

Detection of *Fusarium* spp. on maize kernels

by

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Declaration

I hereby certify that this research, unless specifically indicated to the contrary in the text, is a result of my own investigation and that no part of this thesis has been submitted to any other university.

Renaan Shane Thompson

Abstract

In terms of amount of tonnes produced each year, *Zea mays* also known as maize and corn is the most important crop. Fungi cause significant destruction of maize in the field as well as during storage rendering the grain unsuitable for human consumption by decreasing its nutritional value and by producing mycotoxins that are detrimental to both human and animal health. *Fusarium* species are widely distributed and are amongst the most frequently isolated fungal species by plant pathologists and due to the fact that the *Fusarium* species involved in maize ear rot vary in fungicide sensitivity, pathogenicity as well as in their capability to produce mycotoxins, accurate quantification and identification is of paramount significance. There is currently no method developed for *Fusarium* detection in maize seed that has been validated by ISTA (the International Seed Testing Association). Malachite green agar 2.5 ppm (MGA 2.5) is a potent selective medium for isolation and enumeration of *Fusarium* spp. In this study, eight different media compositions, potato dextrose agar (PDA), PDA + malachite green oxalate, corn meal agar, ½ PDA + malachite green oxalate, 1% malt agar, carnation leaf agar supplemented with potassium chloride (KCLA), malachite green agar (MGA 2.5) and MGA 2.5 + sterile carnation leaf pieces (MGA 2.5 +) were compared using four *Fusarium* species (*F. graminearum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides*) and five commonly encountered saprophytic fungi (*Aspergillus niger*, *Penicillium crustosum*, *P. digitatum*, *Trichoderma harzianum* and *Rhizopus stolonifer*). The maize kernels were surface disinfected using three concentrations of sodium hypochlorite (0.5%, 1% and 1.5%) and for different time intervals (1min, 3min, 5min and 10min). The effect of black-blue light (365nm) on sporulation of the fungi was also investigated. 200 maize seeds from two seed lots were surface disinfested and plated out on PDA, KCLA, MGA 2.5 and MGA 2.5 and incubated at 25°C for 7d under 12h black-blue light/12h darkness. PDA, ½ PDA, 1% malt agar and KCLA allowed profuse growth of the *Fusarium* species as well as saprophytes. Media that contained malachite green oxalate was most inhibitory to the radial colony growth of the saprophytes and the *Fusarium* species. *Fusarium* species growing on these media formed under-developed morphological structures, thereby obscuring accurate identification. MGA 2.5 supplemented with sterile carnation leaves was the most satisfactory medium in hindering saprophytic growth while allowing adequate sporulation of the *Fusarium* species to permit correct identification. The media also

had a higher *Fusarium verticillioides* and less saprophytic fungal isolation frequency when compared to the other media tested. The location of *Fusarium verticillioides* within maize seed was also investigated and found to be only associated within the upper pedicel part of the kernel.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Motivation for the study

Zea mays (L.), known also as corn and maize, belongs to the family Poaceae (Farnham, 2003). FAOSTAT database (2012) revealed that the crop is the leading crop according to the amount of tonnes produced each year with 12 815 000 tonnes produced in South Africa in 2008 followed by wheat with 1 464 970 tonnes. Based on molecular, isozyme and cytological data collected on the origin of maize, many investigators are convinced that maize is most likely a descendant of teosinte (*Zea mays* ssp. *Parviglumis*) native to Mexico, although other evidence suggests that the crop may have originated from Asia or Africa (Inglett, 1970).

Troxell (1996) revealed that maize is regarded as the most important agricultural crop since it is considered the key ingredient in animal feed all over the world and the human dietary staple in many regions of Asia, Africa and central South America. Until the past decade, the plant was the most studied of the plant species that encompasses a significant economic value and serves as the main staple food for millions of people (Messing and Dooner, 2006). Fungi cause significant destruction of maize in the field as well as during storage (Shurtleff, 1980) rendering the grain unsuitable for human consumption by decreasing its nutritional value (Velluti *et al.*, 2004) and by producing mycotoxins that are detrimental to both human and animal health (Nicolaisen *et al.*, 2008).

