to overcome the bakanae disease problem of rice in South Africa as a part of economic and environmentally friendlly method of disease control.

5.3 Materials and Methods

5.3.1 Collection of rice seeds and inoculation procedure

Fifty four rice cultivars and lines were screened against most virulent isolates of *F. fujikuroi* (MRC 5807) and *F. anthophilum* (MRC 5806) using the standardized test tube inoculation method of Ahmed *et al*. (1986). This was an improvement over the method of Haque *et al.* (1979), where they did not standardize the spore suspension of the test fungus.The susceptible cultivar Hsinchu 56 was used as test material.The spore suspension of each test fungus isolate was prepared by pouring 5 ml of sterilised water in a 90 mm Petri dish containing a 10 day old culture growing on potato dextrose agar (PDA) medium and then scraping the culture surface with a sterilised glass slide and filtering through sterilised cheese cloth.The spore suspension of each test fungus was standardized at 125 X 10^3 spores/ml.

Rice seeds of various cultivars and lines were collected from the Grain Crops Institute of Agriculture Research Council, Potchefstroom and the Provincial Department of Agriculture and Rural Development, North West province, South Africa. Sixty seeds of each rice cultivar or line were pre-soaked in cold water for 4 hours and then soaked in hot water at 54°C for 15 minutes to kill external unwanted micro-organisms.The hot water treated seeds were soaked again for 48 hours and incubated for 24 hours at 28±1°C.Thirty freshly sprouted seeds of each cultivar/ line were equally distributed in 3 test tubes (ten each) and five drops of test fungal spore suspension were added to 10 seeds. A similar set of 3 test tubes were maintained as control and 5 drops of sterilised water were added to10 seeds. Inoculated and control sets of each cultivar/line were arranged side by side in test tube racks and placed in a circulating air growth chamber with 14 hours per day photoperiod of 15,000 lux fluorescent light.Temperature and relative humidity ranged from 20°C to 25°C and 70% to 90% respectively in the growth chamber (Marin- Sanchez and Jimenez- Diaz, 1982; Dhingra and Sinclair, 1985; Saremi *et al*., 2008).They were retained in the growth chamber for a period of 10 days.The test

tubes were not left on the laboratory table at room temperature as mentioned by Haque *et al. (*1979). Counts of diseased and healthy plants were made when plants were 10 days old.

5.3.2 Statistical analysis

The responses of the 54 rice cultivars and lines against *F. fujikuroi* (MRC 5807) and *F. anthophilum* (MRC 5806) were measured and statistically analysed by using the Least Significant Difference (LSD) as an Analysis of Variance (ANOVA), as well as cluster analyses (Mead and Curnow, 1987; Box *et al.,* 2005). The LSD was calculated at $p < 0.05$. Data analysis was performed using STATISTICA software (Stat soft Inc., version 17.0).The raw data sets were expressed as a percentage of diseased plants.This percentage was used for the rest of the analyses. For each isolate, the final disease index was calculated as the mean of the three test tubes in each of the three repetitions. Cluster analysis was performed to assign treatment combinations similar to each other into groups.

5.3.3 Determination of resistance and susceptibility

The resistance or the susceptibility of each cultivar and each line was determined according to the following arbitrarily designed classes as prescribed by Kanjanasoon (1965):

S: susceptible 76-100 percent of seedlings were killed

MS: moderately susceptible 51-75 percent of seedlings were killed

MR: moderately resistant 26-50 percent of seedlings were killed

R: resistant 0- 25 percent of seedlings were killed

5.4 Results

5.4.1 *Fusarium fujikuroi* (MRC 5807)

The responses of the 54 rice cultivars and lines to bakanae disease were induced by standardized test tube inoculation method with isolate MRC 5807 of *Fusarium fujikuroi*. The disease incidence caused by *Fusarium fujikuroi* (MRC 5807) varied from 43% to 100% on the 0-100 disease index scales (Table 5.1).The rice cultivar E7034 and rice line RP 2199-16-2-2-1 showed 43% disease incidence on the 0-100 disease index scales and were found as moderately resistant (26% to 50%) against bakanae disease caused by most virulent isolate MRC 5807 of *F. fujikuroi.* Thirteen cultivars and lines were found as moderately susceptible (51% to 75%) on the 0-100 disease index scales and 39 cultivars and lines were found as susceptible (76% to 100%) on the 0-100 disease index scales against bakanae disease caused by isolate MRC 5807 of *F. fujikuroi*. None of the test cultivars and lines was found resistant against the isolate MRC 5807 of *F. fujikuroi.* Cluster analysis was performed to assign treatment combinations similar to each other into groups and it indicated similarity within the data sets (Figure 5.1).

5.4.2 *Fusarium anthophilum* (MRC 5806)

The responses of the rice cultivars and lines to bakanae disease were also induced by standardized test tube inoculation method with the most virulent isolate MRC 5806 of *Fusarium anthophilum*.The disease incidence caused by *Fusarium anthophilum* (MRC 5806) varied from 40% to 100% on the 0-100 disease index scales (Table 5.2).The rice cultivar E7034 showed 40% disease incidence and was found statistically significant as moderately resistant (26% to 50%) on the 0-100 disease index scales against bakanae disease caused by isolate MRC 5806 of *F. anthophilum*. Seven rice cultivars and lines were found as moderately susceptible (51% to 75%) on the 0-100 disease index scales and 46 rice cultivars and lines were found as susceptible (76% to 100%) on the 0-100 disease index scales against bakanae disease caused by isolate MRC 5806 of *F. anthophilum*. None of the test cultivars and rice lines was found resistant against the

isolate MRC 5806 of *F. anthophilum*. Cluster analysis was performed to assign treatment combnations similar to each other into groups and it indicated similarity within the data set (Figure 5.2).

5.5 Discussion and conclusion

Fusarium fujikuroi (MRC 5807) isolated from rice plants with bakanae disease in South Africa proved to be highly pathogenic to rice plants, as 13 rice cultivars and lines were found moderately susceptible (51% to 75%) and 39 rice cultivars and lines were found susceptible (76% to 100%) on the 0-100 disease index scales against bakanae disease of rice caused by the isolate MRC 5807. Only rice cultivar (E7034) and rice line (RP 2199-16-2-2-1) with 43% disease incidence were found statistically significant to be moderately resistant (26% to 50%) on the 0-100 disease index scales against bakanae disease of rice caused by isolate MRC 5807 of *F. fujikuroi*. No rice cultivars and lines proved to be resistant against the isolate MRC 5807 of *F. fujikuroi*.

Similarly *Fusarium anthophilum* (MRC 5806) isolated from rice plants with bakanae disease also proved to be highly pathogenic to rice plants, as most of the rice cultivars and lines were found moderately susceptible to susceptible (51% to 100%) on the 0-100 disease index scales against bakanae disease caused by the isolate MRC 5806. The rice cultivar E7034 was also found statistically significant to be moderately resistant with 40% disease incidence against bakanae disease of rice caused by the isolate MRC 5806 of *F. anthophilum*. No rice cultivars and lines were found resistant against bakanae disease of rice caused by isolate MRC 5806 of *F. anthophilum*. The experiment was conducted under controlled environmental condition.Therefore, it is clear that environment had little influence on the variables.The data was calculated by ANOVA/ Least Significant Difference test and cluster analyses.The mean values of similar superscripts were not very different. Cluster analyses on different rice cultivars and lines were not based on most significant differences but purely and indication of what is similar within full data set on isolate MRC 5807 of *F. fujikuroi* and on isolate

MRC 5806 of *F. anthophilum*. The different clusters of rice cultivars and lines are therefore an indication of intrinsic genetic differences in the rice cultivars and lines. Rice seeds of most cultivars and lines were received as part of the Rice Improvement Programme of the International Rice Research Institute (IRRI), Manila, Philippine. International Rice Research Institute releases rice varieties, cultivars and lines for growing in many countries which are high yielding and resistant against various diseases such as tungro virus, bacterial leaf blight and a major rice disease blast caused by fungus *Pyricularia oryzae* Cavara. In this study, all the rice cultivars and lines failed to prove to be resistant against bakanae disease caused by *Fusarium* spp.

No other research has established this fact in rice and specifically on the disease resistance and susceptibility of rice cultivars and lines caused by bakanae disease in South Africa other than work by Gorter (1977) who only reported the isolation of some fungi from different geographical regions of South Africa from various disease symptoms. It was revealed that the best method for controlling bakanae disease is the cultivation of resistant varieties but a few accessions were reported to have high resistance to bakanae disease (Li *et al*., 1993; Lv, 1994; Khokhar and Jaffrey, 2002). The findings of this study in South Africa corroborate the findings of Kanjanasoon (1965), Li *et al.* (1993), Lv (1994) and Khokhar and Jaffrey (2002). However, Iqbal *et al*. (2011) successfully screened rice germplasm consisting of 9 cultivars for resistance against bakanae disease in Pakistan and they found 2 cultivars as resistant and 3 cultivars as moderately resistant. Hashioka (1952) revealed, after screening 200 cultivars of rice, that cultivars from temperate regions are less affected by bakanae disease than those from tropics. Coarse rice varieties were found more resistant to bakanae disease as compared to fine rice varieties (Ghazanfar *et al*., 2013).These studies also suggested that among various methods applied for the management of this disease, host plant resistance is the cheapest and ideal one.This is important knowledge that can potentially assist in dealing with the problem of bakanae disease of rice.Therefore, it is necessary to screen some more rice varieties, cultivars and lines for resistance for controlling bakanae disease of rice caused by different species of *Fusarium* in South Africa.

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Table 5.1: Number of infected cultivar/lines seedlings after artificial inoculations with *F. fujikuroi* (MRC 5807)

	Diseased seedlings			Mean	%	Classification
Replicates	**1**	**2**	**3**			
Cultivars/lines						
1 AS 34011						
Treatment	7	8	9	8	80^{defg}	S
Control	0	0	0	0	0	
2 BG 1165-2						
Treatment	8	7	9	8	80^{defg}	S
Control	0	0	0	0	0	
3 BG 915						
Treatment	6	8	7	7	70^{cde}	MS
Control	0	0	0	0	0	
4 BR 1067-84-1-3-2-1						
Treatment	10	8	9	9	90^{gh}	S
Control	0	0	0	0	0	
5 SKY Bonnet						
Treatment	8	9	7	8	80^{defg}	S
Control	0	0	0	0	0	
6 E 7034						
Treatment	4	5	4	4	43^{a}	MR
Control	0	0	0	0	0	
7 IET 11755						
Treatment	8	7	8	8	77^{defg}	S
Control	0	0	0	0	0	
8 IR 29725-117-2-3-3						
Treatment	10	8	9	9	90^{gh}	S
Control	0	0	0	0	0	
9 IR 52341-60-1-2-1						
Treatment	8	7	8	8	77^{defg}	S
Control	0	0	0	0	0	
10 IR 53292-159-1-2-3						
Treatment	9	8	8	8	83^{efg}	S
Control	0	0	0	0	0	
11 IR 53970-100-3-3-2						
Treatment	10	10	10	10	100^{h}	S

	Control	0	0	0	0	0	
12	IR 56382-17-3-2						
	Treatment	7	8	8	8	77^{defg}	S
	Control	0	0	0	0	0	
13	IR 56446-94-3-1-2						
	Treatment	8	6	7	7	70^{cde}	MS
	Control	0	0	0	0	0	
14	IR 56450-4-2-2						
	Treatment	8	10	7	8	83^{efg}	S
	Control	0	0	0	0	0	
15	IR 57301-195-3-3						
	Treatment	7	8	7	7	73^{cdef}	MS
	Control	0	0	0	0	0	
16	IR 57311-95-2-3						
	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
17	IR 59606-119-3						
	Treatment	7	8	6	7	70^{cde}	MS
	Control	0	0	0	0	0	
18	Kalyani II (CR 666)						
	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
19	NJ 70507						
	Treatment	7	6	8	7	70^{cde}	MS
	Control	0	0	0	0	0	
20	TC 10 (Local)						
	Treatment	10	7	8	8	83^{efg}	S
	Control	0	0	0	0	0	
21	RP2199-16-2-2-1						
	Treatment	5	4	4	4	43^{a}	MR
	Control	0	0	0	0	0	
22	RP 2434-22-3-3						
	Treatment	6	7	5	6	60^{bc}	MS
	Control	0	0	0	0	0	
23	ZHONG 83-49						
	Treatment	7	8	8	8	77^{defg}	S
	Control	0	0	0	0	0	
24	BLUE BONNET						
	Treatment	7	5	8	7	67^{bcd}	MS
	Control	0	0	0	0	0	