With thousands of diverse species that infect different agricultural commodities and dried grains used for food or feed staples, *Fusarium* species are widely distributed and are amongst the most frequently isolated fungal species by plant pathologists (Mohamed *et al.*, 2003). Due to the prevalence of *Fusarium* species on agricultural crops worldwide (Leslie and Summerell, 2006; Robledo-Robledo, 1991), a number of different media were developed for the isolation and enumeration of *Fusarium* species from seed, soil as well as plant material (Tschanz, 1975; Nelson *et al.*, 1981; Nelson *et al.*, 1983; Dhingra and Sinclair, 1995; Castellà *et al.*, 1997; Gamanya and Sibanda, 2001; Castellà *et al.*, 2004; Leslie and Summerell, 2006).

ISTA (the International Seed Testing Association) currently does not have a method to test for any *Fusarium* species in maize seed (ISTA 2011). As maize needs to be tested in under-developed countries that often do not have the resources for molecular equipment and PCR methods, ISTA requested that the University of Pretoria develop an agar medium-based method for the detection of *Fusarium* spp. on maize that can be used worldwide.

Due to the fact that the *Fusarium* species involved in maize ear rot vary in fungicide sensitivity, pathogenicity as well as in their capability to produce mycotoxins, accurate quantification and identification is of paramount significance (Nicolaisen *et al.*, 2008).

1.2 Aim of the study

The aim of this study was to evaluate different media that are used for the isolation and enumeration of *Fusarium* species and to develop a method to test for the presence or absence of certain *Fusarium* spp. from maize seed. The method has to be within ISTA's operating procedures since the method has to go through ISTA's validation process. The location of *Fusarium verticillioides* within maize seed was also investigated as well as molecular identification of the *Fusarium* species tested for.

1.3 Objectives of the study

The specific objectives of the study were to:

*Identify the isolates of *Fusarium graminearum*, *Fusarium subglutinans*, *Fusarium proliferatum* and *Fusarium verticillioides* using their morphological characteristics and confirm their identification by using molecular techniques.

*Evaluate and develop an *in vitro* method to detect the presence or absence of the above mentioned *Fusarium* species within maize seed.

*To investigate the location of *Fusarium verticillioides* within maize seed using scanning electron microscopy.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Zea mays (L.), commonly known as maize and referred to as Indian corn in the United States of America, belongs to the *Poaceae* family (Farnham *et al.*, 2003). The crop was introduced to Africa from South America in the 16th century (FAO, 1996) and although it is widely distributed throughout the world, the origin of the crop is still unknown. There has been many hypotheses formulated over the years in an attempt to try and indicate the botanical origin of maize and due to a considerable amount of molecular, cytological and isozyme data gathered on the descent of maize, many investigators are convinced that there is a strong probability that maize is a descendant from teosinte (*Zea mays* ssp. *Parviglumis*) native to the Balsas River Valley on the Pacific slopes of the states of Michoacán and Guerrero, Mexico (Galinat, 1977; Piperno and Flannery, 2001).

Zea mays is cultivated in a wide range of climatic conditions ranging from warm temperate areas in the humid sub-tropical regions in addition to the tropics (Berger, 1962) where the bulk of maize is produced between 30 and 55°C (Shaw, 1988). The production of maize in 2008 was estimated at 823 million tonnes making it the most important cereal crop in terms of tonnes of grain produced (FAO, 2010).

It is a cereal crop that is cultivated throughout the world, serves as a basis and forms a vital role in the diet of millions of people in Africa due to its ease of cultivation and adaptableness to diverse agro-ecological zones, significantly high yields per hectare, multipurpose food usages and storage potential (Asiedu, 1989). The increase of the availability of this commodity becomes a real necessity in rural areas where there is a high reliance on basic grains such as maize (Moreno-Martinez *et al.*, 1998).

Maize is subjected to a wide range of pathogens in the field as well as in storage which include bacteria, nematodes, viruses, parasitic plants, insects and fungi, and of all the pathogens that attack maize, fungi are mainly responsible for the majority of the diseases in maize (Shurtleff, 1980). It was reported by Kossou and Aho (1993) that during the

storage of maize, fungi can cause 50 - 80% damage of the maize if the environments are encouraging for their development. The most commonly encountered genera in tropical regions are *Penicillium*, *Aspergillus* and *Fusarium* (Samson, 1991).

2.2 The Pathogens: *Fusarium graminearum*, *Fusarium subglutinans*, *Fusarium proliferatum* and *Fusarium verticillioides*

The genus *Fusarium* was established by Link in 1809 (Leslie and Summerell, 2006), more than 200 years ago and includes a various array of species of importance for being devastating plant pathogens that frequently produce an extensive range of secondary metabolites (Desjardins, 2006). This genus embodies one of the most important groups of ascomycetous fungi (Kvas *et al.*, 2008).

This genus has been given predominantly more attention (Booth, 1971, as cited by Liddell, 1991), and according to Robledo-Robledo (1991), they invade 50% or more of maize grains before harvest and are considered field fungi. Their population in agricultural fields can exceed 100 000 propagules per gram of soil and even more (Nelson *et al.*, 1981).

Fusarium species are widely distributed in plants, soil and air (Nelson *et al.*, 1981). They are commonly encountered in temperate and tropical regions but are also found in alpine, arctic and desert areas (Dignani and Anaissie, 2004).

No less than 80% of all cultivated plants are coupled with at least one disease caused by a *Fusarium* species (Leslie and Summerell, 2006). The genus has a worldwide distribution and numerous species within the genus infect a widespread array of crops including cereals such as oats, wheat, barley and maize (Nicolaisen *et al.*, 2008) resulting in affected growth and yield loss that leads to losses of billions of dollars to farmers each year throughout the world (Fry, 2004).

Fusarium contamination is a key agricultural difficulty, although more notably many species within the genus are prolific producers of mycotoxins that are implicated for severe diseases in farm animals as well as in humans (Nicolaisen *et al.*, 2008).

Fusarium species are agents of hyalohyphomycosis, which is a term that describes fungal infections caused by moulds whose basic tissue form is in the nature of hyaline, light-coloured, hyphal elements that are branched or unbranched, with or without pigmentation in their wall and are occasionally toruloid (Ajello, 1986).

The discovery of *Fusarium* mycotoxicology began in 1961 with the discovery of the structure of the trichothecene-diacetoxyscirpenol and was confirmed in 1991 with verification of the carcinogenicity of fumonisins in experimental rodents (Desjardins, 2006).

The key mycotoxin classes of concern produced by *Fusarium* species include, but are not limited to the zearalenone, trichothecenes, fumonisins and as well as other minor mycotoxins such as beauvericin, fusaproliferin, fusarins and moniliformin (Desjardins, 2006; Leslie and Summerell, 2006 Glenn, 2007). The mycotoxins induce a range of symptoms in animals that include equine leucoencephalomalacia, tremors, staggers, convulsions paralysis and even death (Morgavi and Riley, 2007).

The relationship amongst some of these metabolites with negative effects on growth and development of animals, cellular toxicity and cancer in domesticated animals and humans is of specific interest to agriculture and food safety (Glenn, 2007). The role of fumonisins in equine leucoencephalomalacia, swine pulmonary edema, high incidence of esophageal cancer in humans consuming contaminated maize in some regions of South Africa and China, carcinogenesis and neural tube defects in experimental rodents ; of trichothecenes in hemorrhagic syndrome and feed refusal in several species of animal as well as dermatitis in humans; and of zearalenones in swine estrogenic syndrome, has been proved (Marasas *et al.*, 1984; Marasas *et al.*, 1988a; Thiel, 1992; Nelson *et al.*, 1992; Marasas, 1995; Gooding and Davies, 1997; Blechl *et al.*, 2005; Leslie and Summerell, 2006; Desjardins, 2006; Logrieco *et al.*, 2007; Pestka, 2007).

Fumonisins are polyketide mycotoxins that are produced by numerous *Fusarium* species (Glenn, 2007). Fumonisins B1 are sufficiently thermo stable and are not destroyed by conventional heat during cooking as well as processing of the maize grain (Dupuy *et al.*, 1993). Fumonisins are sphinganine analog mycotoxins that have a basic similarity to free sphingoid bases that exists in

all classes of sphingolipids, which are a diverse class of lipids consisting of in excess of 300 individual compounds (Merrill *et al.*, 2001).

The structural correlation between free sphinganine and fumonisins lead to the discovery that fumonisins are potent and precise inhibitors of the mammalian acyl CoA-dependent ceramide synthase (Wang *et al.*, 1991). Even though fumonisins have a moderately simple chemical structure, their ability to inhibit sphingolipid metabolism can have complex as well as diverse effects on animal systems (Desjardins and Proctor, 2007).