25	IR 50						
	Treatment	6	7	8	7	70^{cde}	MS
	Control	0	0	0	0	0	
26	RP 1451-92-21-9						
	Treatment	5	7	4	5	53^{ab}	MS
	Control	0	0	0	0	0	
27	AS 25370						
	Treatment	8	7	6	7	70^{cde}	MS
	Control	0	0	0	0	0	
28	AT 85-2						
	Treatment	9	8	8	8	83^{efg}	S
	Control	0	0	0	0	0	
29	BR 4676-72-2-4						
	Treatment	8	7	8	8	77^{defg}	S
	Control	0	0	0	0	0	
30	BR 4689-17-1-5						
	Treatment	7	8	9	8	80^{defg}	S
	Control	0	0	0	0	0	
31	CCI 22-23-4-301						
	Treatment	9	10	8	9	90^{gh}	S
	Control	0	0	0	0	0	
32	CCI 38-11-6-F-314						
	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
33	CR 294-548						
	Treatment	8	9	8	8	83^{efg}	S
	Control	0	0	0	0	0	
34	Hsinchu 64						
	Treatment	8	8	9	8	83^{efg}	S
	Control	0	0	0	0	0	
35	IR 53970-21-2-3-2						
	Treatment	8	8	8	8	80^{defg}	S
	Control	0	0	0	0	0	
36	IR 54883-152-3-3						
	Treatment	9	5	8	7	73^{cdef}	MS
	Control	0	0	0	0	0	
37	IR 54950-181-2-1-2-3						
	Treatment	8	9	8	8	83^{efg}	S
	Control	0	0	0	0	0	
38	IR 56453-184-2-1-2						
	Treatment	8	9	8	8	83^{efg}	S

	Control	0	0	0	0	0	
39	IR 59682-132-1-1-2						
	Treatment	7	6	8	7	70^{cde}	MS
	Control	0	0	0	0	0	
40	IR 60133-184-3-2-2						
	Treatment	8	7	8	8	77^{defg}	S
	Control	0	0	0	0	0	
41	IR 60832-187-2-2-2						
	Treatment	9	8	9	9	87^{fgh}	S
	Control	0	0	0	0	0	
42	IR 61009-37-2-1-2						
	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
43	IR 62030-18-2-2						
	Treatment	9	9	8	9	87^{fgh}	S
	Control	0	0	0	0	0	
44	IR 62164-14-2-2-2-3						
	Treatment	7	8	6	7	70^{cde}	MS
	Control	0	0	0	0	0	
45	MR 123						
	Treatment	9	8	9	9	87^{fgh}	S
	Control	0	0	0	0	0	
46	PK 2480-7-31						
	Treatment	9	8	7	8	80^{defg}	S
	Control	0	0	0	0	0	
47	PK 2557-24-2-1						
	Treatment	8	7	8	8	77^{defg}	S
	Control	0	0	0	0	0	
48	RAVI (RP 1664-1529-4254)						
	Treatment	9	10	8	9	90^{gh}	S
	Control	0	0	0	0	0	
49	RP 1670-1418-2205-1585						
	Treatment	7	8	9	8	80^{defg}	S
	Control	0	0	0	0	0	
50	S 972B-22-1-3-1-1						
	Treatment	9	8	8	8	83^{efg}	S
	Control	0	0	0	0	0	
51	S 976B-PN-25-1						

	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
52	IR 36						
	Treatment	9	7	8	8	80^{defg}	S
	Control	0	0	0	0	0	
53	Chainan 8						
	Treatment	9	8	9	9	87^{fgh}	S
	Control	0	0	0	0	0	
54	Hsinchu 56						
	Treatment	8	7	8	8	77^{defg}	S
	Control	0	0	0	0	0	

*LSD (ANOVA at p<0.05.) of 54 rice cultivars and lines disease index after inoculation with *Fusarium fujikuroi* (MRC 5807). The values with similar superscript are not significantly different at P< 0.05 (T-test).

S: susceptible 76-100% infected
MS: moderately susceptible 51-75% infected
MR: moderately resistant 26-50% infected
R: resistant 0- 25% infected

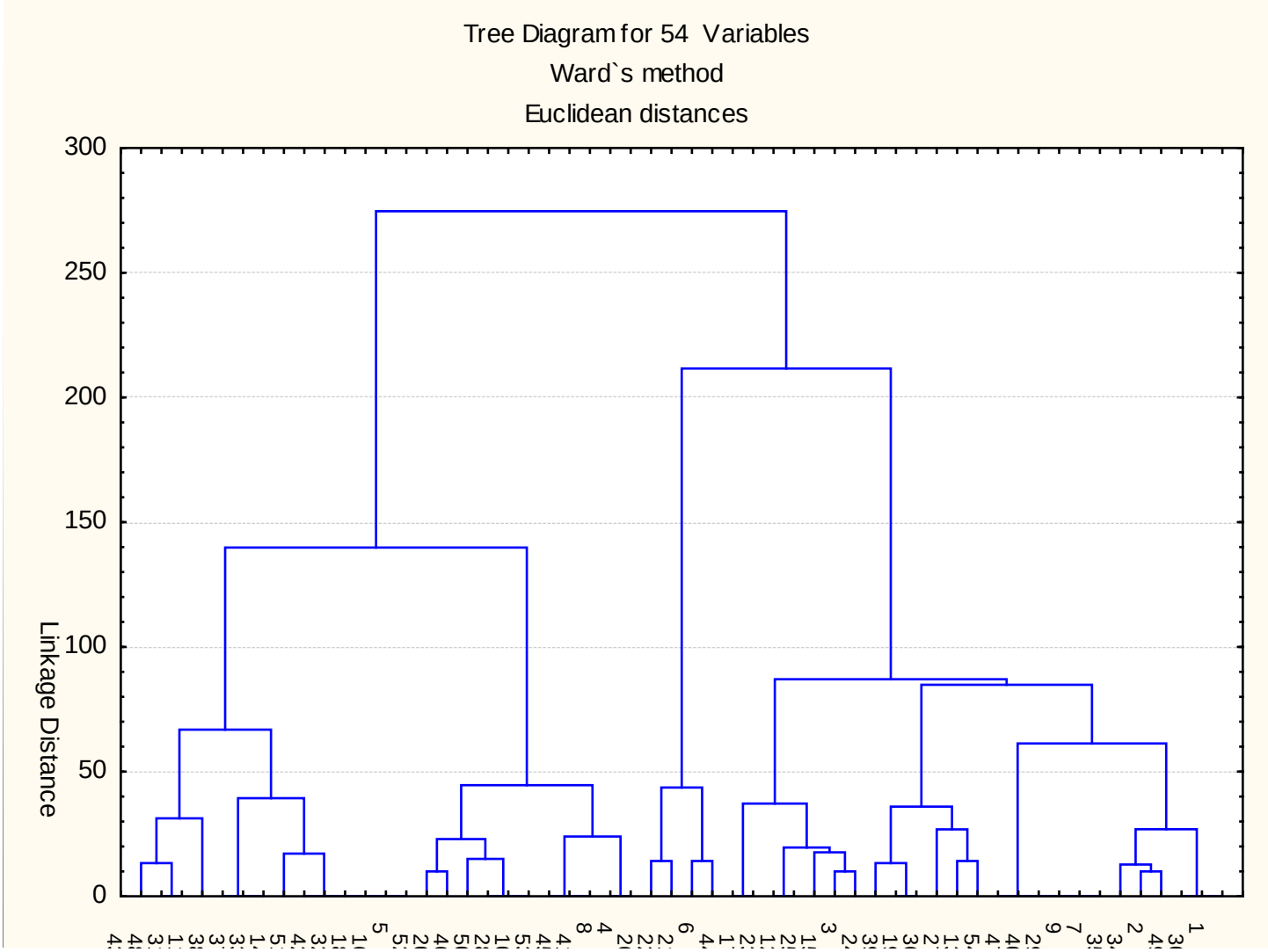

Figure 5.1: Cluster analyses of disease severity of cultivars and lines after inoculation with *F. fujikuroi* (MRC 5807)

Key to the legend for Figure 5.1

1	AS 34011
2	BG 1165-2
3	BG 915
4	BR 1067-84-1-3-2-1
5	SKY Bonnet
6	E 7034
7	IET 11755
8	IR 29725-117-2-3-3
9	IR 52341-60-1-2-1
10	IR 53292-159-1-2-3
11	IR 53970-100-3-3-2
12	IR 56382-17-3-2
13	IR 56446-94-3-1-2
14	IR 56450-4-2-2
15	IR 57301-195-3-3
16	IR 57311-95-2-3
17	IR 59606-119-3
18	Kalyani II (CR 666)
19	NJ 70507
20	TC 10 (Local)
21	RP2199-16-2-2-1
22	RP 2434-22-3-3
23	ZHONG 83-49
24	BLUE BONNET
25	IR 50
26	RP 1451-92-21-9
27	AS 25370
28	AT 85-2
29	BR 4676-72-2-4
30	BR 4689-17-1-5
31	CCI 22-23-4-301
32	CCI 38-11-6-F-314
33	CR 294-548
34	Hsinchu 64
35	IR 53970-21-2-3-2
36	IR 54883-152-3-3
37	IR 54950-181-2-1-2-3
38	IR 56453-184-2-1-2

39	IR 59682-132-1-1-2
40	IR 60133-184-3-2-2
41	IR 60832-187-2-2-2
42	IR 61009-37-2-1-2
43	IR 62030-18-2-2
44	IR 62164-14-2-2-2-3
45	MR 123
46	PK 2480-7-31
47	PK 2557-24-2-1
48	RAVI (RP 1664-1529-4254)
49	RP 1670-1418-2205-1585
50	S 972B-22-1-3-1-1
51	S 976B-PN-25-1
52	IR 36
53	Chainan 8
54	Hsinchu 56

Table 5.2 Number of infected cultivar/lines seedlings after artificial inoculations with *F. anthophilum* (MRC 5806)

		Diseased seedlings			Mean	%	Classification
Replicates		**1**	**2**	**3**			
Cultivars/lines							
1	AS 34011						
	Treatment	10	10	10	10	100^{i}	S
	Control	0	0	0	0	0	
2	BG 1165-2						
	Treatment	8	10	10	9	93^{ghi}	S
	Control	0	0	0	0	0	
3	BG 915						
	Treatment	9	10	8	9	90^{fghi}	S
	Control	0	0	0	0	0	
4	BR 1067-84-1-3-2-1						
	Treatment	10	10	10	10	100^{i}	S
	Control	0	0	0	0	0	
5	SKY Bonnet						
	Treatment	10	10	10	10	100^{i}	S
	Control	0	0	0	0	0	
6	E 7034						
	Treatment	3	4	5	4	40^{a}	MR
	Control	0	0	0	0	0	
7	IET 11755						
	Treatment	8	7	6	7	70^{bcde}	MS
	Control	0	0	0	0	0	
8	IR 29725-117-2-3-3						
	Treatment	6	7	5	6	60^{b}	MS
	Control	0	0	0	0	0	
9	IR 52341-60-1-2-1						
	Treatment	7	6	8	7	70^{bcde}	MS
	Control	0	0	0	0	0	
10	IR 53292-159-1-2-3						
	Treatment	8	7	8	8	77^{cdef}	S
	Control	0	0	0	0	0	
11	IR 53970-100-3-3-2						
	Treatment	7	6	8	7	70^{bcde}	MS