The infection of maize by means of *Fusarium* species and its contamination with mycotoxins are largely influenced by many different elements which include environmental conditions (climate, temperature and humidity), pre- and post-harvest handling and insect infestation, and higher amounts of fumonisins production was linked with drier and warmer climatic conditions worldwide (Fandohan *et al.*, 2003). High temperatures together with humidity, unseasonal rain in the course of harvest, monsoons and flash floods cause fungal proliferation and the production of mycotoxins (Wagacha and Muthomi, 2008).

Fusarium species have become known as human pathogens where the fungi are linked with intrusive infections of immunocompromised patients (Nelson *et al.*, 1994; Dignani and Anaissie, 2004). They are also known to be associated with human esophageal cancer in areas where there is a strong dependency on cereals such as maize (Marasas, 1995).

2.2.1 Taxonomy, origin and characteristics

Fry (2004) reported that *Fusarium* belongs to the Kingdom: Fungi, Order: Hypocreales, Family: *Hypocreaceae* and the Genus: *Fusarium* with it now approaching its third century as a genus (Leslie and Summerell, 2006).

Fusarium species are varied in their mycotoxin profiles and host-associations (Table 2.1), visibly identifying one species from another based on an array of molecular, metabolic, and morphological data is crucial given the need for proper control and precautionary measures that will ensure a harmless, valuable, high-quality and high-yielding grain produce (Nelson *et al.*,

1981; Glenn, 2007). *Fusarium* species produce long, multi-cellular, canoe-shaped macroconidia which are asexual conidia and are the defining morphological feature of the genus (Nelson *et al.*, 1981; Nelson *et al.*, 1983; Nelson, 1992; Nelson *et al.*, 1994; Glenn, 2007).

Table 2.1: *Fusarium* species, known sexual state (teleomorph), primary agronomic hosts and major mycotoxins known to be produced (Nelson *et al.*, 1981; Logrieco 2002; Glenn, 2007; Logrieco *et al.*, 2007).

<i>Fusarium</i> species	Teleomorph	Hosts of primary concern	Mycotoxin profile
<i>F. graminearum</i>	<i>Gibberella zeae</i>	Maize; small grains	DON, ZEA, NIV, FUS
<i>F. proliferatum</i>	<i>Gibberella intermedia</i>	Maize	FB, MON, BEA, FP
<i>F. subglutinans</i>	<i>Gibberella subglutinans</i>	Small grains	MON, BEA, FP
<i>F. verticillioides</i>	<i>Gibberella moniliformis</i>	Maize	FB, FUS, MON

“Small grains” refers mainly to wheat, barley, and oats.

DON, deoxynivalenol or its acetylated derivatives; FB, fumonisin B1, B2, and B3; NIV, nivalenol or its acetylated derivatives; BEA, beauvericin; FUS, fusarin C; MON, moniliformin; ZEA, zearalenone; FP, fusaproliferin.

There is difficulty in the identification of *Fusarium* species due to a wide variation in morphological and non-morphological characteristics (Wagacha and Muthomi, 2007). Separation in this genus is based on primary characteristics such as the shape of macroconidia, presence or absence of macroconidia, type of microconidiophores and whether or not the microconidia are borne in chains (Windels, 1991). They are also separated on secondary characteristics which include the absence or presence of sporodochia, presence or absence of chlamydospores and their position and configuration (Wagacha and Muthomi, 2007). Both physiological and physical characters have been used as morphological characteristics to differentiate between *Fusarium* species, with the main problem now being that the number of readily detectable characters is far smaller than the number of species that are needed to be distinguished (Leslie and Summerell, 2006).

Nelson *et al.* (1983) and Burgess *et al.* (1988) also reported that pigmentation, growth rate as well as colony morphology can be useful if it is based on procedures that are standardized.

The requirement to identify strains and attach names to the *Fusarium* genus is as strong, if not stronger, than it is in any fungal genus and depending on the era and identification scheme being followed, the number of *Fusarium* species can range from a few as nine to well over a thousand (Leslie and Summerell, 2006).

No single taxonomic system that is in use today is entirely satisfactory for the classification of all the *Fusarium* species and the continued increase of modern or new systems for the nomenclature of *Fusarium* species will fail to solve the problem (Nelson *et al.*, 1983). This problem has been, to some extent, alleviated by the use of molecular techniques and identification which follow standardized and published DNA sequences (Bridge *et al.*, 2003).