	Control	0	0	0	0	0	
12	IR 56382-17-3-2						
	Treatment	8	6	9	8	77^{cdef}	S
	Control	0	0	0	0	0	
13	IR 56446-94-3-1-2						
	Treatment	10	10	8	9	93^{ghi}	S
	Control	0	0	0	0	0	
14	IR 56450-4-2-2						
	Treatment	9	8	10	9	90^{fghi}	S
	Control	0	0	0	0	0	
15	IR 57301-195-3-3						
	Treatment	8	10	9	9	90^{fghi}	S
	Control	0	0	0	0	0	
16	IR 57311-95-2-3						
	Treatment	6	5	8	6	63^{bc}	MS
	Control	0	0	0	0	0	
17	IR 59606-119-3						
	Treatment	7	6	8	7	70^{bcde}	MS
	Control	0	0	0	0	0	
18	Kalyani II (CR 666)						
	Treatment	8	6	6	7	67^{bcd}	MS
	Control	0	0	0	0	0	
19	NJ 70507						
	Treatment	8	10	9	9	90^{fghi}	S
	Control	0	0	0	0	0	
20	TC 10 (Local)						
	Treatment	10	10	9	10	97^{hi}	S
	Control	0	0	0	0	0	
21	RP2199-16-2-2-1						
	Treatment	7	8	8	7	77^{cdef}	S
	Control	0	0	0	0	0	
22	RP 2434-22-3-3						
	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
23	ZHONG 83-49						
	Treatment	8	10	9	9	90^{fghi}	S
	Control	0	0	0	0	0	
24	BLUE BONNET						
	Treatment	9	8	9	9	87^{fghi}	S
	Control	0	0	0	0	0	

25	IR 50						
	Treatment	8	9	7	8	80^{defg}	S
	Control	0	0	0	0	0	
26	RP 1451-92-21-9						
	Treatment	9	7	8	8	80^{defg}	S
	Control	0	0	0	0	0	
27	AS 25370						
	Treatment	7	8	9	8	80^{defg}	S
	Control	0	0	0	0	0	
28	AT 85-2						
	Treatment	8	8	9	8	83^{efgh}	S
	Control	0	0	0	0	0	
29	BR 4676-72-2-4						
	Treatment	9	8	8	8	83^{efgh}	S
	Control	0	0	0	0	0	
30	BR 4689-17-1-5						
	Treatment	7	8	9	8	80^{defg}	S
	Control	0	0	0	0	0	
31	CCI 22-23-4-301						
	Treatment	8	9	8	8	83^{efgh}	S
	Control	0	0	0	0	0	
32	CCI 38-11-6-F-314						
	Treatment	8	8	9	8	83^{efgh}	S
	Control	0	0	0	0	0	
33	CR 294-548						
	Treatment	9	8	8	8	83^{efgh}	S
	Control	0	0	0	0	0	
34	Hsinchu 64						
	Treatment	8	7	9	9	80^{defg}	S
	Control	0	0	0	0	0	
35	IR 53970-21-2-3-2						
	Treatment	8	10	8	9	87^{fghi}	S
	Control	0	0	0	0	0	
36	IR 54883-152-3-3						
	Treatment	7	8	9	8	80^{defg}	S
	Control	0	0	0	0	0	
37	IR 54950-181-2-1-2-3						
	Treatment	7	8	8	8	77^{cdef}	S
	Control	0	0	0	0	0	
38	IR 56453-184-2-1-2						

	Treatment	10	10	10	10	100[i]	S
	Control	0	0	0	0	0	
39	IR 59682-132-1-1-2						
	Treatment	8	7	8	8	77[cdef]	S
	Control	0	0	0	0	0	
40	IR 60133-184-3-2-2						
	Treatment	9	10	7	9	87[fghi]	S
	Control	0	0	0	0	0	
41	IR 60832-187-2-2-2						
	Treatment	7	8	9	8	80[defg]	S
	Control	0	0	0	0	0	
42	IR 61009-37-2-1-2						
	Treatment	8	9	8	8	83[efgh]	S
	Control	0	0	0	0	0	
43	IR 62030-18-2-2						
	Treatment	7	8	9	8	80[defg]	S
	Control	0	0	0	0	0	
44	IR 62164-14-2-2-2-3						
	Treatment	9	8	8	8	83[efgh]	S
	Control	0	0	0	0	0	
45	MR 123						
	Treatment	7	8	9	8	80[defg]	S
	Control	0	0	0	0	0	
46	PK 2480-7-31						
	Treatment	10	10	10	10	100[i]	S
	Control	0	0	0	0	0	
47	PK 2557-24-2-1						
	Treatment	9	8	8	8	83[efgh]	S
	Control	0	0	0	0	0	
48	RAVI (RP 1664-1529-4254)						
	Treatment	9	8	9	9	87[fghi]	S
	Control	0	0	0	0	0	
49	RP 1670-1418-2205-1585						
	Treatment	8	9	8	8	83[efgh]	S
	Control	0	0	0	0	0	
50	S 972B-22-1-3-1-1						
	Treatment	8	10	9	9	90[fghi]	S
	Control	0	0	0	0	0	

51	S 976B-PN-25-1						
	Treatment	9	7	8	8	80^{defg}	S
	Control	0	0	0	0	0	
52	IR 36						
	Treatment	8	7	8	8	77^{cdef}	S
	Control	0	0	0	0	0	
53	Chainan 8						
	Treatment	8	8	7	8	77^{cdef}	S
	Control	0	0	0	0	0	
54	Hsinchu 56						
	Treatment	9	8	6	8	77^{cdef}	S
	Control	0	0	0	0	0	

*LSD (ANOVA at p<0.05) of 54 rice cultivars and lines disease index after inoculation with *F. anthophilum* (MRC 5806). The values with similar superscript are not significantly different at P< 0.05 (T-test)

S: susceptible 76-100% infected
MS: moderately susceptible 51-75% infected
MR: moderately resistant 26- 50% infected
R: resistant 0- 25% infected

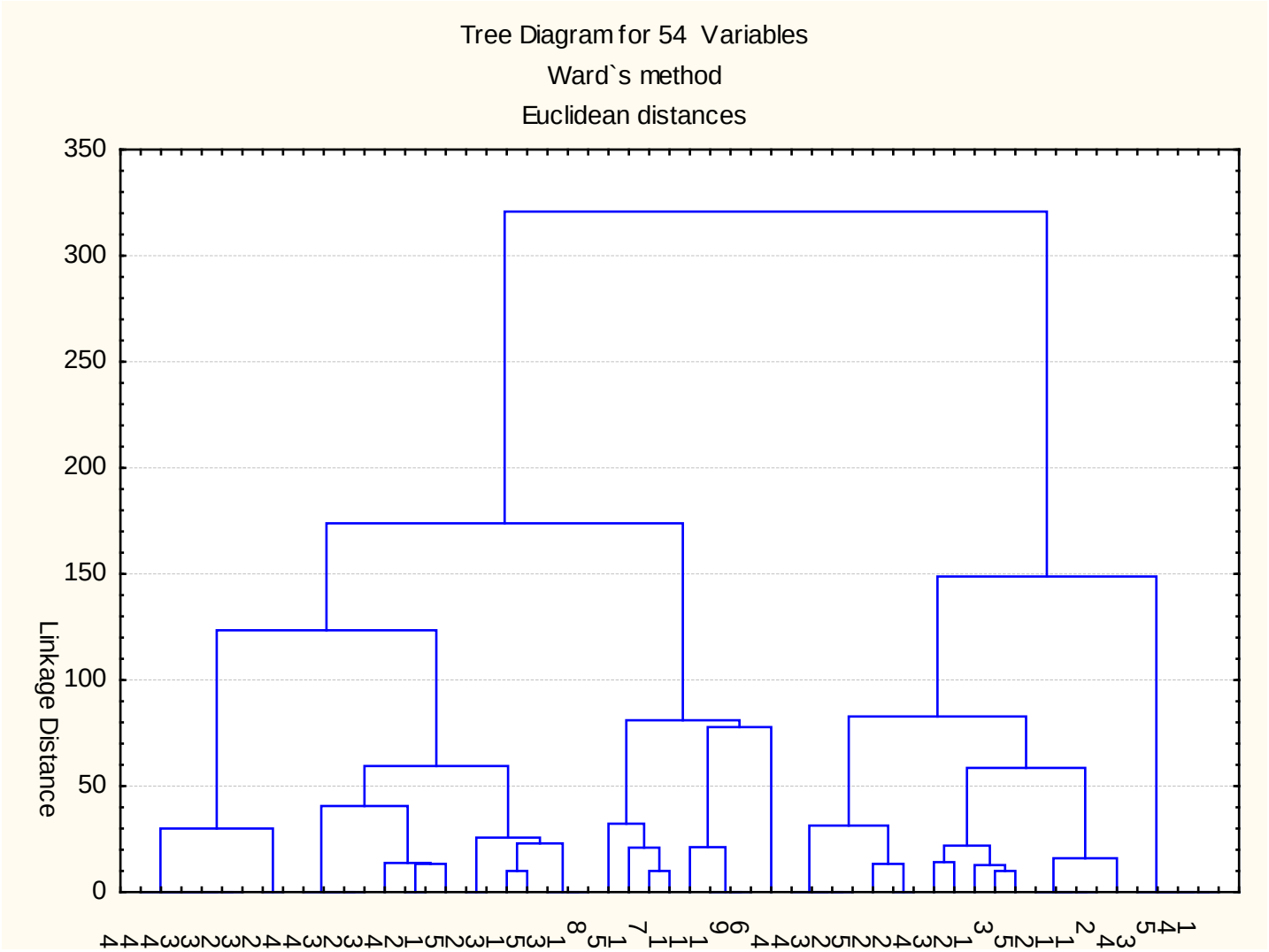

Figure 5.2 Cluster analyses of disease severity of rice cultivars and lines after inoculation with *F. anthophilum* (MRC 5806)

Key to the legend for Figure 5.2

1	AS 34011
2	BG 1165-2
3	BG 915
4	BR 1067-84-1-3-2-1
5	SKY Bonnet
6	E 7034
7	IET 11755
8	IR 29725-117-2-3-3
9	IR 52341-60-1-2-1
10	IR 53292-159-1-2-3
11	IR 53970-100-3-3-2
12	IR 56382-17-3-2
13	IR 56446-94-3-1-2
14	IR 56450-4-2-2
15	IR 57301-195-3-3
16	IR 57311-95-2-3
17	IR 59606-119-3
18	Kalyani II (CR 666)
19	NJ 70507
20	TC 10 (Local)
21	RP2199-16-2-2-1
22	RP 2434-22-3-3
23	ZHONG 83-49
24	BLUE BONNET
25	IR 50
26	RP 1451-92-21-9
27	AS 25370
28	AT 85-2
29	BR 4676-72-2-4
30	BR 4689-17-1-5
31	CCI 22-23-4-301
32	CCI 38-11-6-F-314
33	CR 294-548
34	Hsinchu 64
35	IR 53970-21-2-3-2
36	IR 54883-152-3-3
37	IR 54950-181-2-1-2-3
38	IR 56453-184-2-1-2
39	IR 59682-132-1-1-2

40	IR 60133-184-3-2-2
41	IR 60832-187-2-2-2
42	IR 61009-37-2-1-2
43	IR 62030-18-2-2
44	IR 62164-14-2-2-2-3
45	MR 123
46	PK 2480-7-31
47	PK 2557-24-2-1
48	RAVI (RP 1664-1529-4254)
49	RP 1670-1418-2205-1585
50	S 972B-22-1-3-1-1
51	S 976B-PN-25-1
52	IR 36
53	Chainan 8
54	Hsinchu 56

CHAPTER 6

COMPARATIVE EFFECTIVENESS OF TWO DIFFERENT FUNGICIDES IN CONTROLLING BAKANAE DISEASE OF RICE

6.1 Abstract

Two fungicides (benomyl 50% wettable powder (WP) and thiram 50% WP) at four different concentrations (0.10%, 0.15%, 0.20% and 0.25%) were tested for their effectiveness for controlling bakanae disease of rice caused by *Fusarium anthophilum* (MRC 5806) and *Fusarium fujikuroi* (MRC 5807). Untreated seeds (0%) were used as the experimental control. Benomyl was found most effective for controlling bakanae disease when applied as a seed treatment. Thiram was found relatively in effective for controlling bakanae disease of rice when applied as a seed treatment.

6.2 Introduction

Bakanae disease on rice has been reported in almost all countries where rice (*Oryza sativa*) is grown commercially especially in Asian countries such as Malaysia and Indonesia (Zainudin *et al*., 2008). Although bakanae disease was first described over 100 years ago in Japan, it is still not clear which *Fusarium* species are associated with different symptoms of the disease (Amatulli *et al*., 2010). Early work in Japan identified the pathogen as *F. moniliforme* (current valid name *F*. *verticilioides*) in a broad sense (Ou, 1985; Amatulli *et al*., 2010).This taxon comprises a number of distinct species, collectively called as the *Gibberella fujikuroi* species complex.Three mating populations of *G. fujikuroi* complex have been associated with bakanae diseased rice plants (Desjardins *et al*., 2000).

Mating population-C (anamorph *F. fujikuroi*) was first identified in 1977 among isolates from Taiwan rice (Hsieh *et al*., 1977). Mating population-A (anamorph *F. verticillioides*) and mating population-D (anamorph *F. proliferatum* were isolated from rice in Asia and mating population-D was also isolated from rice in Africa, Australia and USA (Voight *et al*., 1995; Desjardins *et al*., 1997; Amatulli *et al.,* 2010). Wulff *et al*. (2010) reported that *F. fujikuroi* and *F. proliferatum* both cause bakanae disease. Carter *et al*. (2008) showed that *F. fujikuroi* as the causal pathogen of bakanae disease of rice (*Oryza sativa*), water grass (*Echinochloa oryzoidis*) and barnyard grass (*Echinochloa crus-galli*) in California of USA.

In the present study, both *F. anthophilum* and *F. fujikuroi* were isolated from diseased rice plants with bakanae symptoms. Pathogenicity tests indicated that both *F. anthophilum* and *F. fujikuroi caused* bakanae disease. Bakanae disease has been reported from most rice producing countries such as Thailand (Kanjanasoon,1965), Bangladesh (Mia and Zaman,1973), Southern Spain (Marin-Sanchez and Jimenez-Diaz, 1982), Turkey (Copcu and Karaca, 1983), Nepal (Desjardins *et al*., 2000), Italy (Amatulli *et al*., 2010), Malaysia and Indonesia (Zainudin *et al*., 2008; Heng *et al.,* 2011), and Pakistan (Bhalli *et al*., 2001; Iqbal *et al*., 2011; Habib *et al*., 2012; Ghazanfar *et al*., 2013).

The control of bakanae disease of rice was suggested by cultivation of resistant varieties (Li *et al.*, 1993; Lv, 1994; Khokhar and Jaffrey, 2002). These scientists at the same time complained that only a few rice germplasms have high resistance against bakanae disease. Similarly in this study, screening of 54 rice cultivars and lines to bakanae disease caused by *Fusarium* species in South Africa showed that only 2 cultvars and lines were moderately resistant. Ou (1985) reported that evaluation of varietal resistance to rice blast, an important disease of rice caused by *Pyricularia oryzae* Cavara is complicated because of the high potential variability of the fungus in terms of pathogenic races in the field, differences in the level and type of resistance in rice cultivars and environmental factors affecting the expression of resistance. He also reported that 60 Philippines races or 34 international race groups were detected in the

IRRI (International Rice Research Institute) blast nursery during the period of 21 months when samples were taken.

Bakanae disease is seed-borne and primarily seed transmitted (Desjardins *et al*., 1997; Desjardins *et al*., 2000; Amatulli *et al*., 2010).The fungus of bakanae disease was isolated from rice seeds (Hemmi *et al*., 1931; Kanjanasoon, 1965; Ou, 1985; Amatulli *et al*., 2010). Hoschino (1955) emphasized the importance of seed treatment to control bakanae disease of rice. Sawada and Kurosuwa (1924) recommended brine water for seed treatment in order to eliminate the light weight diseased seed and formalin was recommended for seed disinfection. Before 1970, several mercuric compounds such as Granoson and Cereson lime were widely used in Japan and Taiwan in rice cultivation. Since the ban on organo-mercurics, other fungicides have been tested, particularly benomyl and thiram/benomyl combinations.These fungicides have been studied in Japan (Umehara *et al*., 1973; Ito *et al.,* 1974) and in Taiwan (Yu and Yang, 1978). They revealed that benomyl and thiram/benomyl combinations are effective against bakanae disease of rice caused by *F. moniliforme* (*F. vertcillioides)*. Sarkar (1986) reported that thiram reduced bakanae disease significantly in India as seed treatment. Sasaki (1987) confirmed the control of bakanae disease of rice in Taiwan by treating seeds with benomyl or thiram/benomyl combinations. Ahmad (1991) also reported that benomyl had shown good results in inhibiting mycelial growth and sporulation of *F. moniliforme (G. fujikuroi*). Bakanae disease of rice was controlled effectively with benomyl (Illyas and Iftikhar, 1997). Bhalli *et al.* (2001) evaluated 8 fungicides viz. Apron, Benlate (benomyl), Derosal, copperoxychloride, Ridomil, Seore, Topaz and Topsin-M to control the mycelial growth of *F*. *moniliforme*.They revealed that the fungicides benomyl and Derosal caused complete suppression of the growth of *F. moniliforme* (*F. verticillioides*), the causal fungus of bakanae disease of rice when used at 100 ppm. Habib *et al.* (2012) also confirmed the fact that benomyl and Derosal had the ability to suppress completely the growth of the bakanae fungus.

The objective of this investigation was to evaluate the effectiveness of different treatments of benomyl and thiram as seed treatment to control bakanae disease of rice

due to strains of *F. anthophilum* (MRC 5806) and *F. fujikuroi* (MRC 5807) under South African conditions. This investigation was carried out with availalble resources and fund. These 2 commonly used fungicides in South Africa were selected as they were found effective to control bakanae disease of rice in other countries such as India, Japan and Pakistan (Sarkar, 1986; Sasaki, 1987; Ahmad, 1991; Bhalli *et al*., 2001; Habib *et al*., 2012).

6.3 Materials and Methods

Seeds of rice cultivar Hsinchu 56 (known susceptible cultivar in the fields) were pre-soaked in cold water for 4 hours and soaked in hot water at 54ºC for 15 minutes to eliminate external micro-organisms.The hot water treated seeds were then dried at room temperature for future use.The hot water treated seeds were inoculated by immersing them in the spore suspension of test isolate of *F. anthophilum* (MRC 5806) and test isolate of *F.fujikuroi* (MRC 5807) under reduced pressure 200 mm Hg, so as to infiltrate the suspension inside the husk following the protocol of Haque *et al*. (1979), Ahmed *et al*. (1986) and Dhingra and Sinclair (1985).

Dry, powdered formulations of the respective fungicides were used to treat the inoculated seeds with Benomyl 50% WP and Thiram 50% WP at 4 different treatments/ concentrations (0.10%, 0.15%, 0.20% and 0.25%). Inoculated, but untreated seeds (0%) were used as the experimental control. Five days after treatment, 10 sprouted seeds were planted into 12.5 cm diameter pots containing sterilized sand. This was done for each of the different treatment concentrates mentioned above. At least 16 potted seedlings were grown per treatment for each of the two respective test isolates. Sixteen potted seedlings were grown as controls in a growth chamber. The growth chamber was set for 14 hours per day photo-period of about 15 000 lux fluorescent light. Temperature and relative humidity ranged from 20°C to 25°C and 70% to 90% respectively in the growth chamber as recommended by Marin-Sanchez and Jimenez-Diaz (1982). A half strength Hoagland's nutrient solution (Hoagland and Aron, 1950;

Dhingra and Sinclair, 1985) was used to provide nutrients for the growth of plants.The pots were arranged in a randomized block design with four replicates (Mead and Curnow, 1987; Box *et al*., 2005). Final counts of diseased and healthy seedlings were made when plants were 28 days old.

Statistical analysis:

The effect of different concentrations (0.10%, 0.15%, 0.20% and 0.25%) of benomyl and thiram against *F. anthophilum* (MRC 5806) and (*F. fujikuroi* (5807) were measured and statistically analysed by using the Least Significant Difference (LSD) as an Analysis of Variance (ANOVA). Least Significant Difference was calculated at $p<0.05$. Data analysis was performed using STATISTICA software (Stat soft Inc., version 17.0). The raw data sets were expressed as percentage of diseased plants. For each concentration of treatment fungicide, the final disease index was calculated as the mean of the four pots in each of the four replicates.

6.4 Results

Efficacy results of the different treatments of benomyl and thiram against bakanae disease caused by *F. anthophilum* (MRC 5806) have been summarized in Table 6.1 showing statistical significant difference of treatments of two fungicides.This shows that benomyl gave complete control of the disease at concentrations of 0.15%, 0.20% and 0.25%. Benomyl gave 97% significant control of the disease at concentration of 0.10. At concentrations of 0.10% and 0.15%, thiram gave only 17% disease control. At 0.20%, 50% disease control was achieved and 75% disease control at a concentration of 0.25%.

Efficacy results of the different treatments of benomyl and thiram against bakanae disease caused by *Fusarium fujikuroi* (MRC 5807) have been summerized in Table 6.2 showing statistical significant differences of treatments of two fungicides.This shows

that benomyl gave complete control of the disease at concentrations of 0.15; 0.20 and 0.25. It gave 90% disease control at the concentration of 0.10. Thiram completely failed to control the disease at 0.10% and 0.15%. It gave only 15% disease control at 0.20% and it gave 40% disease control at 0.25%.

Benomyl was found as an effective seed treatment fungicide to control bakanae disease of rice. Thiram was found relatively as an ineffective seed treatment fungicide against both test isolates.

6.5 Discussion and conclusion

The control measures of bakanae disease of rice have been complicated due to the involvement of different species of *Fusarium* with the disease. Due to lack of proper control measures, bakanae disease incidence threat is an increasing trend and this has resulted in a serious disease problem in the main rice growing countries of the world (Wahid *et al*., 1991; Ma *et al.,* 2008; Iqbal *et al.,* 2011; Ghazanfar *et al*., 2013). In our previous experiments on the reactions of 54 rice cultivars/lines to bakanae disease, none of the cultivars and lines were found resistant against the disease caused by *F. anthophilum* (MRC 5806) or *F. fujikuroi* (MRC 5807). For this reason, there was a need to investigate other method of disease control such as seed treatment with fungicides such as benomyl and thiram to control bakanae disease of rice.

The conclusion might be drawn from the results that seed treatment with benomyl prior to planting is highly significant in controling bakanae disease of rice caused by both isolates of *F. anthophilum* (MRC 5806) and *F. fujikuroi* (MRC 5807). The results of this study on the effectiveness of benomyl as seed treatment fungicide to control bakanae disease corroborate the conclusions of Sasaki (1987), Ahmad (1991), Illyas and Iftikhar (1997), Bhalli *et al*. (2001), Iqbal *et al*. (2011) and Habib *et al*. (2012) who suggested benomyl as good seed treatment fungicide to control bakanae disease of rice. It is also clear that thiram was relatively ineffective as a seed treatment against both test isolates.