Fusaria are found in soil, subterranean and aerial plant parts (Nelson *et al.*, 1981). Several *Fusarium* species occur extensively on maize (Desjardins and Plattner, 2000; Logrieco *et al.*, 2002) in semi-tropical and temperate areas where they cause ‘*Fusarium* ear rot’ that result in severe reductions in crop yield (Logrieco *et al.*, 2007).

Fusarium species can also be found in non-cultivated land (Mandeeel, 2006) and are predominant in the top 5- 15cm of the cultivated soil which encompasses the zone most affected by agricultural practices such as fertilizing, tillage, liming, irrigation and herbicide application (Nelson *et al.*, 1981).

The prevalence and distribution of *Fusarium* species that cause ‘ear rot’ are largely governed by environmental conditions, particularly humidity and temperature, and other factors such as insect herbivory (Munkvold *et al.*, 1997a; Bottalico, 1998).

In general, *Fusarium* ear rot has been distinguished into two distinct diseases called “pink ear rot” or pink fusariosis and “red ear rot” or red fusariosis (Bottalico, 1998). Bottalico (1998) further reported that, generally, pink fusariosis is prevalent in drier warmer climates whereas red fusariosis is mostly severe in locations and years with frequent rainfalls and low temperatures during summer and early autumn.

Red ear rot is primarily caused by *Fusarium* species (*F. graminearum*) within the *Discolor* section whereas pink ear rot is primarily caused by *Fusarium* species (*F. proliferatum*, *F. subglutinans* and *F. verticillioides*) within the *Liseola* section (Leslie and Summerell, 2006; Logrieco *et al.*, 2007).

Infection of the maize by *Fusarium* species can occur via numerous routes (Oren *et al.*, 2003). The means of kernel contamination through airborne conidia that infect the silks are most commonly reported (Headrick and Pataký, 1991; Munkvold *et al.*, 1997a; Munkvold *et al.*, 1997b).

2.3 *Fusarium graminearum*

Two populations of *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) were formerly recognized based on cultural and morphological characteristics (Glenn, 2007). The key diagnostic feature for distinguishing Group 1 from Group 2 strains was the capability of Group 2 strains to form abundant homothallic perithecia in single-spore derived cultures on carnation leaf agar, whereas those of Group 1 do not form perithecia in culture and are presumed to be heterothallic (Francis and Burgess, 1977; Burgess *et al.*, 1988).

Combined molecular and morphological examination of Group 1 and Group 2 strains formerly identified as *F. graminearum* have recognized the Group 1 strain as the distinct species *F. pseudograminearum* (Aoki and O'Donnell, 1999).

Group 1 and Group 2 appears to have geographic distributions that are distinct and they induce different pathological symptoms on agronomically important cereals where Group 1 has been reported to be soil-borne pathogens in Australia (Francis and Burgess, 1977) and Africa (Marasas *et al.*, 1988b) where they cause foot and crown rot of barley, oats and wheat. Group 2 members normally cause ear and stalk rot of maize (Stack, 2003), head scab of barley, oats and wheat (Glenn, 2007) and have been reported to be seed-borne pathogens in the northern temperate regions of the northern hemisphere (McMullen *et al.*, 1997).

F. graminearum causes gibberella ear rot of maize in many maize-producing areas throughout the world with cool and wet growing seasons (Sutton, 1982).

Strains of *F. graminearum* produce the potent trichothecene mycotoxins deoxynivalenol (and its derivatives), in addition to the polyketide estrogenic metabolite zearalenone (Sydenham *et al.*, 1991; Blaney and Dodman, 2002). Deoxynivalenol, a cytotoxic trichothecene, has been correlated with oesophageal cancer, oestrogenic disorders, liver diseases and immunotoxic effects (Marasas *et al.*, 1984; Lou *et al.*, 1990). All animal species evaluated to date are susceptible to DON according to the rank order of pigs > mice > rats > poultry \approx ruminants (Pestka, 2007). Of all the mycotoxins that have been discovered to date, trichothecenes have been the toxins strongly linked with fatal and chronic toxicoses of animals and humans (Desjardins, 2006).