In this investigation, thiram at 0.25% reduced bakanae disease incidence to a certain extent under controlled environmental conditions but the disease control was not very effective. Sarkar (1986) reported that thiram reduced bakanae disease incidence significantly in India as a seed treatment fungicide at 0.25% in field conditions, but it did not give complete control.The findings of the present study do not support these findings. Good protection against isolates of *F. moniliforme* (*F. verticillioides*) causing bakanae disease of rice in Japan was obtained by dipping seeds for 24 hours in ipconazole (Tateishi and Chida, 2000). In this study Benomyl was found as an effective fungicide for controlling bakanae disease of rice. Benomyl was also found effective to control bakanae disease of rice in Turkey (Surek and Gumustekin, 1994). Sinohin (2011) reported that benomyl or thiophanate methyl as seed treatment is very effective to control bakanae disease of rice in the Philippines. Thiram-benomyl wettable powder (active ingredient: thiram 20%, benomyl 20%) was found as an effective seed treatment fungicides to control loose smut a seed borne disease of wheat caused by *F. roseum* Lk. Emend. Snyd. & Hans. Pro parte [S&H] in Japan (Nakagawa and Yamaguchi, 1989). They also suggested that same fungicides would be useful to control loose smut of barley caused by *F. roseum.* However, an isolate of *G. fujikuroi* the causal agent of bakanae disease of rice has been reported as highly resistant to benomyl and was first discovered in 1980. In 1987 most of the isolates of *G. fujikuroi* were highly resistant to benomyl (Ogawa and Takeda, 1990).This development of resistance was suspected due to successive applications of benomyl as a seed treatment (Ogawa and Takeda (1990). Low and high levels of resistance to the benzimidazole fungicides benomyl and thiophanate-methyl were observed in the field isolates of *Monilinia fructicola* (G. Wint.) Honey, the causal agents of brown rot of stone fruits (Ma *et al.,* 2003).

Fusarium fujikuroi the causal agent of bakanae disease of rice is highly diverse on the basis of DNA fingerprinting analysis and due to high diversity the fungus poses a risk in terms of the development of fungicide resistance and susceptibility of new cultivars (Cumagun, 2012). The fungus has been reported to produce a considerable amount of fumonisins in corn but fortunately is minimal in rice (Cumagun, 2012). However, the minimal risk can not be underestimated, because rice is one of the world's most

important crops.There is a need to screen more fungicides to be used as seed treatment in South Africa. There is a need to screen more rice varieties/cultivars to find resistant one to control bakanae disease. Development of resistant varieties must be based on the information of genetic diversity, aggressiveness and the production of mycotoxins by *Fusarium* spp. The control strategy should also include biological option. Bacterization of naturally infected rice seeds reduced bakanae disease incidence in seed box and seedbed tests (Rosales and Mew, 1997).

Reference

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Table 6.1 Efficacy of Benomyl and Thiram as seed treatment fungicides for controlling bakanae disease caused by *F. anthophilum* (MRC 5806)

	Diseased seedlings											
Concentration	**Benomyl**						**Thiram**					
	1	**2**	**3**	**4**	**Mean**	**%**	**1**	**2**	**3**	**4**	**Mean**	**%**
0.10%	**0**	**0**	**1**	**0**	**0.25**	**3[a]**	**8**	**9**	**8**	**8**	**8.25**	**83[f]**
0.15%	**0**	**0**	**0**	**0**	**0**	**0[a]**	**9**	**8**	**7**	**9**	**8.25**	**83[f]**
0.20%	**0**	**0**	**0**	**0**	**0**	**0[a]**	**5**	**4**	**6**	**5**	**5**	**50[d]**
0.25%	**0**	**0**	**0**	**0**	**0**	**0[a]**	**4**	**3**	**2**	**1**	**2.5**	**25[c]**
0%	**10**	**10**	**10**	**10**	**10**	**100[g]**	**10**	**10**	**10**	**10**	**10**	**100[g]**

*LSD (ANOVA at $p<0.05$) on efficacy of Benomyl and Thiram as seed treatment fungicides for controlling bakanae disease caused by *F. anthophilum* (MRC 5806).

Table 6.2 Efficacy of Benomyl and Thiram as seed treatment fungicides for controlling bakanae disease caused by *F. fujikuroi* (MRC 5807)

	Diseased seedlings											
Concentration	**Benomyl**						**Thiram**					
	1	**2**	**3**	**4**	**Mean**	**%**	**1**	**2**	**3**	**4**	**Mean**	**%**
0.10%	**1**	**1**	**2**	**0**	**1**	**10[b]**	**10**	**10**	**10**	**10**	**10**	**100[g]**
0.15%	**0**	**0**	**0**	**0**	**0**	**0[a]**	**10**	**10**	**10**	**10**	**10**	**100[g]**
0.20%	**0**	**0**	**0**	**0**	**0**	**0[a]**	**9**	**8**	**9**	**8**	**8.5**	**85[f]**
0.25%	**0**	**0**	**0**	**0**	**0**	**0[a]**	**5**	**6**	**7**	**6**	**6**	**60[e]**
0%	**10**	**10**	**10**	**10**	**10**	**100[g]**	**10**	**10**	**10**	**10**	**10**	**100[g]**

*LSD (ANOVA at $p<0.05$) on efficacy of Benomyl and Thiram as seed treatment fungicides for controlling bakanae disease caused by *F. fujikuroi* (MRC 5807).

CHAPTER 7

STUDIES ON THE PRODUCTION OF MYCOTOXINS BY SELECTED *FUSARIUM* SPECIES ISOLATED FROM RICE (*ORYZA SATIVA*)

7.1 Abstract

The ability of 3 representative isolates of *Fusarium anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and 3 isolates of *Fusarium fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) to produce mycotoxins were studied. All test isolates of *Fusarium* species were obtained from rice plants exhibiting bakanae disease in the fields of South Africa. All 3 isolates of *F. anthophilum* tested produced the mycotoxin moniliformin in quantities ranging from 32µg/g to 51µg/g. All 3 isolates of *F. fujikuroi* produced mycotoxin moniliformin in quantity ranging from 572µg/g to731 µg/g. None of the representative isolates of *F. anthophilum* and *F. fujikuroi* produced fumonisins (FB_1, FB_2, and FB_3). This is the first report on the production of moniliformin by *F. anthophilum* and *F. fujikuroi* isolated from rice plants with bakanae disease under South African field conditions.

7.2 Introduction

Fusarium species are found worldwide in geographical distribution and are well known as plant pathogenic and toxigenic fungi (Smith, 1971; Nelson *et al*., 1983; Marasas *et al*., 1984; Leslie *et al.,* 1996; Desjardins *et al*., 2000; Desjardins, 2006; Zainudin, 2008; Nayaka *et al*., 2011; Ghazanfar *et al*., 2013). They produce mycotoxins that negatively affect animal and human health (Scott *et al*., 1987; Peraica *et al*., 1999; Desjardins *et al*., 2000; Hussein and Brasel, 2001; Desjardins, 2006; Glenn, 2007; Nayaka *et al*.,

2011; Van Rensburg, 2012). Murray *et al*. (2005) reported that mycotoxins have been implicated in a variety of illnesses and clinical syndromes in humans and animals. Mycotoxins are secondary metabolites which are produced by various fungi and pose a continuous challenge to the safety and quality of food commodities in South Africa (Lezar and Barros, 2010). These toxins can cause acute or chronic toxicity in humans and animals that eat contaminated foods or crops. Manifestation of these symptoms however, depend on the quantities produced and consumed (Barrett, 2000; Lezar and Barros, 2010). Mycotoxins that adversely affect human or animal health are found on virtually any food or animal feeds which support fungal growth including cereal grains such as maize, rice, sorghum, wheat and oil seeds, ground nuts, cotton seeds, oil-palm kernels and copra (Marasas *et al*., 1984; Marasas and Nelson, 1987; Sydenham and Thiel, 1996; Desjardins *et al*., 2000; Glenn, 2007; Lezar and Barros, 2010; Van Rensburg, 2012).

The economic impact of mycotoxins include loss of human and animal life, increased health care and veterinary care costs, reduced livestock production, disposal of contaminated food and feeds, and investment in research and applications to reduce severity of the mycotoxin problem (Hussein and Brasel, 2001; Glenn, 2007).It is estimated that 25% of the world's food crops are affected by mycotoxins each year, which means that *Fusarium* species are playing an important role and substantially contribute to food contamination (Charmley *et al*., 1994; Mellor, 2003; Lezar and Barros, 2010; Van Rensburg, 2012).

The mycoflora are well known to produce various mycotoxins such as aflatoxin, beauvericin, chlamydosporol, enniatins, equistin, fumonisins, moniliformin, neosolaniol, trichothecene, ochratoxin A and zearalenone that negatively affect human and animal health (Marasas *et al*., 1984; Marasas and Nelson, 1987; Peraica *et al.,* 1999; Desjardins *et al*., 2000; Desjadins *et al*., 2006; Van Rensburg, 2012).

Fumonisins and Moniliformin Production

Fumonisins are a group of mycotoxins originally characterized from culture material of *Fusarium verticillioides* (*F. moniliforme*) strain MRC 826) (Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988). The strain was isolated from maize in the Transkei region of South Africa and was known to be hepatotoxic to ducklings and rats (Kriek *et al*., 1981; Colvin and Harrison, 1992). Fumonisins B_1 and B_2 have toxicological significance and while others (B_3, B_4, A_1 and A_2) have low concentrations and are less toxic (Peraica *et al*., 1999). It was shown that fumonisin B_1 is responsible for leukoencephalomalacia (ELEM) disease in horses (Marasas *et al*., 1988; Kellerman *et al.* 1990; Wilson *et al*., 1990; Thiel *et al*., 1991; Gelderblom *et al.,* 1992; Rheeder *et al*., 2002) and pulmonary edema in pigs (Harrison *et al*., 1990; Ross *et al.,* 1990; Gelderblom *et al*., 1992; Nelson *et al*., 1994; Sala *et al*., 1994; Maheshwar *et al*., 2009). Fumonisins have been associated with oesophageal cancer in humans (Marasas *et al.,* 1981; Marasas *et al.,* 1982; Sydenham *et al*., 1991; Thiel *et al*., 1992; Dutton, 1996; Van Rensburg, 2012). Fumonisins have been associated with a high incidence of oesophageal cancer in people living in South Africa, China and Italy (Murray *et al*., 2005). The fungi that produce fumonisins are well known pathogens of a variety of plants (Bhargava *et al*., 1978; Nelson *et al.,* 1983; Cartwright *et al*., 1995; Abbas *et al*., 1998; Desjardins *et al*., 2000; Desjardins, 2006). Fumonisins have been isolated in *Fusarium* spp. and *Alternaria spp*. (Abbas and Riley, 1996; Deasjardins, 2006). Frisvads *et al*. (2007) detected fumonisin B_2 in cultures of *Aspergillus niger* for the first time. Fumonisin B_2 production by strains of *A. niger* was shown again by Noonim *et al*. (2009) in Thai coffee beans. Morgensen *et al*. (2010) showed the production of fumonisin FB_2 and FB_4 by *A. niger* on grapes and raisins. Recently Soares *et al*. (2013) showed the production of FB_2 by *A. niger* aggregate strains isolated from harvested maize in three Portuguese regions.

The presence of fumonisins has been confirmed in at least twenty five countries of the world (Mazzani *et al*., 2001; Van Rensburg, 2012). Fumonisin B_1 accounts for 70-80% of total fumonisins produced, FB_2 usually makes up to 15-25% and FB_3 3-8% (Dilkin *et al*., 2002; Rheeder *et al*., 2002; Van Rensburg, 2012). International Agency for Research on Cancer (IARC) evaluated fumonisin B_1 (FB_1) as a possible carcinogen

(Group 2B) to humans (Domijan *et al*., 2005). International Agency for Research on Cancer grouped carcinogens into different categories such as Group 1(carcinogenic to humans), Group 2A (probably carcinogenic to humans), Group 2B (possibly carcinogenic to humans), Group 3 (not classifiable as to its carcinogenicity to humans) and Group 4 (probably not carcinogenic to humans). Carcinogens are classified as 2B on the basis of strong evidence from mechanistic and other relevant data (IARC, 1993; IARC, 2011).

Moniliformin production was reported from infected rice plants and rice seeds with bakanae disease caused by *Fusarium* spp. (Desjardins *et al.,* 1997; 2000; Desjardins, 2006). Moniliformin production in food and feed products has been reported from various *Fusarium* spp. world wide (Nelson *et al*., 1993; Rheeder *et al*., 2002; Sagaram and Shim, 2007; Saremi *et al*., 2008). Moniliformin was first reported from natural occurrence in mouldy maize from the Transkei region of South Africa (Thiel *et al*., 1982). In South Africa, moniliformin contamination of maize was associated with the presence of *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas (Thiel *et al.,* 1982; Desjardins, 2006). Moniliformin is produced by *F. verticillioides* a member of the section *Liseola* (Cole *et al.,* 1973; Rabie *et al*, 1982; Marasas *et al.,* 1984; Marasas *et al.,* 1986; Leslie *et al.,* 1996; Sydenham *et al*., 1997). There is however, considerable controversy surrounding its production as summarized by Marasas *et al*. (1986).The mycotoxin moniliformin (sodium or potassium salt of 1-hydroxycyclobut-1-ene-3, 4- Dione) is a highly toxic compound which was first isolated by Cole *et al*. (1973) from corn that had been inoculated with *F. verticillioides*. The mycotoxin caused the development of pathological lesions on the tissues of the hearts of experimental animals and subsequent death (Kriek *et al*., 1977; Burmeister *et al*., 1980; Allen *et al*., 1981; Zhao *et al*., 1993). Chelkowski *et al*. (1990) showed the production of moniliformin by *F. anthophilum* but they could not show production of moniliformin by strains of *F. chlamydosporum*, *F. compactum* and *F. equiseti*. Desjardins (2006) however reported that although the isolation of moniliformin was based on toxicity to 1 day old chicks, the mycotoxin has not been associated with any chronic or fatal animal disease outbreak. A strain of *F. fujikuroi* that produced

moniliformin but no fumonisin was crossed with a strain of *F. proliferatum* that produced fumonisin but no moniliformin (Desjardins, 2006).

Due to the regular isolation of *F. anthophilum* and *F. fujikuroi* during this investigation in South Africa, this study aimed to determine the ability of 3 representative isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) obtained from rice plants with bakanae disease to produce the mycotoxins fumonisins (FB_1, FB_2 and FB_3) and moniliformin with available resources and fund. Apart from risks assessment, to determine the ability of isolates of *F. anthophilum* and *F. fujikuroi* to produce the mycotoxins fumonisins and moniliformin may strengthen their identification as an individual biological species (Leslie *et al*., 1992a, Leslie *et al*., 1992b; Leslie *et al*., 1996).

7.3 Materials and Methods

Three representative isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and 3 isolates of *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) obtained from diseased rice plants and preserved in the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council (MRC), Tygerberg, South Africa, were used in this study. The isolates of *F. anthophilum* and *F. fujikuroi* were obtained from diseased rice plants exhibiting bakanae symptoms in the fields of North West province and Northern Cape province of South Africa.

Lyophilised conidia of the respective *Fusarium* isolates were suspended in sterile water and used to inoculate moistened yellow corn kernels (400g of kernels and 400 ml of water) in 2 liter glass fruit jars previously autoclaved at 121°C for 1 hour on each of 2 consecutive days prior to use. The fungal cultures on corn were incubated at 25°C for 21 days, dried overnight at 45°C, ground to a fine meal in a laboratory mill, and stored at 0°C until analysed as recommended by Thiel *et al.* (1982, 1991) and Marasas *et al*.

(1986). Corn was chosen as substrate for the production of fumonisins and moniliformin for the following reasons:

- Both Fumonisins and moniliformin are prevalent on corn and major contaminant of corn (Marasas *et al*., 1984; Sala *et al*., 1994; Dutton, 1996).
- Both mycotoxins were isolated from corn for the first time (Cole *et al*., 1973; Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988).
- Corn is considered as one of the crops more susceptible to mycotoxin in the world (Soares *et al*., 2013).
- It was expected that corn as a substrate would provide better condition for production of fumonisins and moniliformin for the fungal cultures.

Moniliformin Analyses:

Moldy meal prepared from *Fusarium* cultures were analysed by High Performance Liquid Chromatography (HPLC) as described by Thiel *et al*. (1982, 1991) and Marasas *et al*. (1986).The results obtained by employing a strong anion-exchange column was substantiated by separation and quantification of moniliformin using a paired ion chromatography (PIC) technique on a reverse phase column. HPLC analyses were carried out on both water extracts of the meal samples and water extracts prepurified on columns of DEAE-Sephadex. Sample extracts were prepared by shaking 3g of the dry meal with 40 ml of distilled water on a rotary shaker for 2h at room temperature in centrifuge tubes.The contents were then centrifuged for 20 min at 10 000g and the supernatant extracts were filtered through Millipore filters (0.45 µM) and retained for HPLC analyses or for pre-purification on a DEAE-Sephadex column.

Fumonisins Analyses:

Test samples were analysed for fumonisins (FB_1, FB_2, FB_3) by HPLC according to the method of Shepard *et al*. (1990) and Thiel *et al*. (1991). Briefly, samples were extracted

with methanol: water (3:1, vol/vol), centrifuged, and filtered. The filtrate was applied to a Bond-Elut SAX cartridge and eluted with 0.5% acetic acid in methanol. The solution was evaporated, and the residue was derivatized with o-phthaldihyde (OPA) and injected onto the HPLC column. The detection limit was 0.5µg/g.

7.4 Results

Results of the production of mycotoxin by all the test isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) are summarized in Table 7.1. All representative isolates of both *Fusarium* species produced moniliformin.The isolates of *F. anthophilum* such as MRC 5519 produced 32µg/g, MRC 5520 produced 48µg/g and MRC 5806 produced 51µg/g of moniliformin respectively.The isolates of *F. fujikuroi* such as MRC 5807 produced 731µg/g, MRC 5808 produced 669µg/g and MRC 5809 produced 572 µg/g of mycotoxin moniliformin respectively. Isolates of *Fusarium fujikuroi* produced much higher levels (10 fold) of moniliformin than *F. anthophilum*. The highest producer of moniliformin was *F. fujikuroi* (MRC 5807).The lowest producer of moniliformin was *F. anthophilum* (MRC 5519). All the representative 3 isolates of *F. anthophilum* produced moniliformin in quantities ranging from 32µg/g to 51µg/g.Three isolates of *F. fujikuroi* produced moniliformin in quantities ranging from 572µg/g to 731µg/g. None of the representative isolates of *F. anthophilum* and *F. fujikuroi* produced any chemically detectable fumonisins (FB_1, FB_2 and FB_3).

7.5 Discussion and conclusion

Moniliformin

The present study showed that all 3 strains of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and 3 strains of *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) isolated from diseased rice plants showing bakanae symptoms in the fields of North

West and Northern Cape provinces, South Africa could produce moniliformin. Marasas *et al*. (1986) reported that the largest quantities of moniliformin (up to 11717 mg/kg) were produced by isolates of *Fusarium* species isolated from rice in Taiwan, Japan and Philippines with bakanae disease that resembled *F. fujikuroi* (Nirenberg, 1976). The mycotoxin moniliformin was found to be toxic to ducklings. Marasas *et al*. (1986) also reported the production of moniliformin by isolates of *F. anthophilum* isolated from soil debris in New Guinea (up to 830 mg/kg) and from wheat in USA (up to 510 mg/kg).The moniliformin was found to be toxic to ducklings. *Fusarium proliferatum* isolated from various crops in different countries produced moniliformin up to 293 mg/kg (Marasas *et al*., 1986). Desjardins *et al*. (1997) showed high levels of moniliformin production (up to 7000μg/g) by isolates of *F. fujikuroi* obtained from rice. However, these isolates of *F. fujikuroi* could not produce fumonisins. External factors or environment conditions seem to play a lesser role in the production of fumonisins.

The mycotoxin moniliformin production in food and feed product has been reported from *Fusarium* spp. isolated from various countries of the world. (Nelson *et al.,* 1993; Desjardins *et al.,* 2000; Rheeder *et al*., 2002; Glenn, 2007; Sagaram and Shim, 2007; Saremi *et al*., 2008). Desjardins (2006) showed the production of moniliformin by isolates of *F. anthophilum*, *F. chlamydosporum*, *F. equiseti*, *F. fujikuroi and F. semitectum*.The production of moniliformin has also been reported by strains of *F. chlamydosporum*, *F. equiseti* and *F. semitectum* (Rabie *et al*., 1978, 1982; Marasas *et al*., 1984; Scott *et al*., 1987; Farber *et al*., 1988; Lezar and Barros, 2010).

Most studies on the investigation of mycotoxin from bakanae diseased rice plants and rice seeds confirmed the production of moniliformin by *F. fujikuroi* (Marasas *et al*., 1986; Desjardins *et al*., 1997; Desjardins *et al*., 2000; Desjardins, 2006).This finding is in good agreement with my observation with three isolates of *F. fujikuroi* isolated diseased rice plants with bakanae symptoms in South Africa.Three isolates of *F. fujikuroi* from diseased rice plants produced much higher levels (10 folds) of moniliformin than three isolates of *F. anthophilum.*

Three isolates of *F. anthophilum* tested for production of moniliformin confirmed the previous report (Marasas *et al*., 1986) on moniliformin production by isolates of *F. anthophilum* isolated from soil debris in New Guinea and from wheat in USA.This is the first report of production of moniliformin by *F. anthophilum* and *F. fujikuroi* strains isolated from rice (*Oryza sativa*) showing symptoms of bakanae disease in South Africa.

Fumonisins

In this study isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) were not able to produce fumonisins. The production of fumonisins by *Fusarium anthophilum* has been reported by many researchers (Chelkowski and Lew, 1992; Nelson *et al*., 1992; Sydenham *et al*., 1997; Riley *et al*., 1998; Rheeder *et al*., 2002). Fumonisins have been reported by isolates of *F. anthophilum*, *F. fujikuroi* and *F. konzum* (Desjardins, 2006). Lezar and Barros (2010) also reported the production of fumonisins by isolates of *F. anthophilum*, *F. compactum* and *F. semitectum*. However, a strain of *F. anthophilum* isolated from rice grain (*Oryza sativa*) in Australia did not produce fumonisins (Nelson *et al*., 1992). Six isolates of *F. anthophilum* from paddy (*Oryza sativa*) in India did not produce fumonisins (Maheshwar *et al*., 2009). The sole culture of *F. anthophilum* isolated from corn did not produce fumonisins (Sala *et al*., 1994).Thiel *et al*. (1991) reported that isolates of *F. anthophilum*, *F. compactum*, *F. equiseti* and *F. semitectum* failed to produce fumonisins. Therefore, it is clear that not all strains of *F. anthophilum* will produce fumonisins. Various factors operate interdependently to affect fungal colonization and or production of mycotoxins (Hussein and Brasel, 2001). D'Mello and MacDonald (1997) categorized the factors as physical factors including environmental conditions conducive to fungal colonization and mycotoxin productions such as temperature, relative humidity and insect infestation, chemical factors include the use of pesticide and or fertilizers and biological factors based on the interactions between the colonizing toxigenic fungal species and substrates. Therefore, it is not only just the inherent ability of the fungus to produce the specific metabolite.

Similarly the failure of production of fumonisins by the isolates of *F. fujikuroi* confirmed the findings of Desjadins *et al*. (2000) in which 5 isolates of *F. fujikuroi* obtained from rice in Nepal could not produce fumonisins. Recently, Cruz *et al.* (2013) showed the failure of production of fumonisin B_1 by 19 of the 32 strains of *F. fujikuroi* isolated from rice in the Philippines. Hussein and Brasel (2001) suggested that the production of mycotoxins by fungi is depended on the surrounding intrinsic and extrinsic environment.

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Table 7.1 Production of mycotoxins moniliformin and fumonisins by isolates of *Fusarium anthophilum* and *Fusarium fujikuroi* from bakanae diseased rice plants

Fungal isolates	Toxin's concentraion (µg/g)			
F. anthophilum	Mon	FB_1	FB_2	FB_3
MRC 5519	32	ND	ND	ND
MRC 5520	48	ND	ND	ND
MRC 5806	51	ND	ND	ND
F. fujikuroi				
MRC 5807	731	ND	ND	ND
MRC 5808	669	ND	ND	ND
MRC 5809	572	ND	ND	ND

Mon = Moniliformin

FB_1=Fumonisin B_1

FB_2= Fumonisin B_2

FB_3=Fumonisin B_3

ND = Not detected

CHAPTER 8

GENERAL DISCUSSION

This research study was undertaken to investigate pathogenic and toxigenic mycoflora associated with rice plants and rice seeds in South Africa and their negative impact. There is very scant information about the mycoflora associated with rice (*Oryza sativa*) under South African conditions thus prompting a study of this nature. Gorter (1977) only reported some fungal diseases of rice in South Africa but there was no recommendation on the control measures. Moreover, there was no information at all on the toxicity caused by these fungi associated with rice in South Africa. Fungi not only cause disease to plants, they can produce mycotoxins which are hazardous to human and animal health (Lezar and Barros, 2010), and constitute a factor for economic losses in food products worldwide (Omidbeygi *et al*., 2007; Pitt and Hocking, 2009).