Zearalenones were first isolated and structurally characterized from *G. zeae* by Urry *et al.* (1996), as cited by Desjardins (2006) and Glenn (2007). Zearalenone arises in maize that is used in feedstuffs and induces a well-known hyperestrogenic disorder in swine (Velluti *et al.*, 2003; Desjardins, 2006). Zearalenones have not been associated with any fatal mycotoxicoses in humans and animals, and are not acutely toxic and non-steroidal (Desjardins, 2006; Desjardins and Proctor, 2007).

2.4 *Fusarium proliferatum*

Fusarium proliferatum (Matsushima) Nirenberg (*Giberella intermedia*, *Giberella fujikuroi* mating population D) (Samuels *et al.*, 2001), has an extreme broad host range causing major diseases of economically important plants such as maize, asparagus, onion, palm, pine, sorghum and wheat (Desjardins, 2006; Glenn, 2007). The pathogen has been identified as an intruder of maize plants worldwide and is a progressively more significant component of maize ear rot in Europe (Logrieco *et al.*, 2002). *F. proliferatum* has been isolated from various environments worldwide and is a causal agent of stalk and cob rot of maize (Leslie and Summerell, 2006).

F. proliferatum is morphologically similar to *F. verticillioides* (Desjardins, 2006; Glenn, 2007) and both were once grouped together into *F. moniliforme*, although, *F. proliferatum* can be differentiated based on its formation of microconidia in chains, shorter than *F. verticillioides*, from polyphialides (Leslie and Summerell, 2006). *F. proliferatum* is in the same section *Liseola* together with *F. moniliforme* (*verticillioides*) and *F. subglutinans* (Nelson, 1992).

F. proliferatum has been isolated from feed samples associated with leukoencephalomalacia in horses and pulmonary edema in swine, but its significance in causing these toxicoses is masked by the co-occurrence of *F. verticillioides* in the samples (Ross *et al.*, 1990). *F. proliferatum* has not yet been associated with any animal or human toxicoses, however this could be due to *F. proliferatum* not being correctly distinguished from the morphologically similar *F. verticillioides* (Marasas *et al.*, 1984).

F. proliferatum produces a wide range of mycotoxins (Glenn, 2007) and in addition to the production of fumonisins, the fungus also produces moniliformin, beauvericin, and fusaproliferin, all at significant levels (Logrieco *et al.*, 2002). It is reported by Desjardins (2006) that strains of *F. proliferatum* are amongst the highest level of fumonisin producers reported to date.

2.5 *Fusarium subglutinans*

Fusarium subglutinans (Wollenweber & Reinking) Nelson, Toussoun & Marasas (teleomorph *Gibberella subglutinans* (Edwards) P. E. Nelson, Toussoun & Marasas) was first distinguished by Nelson *et al.* (1983) as a separate species. It is a pathogen of maize which is primarily found in cooler areas where maize is cultivated (Marasas *et al.*, 1984; Leslie and Summerell, 2006) and is associated with cob and stalk rot of maize (White, 1999). *F. subglutinans* has a lower optimum temperature and is more predominant in temperate areas when compared to *F. verticillioides* (Nelson *et al.*, 1981). *F. subglutinans* has also been isolated from teosinte which suggests that the relationship between the host and the pathogen is of evolutionary importance (Desjardins *et al.*, 2000).

It is reported by Cotten and Munkvold (1998) that *F. subglutinans* can persist in debris of maize for at least 21 months buried in the field or on the soil surface.

F. subglutinans is morphologically comparable to *F. verticillioides* and *F. proliferatum*; although it can be distinguished by the production of microconidia only in false heads and not in chains as in *F. verticillioides* and *F. proliferatum* (Nelson *et al.*, 1983; Marasas *et al.*, 1984; Leslie and Summerell, 2006). The absence of chlamydospore production also separates *F. subglutinans*

from *F. oxysporum* due to the fact that both fungi produce the same pigment colour when grown in PDA (Nelson *et al.*, 1981; 1983).

F. subglutinans is reported to produce little or no fumonisins (Nelson *et al.*, 1992; Reynoso *et al.*, 2004), moniliformin in high quantities (Marasas *et al.*, 1984; Farber *et al.*, 1988; Leu *et al.*, 1996), fusaric acid (Bacon *et al.*, 1996), beauvericin (Table 2.1) and high levels of fusaproliferin (Gupta *et al.*, 1991; Castellá *et al.*, 1999 Leslie *et al.*, 2004). This fungus is also one of the most prevalent fungi associated with human esophageal cancer in home grown maize in the Transkei (Marasas *et al.*, 1984; Leslie and Summerell, 2006). *F. subglutinans* has been proved to be fatal and acutely toxic to ducklings and caused 100% mortality in mean times of 90 to 136 minutes in an experiment carried out by Marasas *et al.* (1978).

2.6 *Fusarium verticillioides*

Fusarium verticillioides (Sacc.) Nierenberg (= *F. moniliforme* Sheldon) (teleomorph *Gibberella moniliformis*, *G. fujikuroi* mating population A) is a sophisticated ascomyceteous fungus that is constantly linked with maize worldwide (Glenn, 2001; 2007). The fungus causes several major diseases of maize, including root rot, ear rot, stalk rot, seed rot as well as seedling blight (Glenn, 2007), although a symptomless contamination can occur all over the entire plant in leaves, stems, roots and grains (Munkvold and Desjardins, 1997). Oren *et al.* (2003) reported that *F. verticillioides* can be found in just about every maize field after harvest and that the infection of maize by *F. verticillioides* can fluctuate highly in disease symptoms that range from severe rotting and wilting to asymptomatic plants.

There has been an abundance of controversy between taxonomists concerning the name of the fungus, some calling it *F. verticillioides* and others calling it *Fusarium moniliforme* (Leslie and Summerell, 2006). At the 8th International *Fusarium* Workshop held at CABI Bioscience, Egham, United Kingdom, 17-20th August 1998, *F. moniliforme* Sheldon, was renamed *F. verticillioides* (Sacc.) Nirenberg (Summerell *et al.*, 2001).

The name *F. verticillioides* has priority and is now commonly acknowledged that this is the name that should be used for this species and the name *F. verticillioides* should only be used

for strains that have the *G. moniliformis* (*Gibberella fujikuroi* mating population A) teleomorph and not simply as a substitution for *F. moniliforme* (Desjardins, 2006; Leslie and Summerell, 2006).

F. verticillioides has been isolated from teosinte, which suggests that the relationship between the fungus and the maize is of evolutionary importance (Leslie and Summerell, 2006) with microconidia as the predominant inoculum for seed infection.

F. verticillioides produces fumonisins with the most important fumonisin being fumonisin B₁ (FB₁) (Gamanya and Sibanda, 2001).

Mouldy maize associated with animal toxicity has been documented for more than a century in the United States and the fungus implicated with the toxic syndrome was identified by Sheldon (1904, as cited by Glenn, 2007), as the pink mould that grows on maize.

2.7 *In vitro* seed health testing methods for the detection of *Fusarium* in maize seed

There is currently no method developed to test for *Fusarium* species on maize seed that has been validated by the International Seed Testing Association (ISTA) (ISTA, 2011). Due to the fact that the *Fusarium* species involved in maize ear rot vary in fungicide sensitivity, pathogenicity as well as in their capability to produce mycotoxins, accurate quantification and identification is of paramount significance (Nicolaisen *et al.*, 2008).

There are a number of seed health testing methods that are used to detect *Fusarium* species on maize seeds which include the use of modified media such as carnation leaf agar (CLA), synthetic low nutrient agar (SNA) and pentachloronitrobenzene (PCNB) (Nelson *et al.*, 1983; Gamanya and Sibanda, 2001; Leslie and Summerell, 2006).

Nelson *et al.* (1983) used carnation leaf agar in their studies and noted that the value of CLA medium as a growth medium for *Fusarium* lies in the fact that it is low in available carbohydrates. The authors also found that leaves from various grasses, alfalfa, corn and wheat could also be used. Dhingra and Sinclair (1995) further reported that the best identification is carried out on CLA and that the carnation leaves can be substituted with maize leaves.

Carnation leaf disks sterilized with 70% ethanol then rinsed with sterile distilled water gave the most reliable results in a study by Tschanz *et al.* (1975) where they conducted experiments to find a substrate for uniform production of perithecia in *Gibberella zeae*.