Accurate species identification of plant pathogenic and toxigenic fungi is important for the development of effective disease control, management and quarantine policy.The ultimate goal being the protection of agricultural crops and natural resources for food security (Rossman and Palm Hernandez, 2008; Heng *et al*., 2011). Any error can have far reaching consequences impacting on biodiversity assessment, ecological studies and management decision (Bortolus, 2008). Even though rice cultivation is not well adapted in South Africa, rice is a staple food of many countries across the world. The main purpose of this research study was therefore to accurately isolate and identify fungi associated with diseased rice plants and rice seeds, their control measures, production of mycotoxins and to prevent their harmful impact.

Different species of fungi were isolated from diseased rice plants and rice seeds from different regions of South Africa. A total of six species of *Fusarium* were isolated and

identified as *F. anthophilum, F. chlamydosporum, F. compactum, F. equiseti, F. fujikuroi and F. semitectum.* The isolates of *Fusarium* species were initially identified on the basis of their morphological characters and the identifications of the representative isolates of *Fusarium* spp. were confirmed based on the translation elongation factor 1-α (TEF-1-α) gene (O'Donnell *et al*., 1998).

Fusarium species isolated from bakanae diseased rice plants in the warm areas of North West province and Northern Cape province were identified as *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809). Bakanae disease of rice had been described as early as 1828 by Konishi a semiliterate farmer in Japan (Ito and Kimura, 1931). The causal fungus was identified as *F. heterosporum* Nees by Hori (Hori, 1898). Later Fujikuroi discovered the perfect stage and this was described as *Lisea fujikuroi* by Sawada (Sawada, 1917). Sawada was uncertain as to whether the imperfect stage of *Lisea fujikuroi* was identical with *F. heterosporum*. Ito and Kimura (1931), after careful comparison of this fungus with *Gibberella moniliforme*, concluded that it was indeed *Gibberella fujikuroi* (Sawada, 1917).The anamorph was considered to be *F. moniliforme* by Booth (1971) but Nirenberg (1976) differentiated it as a separate species, *F. fujikuroi*. Nelson *et al*. (1983) did not accept *F. fujikuroi* as a separate species but included it in *F. moniliforme* as the "short chained" type of *F. moniliforme*. Marasas *et al*. (1986) reported the presence of polyphialides in *Fusarium* isolates from rice with bakanae disease ("bakanae strains") and excluded them from *F. moniliforme*. These authors concluded, however that the use of the name *F. fujikuroi* (Nirenberg, 1976) for these cultures would create the problem of separating this species from *F. proliferatum* only on the basis of the host plant. On the basis of studies on the ultrastructure of collarette formation, Tiedt and Jooste (1988) concluded that *F. fujikuroi* could be clearly differentiated from *F. moniliforme* (*F. verticillioides*). However, this fungus could not be differentiated from *F. proliferatum*. Phylogenetically, *F. proliferatum* is closely related to the rice pathogen *F. fujikuroi,* teleomorph, *G. fujikuroi*; mating population-C (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998b; Malonek *et al*., 2005; Glenn, 2007). Both *F. fujikuroi* and *F. proliferatum* have been reported to be interfertile, though the level of fertility is generally reduced

compared to intraspecific crosses (Desjardins *et al*., 1997; Leslie *et al.,* 2004; Glenn; 2007). In terms of biological species concept, such interfertility suggests the two species may actually represent variants of the same species (Leslie *et al.,* 2004). The mycotoxin profiles of *F. fujikuroi* and *F. proliferatum* are very distinct. Some strains of *F. fujikuroi* produce low levels of fumonisins but most strains do not produce fumonisins while these (non fumonisins producers) strains of *F. fujikuroi* produce significant quantities of moniliformin (Desjardins *et al*., 1997; Desjardins *et al*., 2000; Proctor *et al.,* 2004; Glenn, 2007). Some strains of *F. proliferatum* were shown to produce significantly greater quantities (6000$\mu g/g$) of fumonisins (Leslie *et al*., 2004; Desjardins, 2006). Based on phylogenetic analysis, the strains of *F. fujikuroi* were found distinct from *F. proliferatum* strains isolated from rice in the Philippines (Cruz *et al*., 2013). These studies showed the production of fumonisin B_1 by 13 of 32 strains of *F. fujikuroi* and found no relationship between fumonisin production and pathogenicity. Where as, Glenn *et al.* (2008) adviced that fumonisin production by *F. verticillioides* is required for development of foliar disease symptoms on maize seedlings.

Marasas of PROMEC Medical Research Council, Tygerberg, South Africa confirmed the identity of the isolates of *Fusarium* spp. from rice in South Africa and expressed a concern that the three isolates MRC 5807, MRC 5808 and MRC 5809 identified as *F. fujikuroi* could not be differentiated morphologically from *F. proliferatum*.Thus these three strains could either be called "*F. fujikuroi*" (because they were isolated from rice plants with bakanae disease) or "short-chained strains of *F. proliferatum"*.

Desjardins *et al*. (2000) reported that bakanae disease of rice is caused by one or more *Fusarium* species and is a complex of disease symptoms that include root and crown rot, abnormal elongation of stems, wilting, stunting and the formation of adventitious roots at nodes on the lower portions of stems (Yamanaka and Honkura, 1978; Ou, 1985; Webster and Gunnell, 1992). Carter *et al*. (2008) reported *F. fujikuroi* as the causal pathogen of bakanae disease of rice (*Oryza sativa*), water grass (*Echinochloa oryzoidis*) and barnyard grass (*Echinochloa cruss- galli*) in California of USA. Apart from *F. fujikuroi* some strains of *F. verticillioides* and *F. proliferatum* have been isolated from

bakanae infected rice seedlings from various geographical areas (Desjardins *et al*., 1997). *Fusarium fujikuroi* has been reported as the only *Fusarium* species involved in bakanae disease of rice in Malaysia and Indonesia (Zainudin *et al*., 2008). Amatulli *et al*. (2010) also reported that *F. fujikuroi* was the only fungus could cause bakanae disease of rice in Italy. *Fusarium fujikuroi, F. proliferatum and F. verticillioides* of *G. fujikuroi* species complex have showed varying ability to cause symptoms of bakanae disease on rice plants (Wulff *et al*., 2010; Heng *et al*., 2011). These studies found *F. fujikuroi* as being more pathogenic than others. *Fusarium fujikuroi* the causal pathogen of bakanae disease of rice can produce the plant hormone gibberellins (Sun and Snyder, 1981; Desjardins, 2006; Zainudin *et al*., 2008; Bomke *et al*., 2009; Amatulli *et al.,* 2010; Wulff *et al*., 2010) which play a role in causing bakanae symptoms. Bomke *et al*. (2008) showed the presence of the gibberellic acid (GA) gene cluster and the ability to produce GAs in the *G. fujikuroi* species complex.These studies could not find any GA- non producing *F. fujikuroi* strain isolated from rice, while almost all the other *Fusarium* species of *G. fujikuroi* species complex with different host plants have lost the ability to produce GAs due to multiple mutations in some GA cluster genes. Other genes in these clusters still encode functional enzymes, as they were able to complement the corresponding mutants of *F. fujikuroi* and restore the GA biosynthesis capability.

Fusarium anthophilum has been isolated from Copra in Trinidad (Gordon, 1956) and from oats in South Africa (Thiel *et al*., 1991). Nelson *et al*. (1992) reported the isolation of *F. anthophilum* from rice in Australia and pearl millet in Zambia. *Fusarium anthophilum* was found in paddy grains in Egypt (Abdel-Hafez *et al*., 1987) and isolated from cultivated rice (*Oryza sativa*) and wild rice (*Zizania palustris*) with head blight symptoms in Minnesota of USA (Nyvall *et al*., 1999), but pathogenicity has not been reported. It has also been found to reduce the height of wheat seedlings in Argentina (Mantecon *et al*., 1984) and cause wilting and damping off on sunflower in Pakistan (Sharfun and Mushtaq, 2006).

In pathogenicity test in the present study, all the representative strains of *F. anthophlum* (MRC 5519, MRC5520, MRC 5806) and *F. fujikuroi* (MRC 5807, MRC 5808, MRC

5809) isolated from rice plants caused bakanae disease in four different rice cultivars/ lines. The disease incidence caused by the strains of *F. anthophilum* varied from 70 to 95% on a 0-100 disease index scales and the disease incidence caused by the strains of *F. fujikuroi* varied from 78 to 100 % on a 0-100 disease index scales.This is the first occurrence of bakanae disease of rice in South Africa. Both *F. anthophilum* and *F. fujikuroi* are new records as pathogens of rice in South Africa, although Gorter (1977) reported "*F. moniliforme*/*Gibberella fujikuroi* to be associated with foot rot of rice in South Africa.To the best of my knowledge, *F. anthophilum* has not been reported before from any other country in the world as a pathogen of rice (*Oryza sativa*) and or as causative fungus of bakanae disease.

All the *Fusarium* species isolated from rice in this research are well known as mycotoxin producers. Representative isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and of *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) produced mycotoxin moniliformin.The isolates of *F. fujikuroi* (MRC 5807, MRC 5808 and MRC 5809) produced much higher levels (10 fold) of mycotoxin moniliformin than the isolates of *F. anthophilum*.None of the isolates of *F. anthophilum* and *F. fujikuroi* produced fumonisins (FB_1, FB_2 and FB_3).The production of mycotoxins by fungi is depended on the surrounding intrinsic and extrinsic environments (Hussein and Brasel, 2001).This is the first report on the production of mycotoxin moniliformin by *F. anthophilum* and *F. fujikuroi* isolated from rice with bakanae disease in South Africa.

Fifty four rice cultivars and lines were tested against the most pathogenic bakanae strains (MRC 5806) of *F. anthophilum* and (MRC 5807) of *F. fujikuroi* for resistance and susceptibility of each cultivar/line as a part of economic and environmental friendly method of disease control of rice in South Africa. Most of the rice cultivars and lines were found susceptible to moderately susceptible against bakanae disease caused by the pathogenic strain (MRC 5806) of *F. anthophilum*. The rice cultivar E7034 was the only cultivar found to be moderately resistant to bakanae disease caused by the pathogenic strain (MRC 5806) of *F. anthophilum*. None of the test cultivars/lines were found to be resistant against this strain. The disease incidence caused by this strain

varied from 40 to 100%. The cultivar E7034 showed 40% disease incidence caused by the strain MRC 5806 of *F. anthophilum*. Other rice cultivars and lines showed disease incidence from 60 to 100%.

Most of the rice cultivars and lines were found susceptible or moderately susceptible to bakanae disease caused by the most pathogenic strain MRC 5807 of *F. fujikuroi*. Only cultivar E7034 and rice line RP2199-16-2-2-1 were found to be moderately resistant to bakanae disease caused by the strain MRC 5807 of *F. fujikuroi*. None of the test rice cultivars and rice lines was found to be resistant to bakanae disease caused by the strain MRC 5807 of *F. fujikuroi*. The disease incidence caused by the strain MRC 5807 of *F. fujikuroi* varied from 43 to 100%. Cultivar E7034 and rice line RP2199-16-2-2-1 showed 43% disease incidence caused by the strain MRC 5807 of *F. fujikuroi*. Other cultivars and lines showed disease incidence from 53 to 100%.

Comparative effectiveness of two fungicides (benomyl 50% WP and thiram 50% WP) at different concentrations (0%, 0.10%, 0.15%, 0.20% and 0.25%) were evaluated as seed treatment to control bakanae disease of rice caused by one of the most pathogenic strains of *F. anthophilum* (MRC 5806) and the pathogenic strains of *F. fujikuroi* (MRC 5807). Benomyl was found most effective to control bakanae disease against the strain MRC 5806 of *F. anthophilum* and against strain MRC 5807 of *F. fujikuroi*. Benomyl is well known as systemic fungicide and comparatively environmental friendly. Thiram was not found effective as a seed treatment fungicide to control bakanae disease caused by both *F. anthophilum* (MRC 5806) and *F. fujikuroi* (MRC 5807).

Fusarium chlamydosporum (MRC 7368) and *F. compactum* (MRC 7369 and MRC 7370) were isolated from rice seeds of warm area of Mpumalanga province. This is the first report of the isolation and identification of these fungi from rice in South Africa. These fungi are well known as both pathogenic and toxigenic to plants. They have a negative impact on human and animal health.

Fusarium equiseti (MRC 5817, MRC 5818 and MRC 5819) was also isolated from diseased rice plants, but those with sheath rot symptoms. The fungus was isolated from rice plants in the warm areas of North West Province, Northern Cape Province and cool areas of Free State Province. The fungus is also well known as being both pathogenic and toxigenic. *Fusarium equiseti* is a member of the *F. incarnatum-equiseti* Species Complex (FIESC). Of the 28 species within the FIESC, 9 species within the *equiseti* clade and 11 within the *Incarnatum* clade were recovered from mycotic infections. Species rich FIESC comprises at least 20 mycoses-associated species among the 28 reciprocally monophyletic lineages resolved by multilocus molecular phylogenetics (O' Donnell *et al.,* 2009).The South African isolates from rice were clustered together in a single clade with the *F. equiseti* and *F. incarnatum* isolates forming two separate sub-clades.The South African isolates of *F. equiseti* from rice clustered with none of 28 phylogenetic lineages in the FIESC (O'Donnell *et al.,* 2009).The fungus has been reported to produce type A trichothecene (T2), zearalenone and moniliformin (Hussein *et al*., 1991).There have been no previous findings of this fungus in rice in South Africa.

The fungus *F. semitectum* (MRC 7363, MRC 7364, MRC 7365, MRC 7366 and MRC 7367) was isolated from rice seeds in the warm areas of Mpumalanga Province of South Africa. It has previously been shown to exist in rice seeds in Argentina (Broggi and Molto, 2001). *Fusarium semitectum* has also been reported by Sergio *et al*. (1997) in Argentina and Paraguay and found to have ability to produce fumonisins and moniliformin (Lezar and Barros, 2010). No previous studies have identified this fungus from rice in South Africa.

Among other fungi, *Alternaria alternata*, *Alternaria longipes*, *Cochliobolus miyabeanus*, *Nigrospora sphaerica*, *Phoma eupyrena*, *Phoma jolyana*, *Phoma sorghina* and *Pithomyces* species that were found in association with diseased rice plants and rice seeds in South Africa. Except, *Phoma eupyrena* and *Phoma jolyana*, all other fungi are also regarded as pathogenic and toxic to plant and human and animal health.

There are risks of contamination of rice with mycotoxins both pre-harvest and in post-harvest storage in South Africa. In this research, a total of six toxigenic *Fusarium* spp. were isolated from diseased rice plants in the fields and from rice seeds in storage. Desjardins *et al*. (2000) was able to isolate 11 *Fusarium* spp. from paddy samples from fields at the foot hills of the Himalayas in Nepal. Apart from *Fusarium* species, other toxigenic fungi were also isolated from the diseased rice plants in the fields and from rice seeds in storage in South Africa.

Mycotoxin contamination in agricultural commodities has been a serious concern for human and animal health.The contamination of foods and feeds with mycotoxins is a significant problem and studies have shown extensive mycotoxin contamination in both developing and developed countries (Hussein and Brasel, 2001). During a review (Fink-Gremmels, 1999; Hussein and Brasel, 2001), it was estimated that 25% of the world's crop may be contaminated with mycotoxins. Surveillance studies (Placinta *et al*., 1999; Hussein and Brasel, 2001; Lezar and Barros, 2010) showed worldwide contamination of cereal grains and other feed with *Fusarium* mycotoxins and which is a global concern.

Mycotoxin contamination is less commonly reported for rice than many other cereal crops (Reddy *et al*., 2008), but rice represents a very good substrates for fungal growth and toxinogenesis since it is used as an ideal culture medium to test the toxigenic potential of fungal isolates (Bars and Bars, 1992; Reddy *et al*., 2008). Among the rice mycotoxins, aflatoxin B_1, fumonisin B_1 and ochratoxin A are the most toxic for mammals and have hepatotoxic, teratogenic and mutagenic activity, causing toxic hepatitis, hemorrhage, edema, immunosuppresssion, hepatic carcinoma equine leukoencephalo malacia, oesophageal cancer and nephrotoxicity (Altuntas *et al*., 2003; Reddy *et al*., 2008). Aflatoxin B_1 has been classified as a group 1 human carcinogen (The agent is carcinogenic to human) and fumonisin B_1 and B_2 as group 2 B carcinogens (The agent is possibly carcinogenic to humans) by the international Agency for research on Cancer (IARC, 1993; IARC, 2011). Several mycotoxicoses in humans and animals have been reported due to the consumption of mycotoxin-contaminated food and feed (Peraica and Domijan, 2001; Reddy and Raghavender, 2007; Reddy *et al*., 2008).

In this investigation, isolates of both *F. anthophilum* and *F. fujikuroi* produced moniliformin which has been implicated in hepatic carcinoma equine leukoencephalo malacia and acute neurotoxicity (Gelderblom and Snyman, 1991; Placinta *et al.,* 1999; Hussein and Brasel, 2001). These two diseases were attributed to consumption of corn contaminated with fumonisin B_1 and moniliformin and have been implicated in hepatic tumor formation in rats (Gelderblom and Snyman, 1991; Hussein and Brasel, 2001).

Mere isolation and confirmation of mycotoxigenic fungal species in foods or feeds does not indicate the presence of mycotoxins. Researchers have found that various factors operate interdependently to affect fungal colonization and/or production of mycotoxins (Hussein and Brasel, 2001). D'Mello and MacDonald (1997) categorized the factors as physical, chemical and biological. Physical factors include the environmental conditions conducive to fungal colonization and mycotoxin production such as temperature, relative humidity and insect infestation. Chemical factors include the use of fungicides and/or fertilizers. Stress such as drought, an increase in temperature, and an increase in relative humidity may selectively alter colonization and metabolism of mycotoxigenic fungi and alter mycotoxin production (Hussein and Brasel 2001; Russel *et al*., 1991). These researchers also indicated that unseasonable conditions may render crops and forages susceptible to mycotoxin production.The biological factors are based on the interactions between the colonizing toxigenic fungal species and substrates. While some plant species are more susceptible to colonization, environmental conditions may increase the vulnerability of other more resistant plant species (Hussein and Brasel, 2001; Russel *et al*., 1991).

Mycotoxin contamination of foods or feeds may result from inadequate storage and/or handling of harvested products. Prevention and control methods have been prescribed for mitigating mycotoxin contamination of feeds (Harris, 1997; Hussein and Brasel, 2001). These methods require that feed handlers and grain mill operators keep grains at less than 14% moisture. Feed ingredients must be dry, oxygen free, fermented or treated with mold growth inhibitors. With regard to silage crops, harvesting at the appropriate moisture content and both packing and sealing the silo (to exclude oxygen

and allow for desirable anaerobic fermentation) are essential for reducing mycotoxin contamination potential. Wet and dry milling processes as well as heat in the cooking process have been shown to reduce aflatoxin in foods (Scott, 1984). Heating and roasting have been shown to significantly decrease aflatoxin content in corn (Conway *et al*., 1978; Hale and Wilson, 1979).

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CHAPTER 9

CONCLUSIONS, RECOMMENDATIONS AND FURTHER AREAS OF RESEARCH

In this research, a total of six species of *Fusarium* were isolated from diseased rice plants and rice seeds and identified as, *F. anthophilum, F. chlamydosporum, F. compactum, F. equiseti, F. fujikuroi and F. semitectum*. All the *Fusarium* species are well known as plant pathogens. However pathogenicity of *F. compactum* with rice (*Oryza sativa* or *Oryza glaberrima*) has not been reported from any other country in the world. Therefore, it is recommended that further research on the pathogenicity of *F. compactum* with rice should be conducted. In the pathogenicity test in this study, representative isolates *F. anthophilum* and *F. fujikuroi* evidently caused bakanae disease. This is the first report of such an occurrence in South Africa. Therefore both *F. anthophilum* and *F. fujikuroi* are new records as pathogens of rice in South Africa.

All the *Fusarium* species isolated from rice in the present investigation are well known as toxin producers which could have negative impact on human and animal health. Representative isolates of *F. anthophilum* (MRC 5519, MRC 5520 and MRC 5806) and *F.fujikuroi* (MRC5807, MRC5808 and MRC 5809) produced mycotoxin moniliformin.The isolates of *F. fujikuroi* produced much higher levels (10 fold) of moniliformin than the isolates of *F. anthophilum*. There is no substantial report of production of gibberellic acid by *F. anthophilum*. However, *F. anthophilum* (as *moniliforme* var. *anthophilum*) has been reported to produce substances with gibberellin-like biological properties (Gordon, 1960; Marasas *et al*., 1984). Gibberellic acid is associated with bakanae symptoms. Therefore, there is a need for further research on the molecular biology of *F. anthophilum* and the potential to produce gibberellic acid. None of the isolates of *F. anthophilum* and *F. fujikuroi* produced fumonisins (FB_1, FB_2, and FB_3). Once again, this

is the first revelation on the production of mycotoxin monilifomin by *F. anthophilum* and *F. fujikuroi* isolated from rice with bakanae disease in the fields of South Africa.

Fifty four rice cultivars and lines were tested by the standardized test tube inoculation method for resistance and susceptibility against bakanae isolate MRC 5807 of *Fusarium fujikuroi* and bakanae isolate MRC 5806 of *Fusarium anthophilum*. No rice cultivars and rice lines were found resistant against bakanae isolates of *Fusarium* spp. Therefore, it is recommended that further research be conducted to find disease resistant rice cultivars suitable for South African conditions. NERICA (New Rice for Africa) is well known rice cultivar for disease and insect control. The ability of NERICA against bakanae disease needs to be established in local field trails.

Benomyl 50% WP was found most effective for controlling bakanae disease of rice due to isolates of *F. anthophilum* and *F. fujikuroi*. Thiram was found most ineffective for controlling bakanae disease of rice due to both the *Fusarium* spp.Therefore, it is recommended to use benomyl 50% WP as seed treatment fungicide for controlling bakanae disease of rice. Even though bakanae disease can be controlled by the correct application of Benomyl, the risk of pesticide use on the environment can be mitigated by investigating alternative methods for disease control such as Integrated Pest Management or biological control.

In the translation elongation factor (TEF) data set, *Fusarium equiseti* isolates from rice in South Africa grouped together within the *F. incarnatum* - *F. equiseti* Species Complex, FIESC. The South African isolates from rice cluster together in a single clade with the *F*. *equiseti* and *F. incarnatum* isolates forming two separate subclades. The South African isolates clustered with none of 28 phylogenetic lineages in the FIESC (O'Donnell *et al.,* 2009). The isolates proved to present a new phylogenetically distinct species in FIESC. The species-rich FIESC comprises at least 20 mycoses associated species among 28 reciprocally monophyletic lineages resolved by the multilocus molecular phylogenetics.Therefore, it is important to do further molecular biology

research on the strains of *F. equiseti* isolated from rice in South Africa and their production of mycotoxins.

Numerous other species of fungi apart from *Fusarium* species that were also important fungi under investigation might also play a significant role in rice disease and toxin production in storage. These aspects require further investigation an example being well known fungal pathogen *Cochliobolus miyabeanus*.The fungus *Cochliobolus miyabeanus* that causes brown spot disease of rice.

Since South Africa imports large quantities of rice from foreign countries for consumption, the risks associated with contamination of rice with mycotoxins under storage can be ameliorated when further research is undertaken to understand the biological/biochemical processes that takes place under storage.

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