Carnation leaf agar is used widely for the isolation of *Fusarium* species as this medium is low in nutrients thereby inhibiting the growth of saprophytes such as *Aspergillus* and *Penicillium*, while stimulating the growth of well-defined morphological characteristics of *Fusarium* species which are used in the identification of the *Fusarium* species (Nelson *et al.*, 1981; 1983; Dhingra and Sinclair, 1995). Kabeere (1995) found that adding a few (5-10) carnation leaf pieces randomly to unsolidified water agar, allowing the media to solidify and plating out surface sterilized maize seeds on this media successfully allowed for the isolation and identification of *F. graminearum*. This method to isolate *Fusarium* successfully represented the transmission rate and was more reliable than using malt extract agar where seed infection was lower than the transmission rate.

Dhingra and Sinclair (1995) reported that the use of potato-dextrose agar (PDA) favours mycelial growth over sporulation.

Nash and Snyder medium (Nash and Snyder, 1962) suppresses most contaminants although it does not provide vivid colour to the colonies, whereas the modified Czapek-Dox medium (Abildgren *et al.*, 1987) provides more colour within the developing colonies, but does not suppress contaminants as well as the Nash and Snyder medium (Nelson *et al.*, 1983).

Castellà *et al.* (1997) noted that the use of malachite green agar 2.5ppm (MGA 2.5) is a potent selective medium for the enumeration and isolation of *Fusarium* species. They tested the medium with mixed and pure cultures, and found that the recoveries of *Fusarium* species in Nash and Snyder medium and MGA 2.5 medium were equivalent, although MGA 2.5 was more selective for *Fusarium* species as it did not allow for the development of colonies belonging to other genera. The authors also reported that MGA 2.5 was less hazardous than other *Fusarium* selective media containing PCNB and that MGA 2.5 was relatively simple to prepare. In an experiment conducted by Richard *et al.* (2007), MGA (0.0025% w/v) was incorporated into malt extract agar (MEA) to limit the growth of proliferous fungi such as *Trichoderma*.

Bragulat *et al.* (2004) tested the selective efficacy of culture media recommended for isolation and enumeration of *Fusarium* spp. In their study they tested the selective efficacy of culture

media such as Nash and Snyder medium (NS), dichloran-chloramphenicol peptone agar (DCPA), modified Czapek-dox agar (MCz), Czapek-doxiproditionedichloran agar (CZID), potato dextrose iprodionedichloran agar (PDID), and malachite green agar (MGA 2.5) which have all been developed for the isolation and enumeration of *Fusarium* species. They concluded that in natural samples, MGA 2.5 was the most potent selective medium among the six recommended selective culture media assayed for *Fusarium* spp., whereas the other recommended selective media allowed the growth of many other different fungal species including Zygomycetes and yeasts. It was furthermore reported by Alborch *et al.* (2010) that MGA 2.5 is a potent selective medium for the detection of *Fusarium* in infected maize kernels where the direct plating technique is used.

2.8 Scanning electron microscopy of *Fusarium verticillioides* within maize seed

Fusarium verticillioides is a major pathogen of maize as well as a constant companion of maize worldwide (Desjardins and Plattner, 2000). *F. verticillioides* infection of maize can occur via several routes with the method of kernel infection through airborne conidia that infect the silks most commonly reported (Headrick and Pataky, 1991; Reid *et al.*, 1992; Munkvold and Carlton, 1997; Munkvold *et al.*, 1997). Due to the fact that a heat treatment is required to eliminate the fungus from maize seed (Dupuy *et al.*, 1993) and that the fungus grows from surface disinfested seed (Marasas *et al.*, 1984; Leslie and Summerell, 2006) it can be concluded that the fungus occurs internally. Bacon *et al.* (1992) concluded that, in sound kernels, *F. verticillioides* was associated with tissue of the upper pedicel and not with the endosperm or embryo. Bacon *et al.* (1992) further reported that the hyphae in this location were viable as evident by the growth through the ruptured scar generated by the emerging radical. The discovery of the fungus in this location confirms an earlier report on the isolation incidence from the pedicel part of the kernel (Zummo and Scott, 1990).

CHAPTER 3

MATERIALS AND METHODS

3.1 Origin of maize seeds and fungi isolates

Maize seed (batch number PAN 6223B) was obtained from Pannar, Greytown, South Africa and Amandhla seed (packed by Dannhauser Malt, Klipkuil, KwaZulu-Natal, South Africa). *Fusarium graminearum*, *Fusarium subglutinans* and *Fusarium verticillioides* were isolated from this seed. Isolates of *Fusarium graminearum* (FCC 4121), *Fusarium subglutinans* (MRC 4145), *Fusarium proliferatum* (FCC 4131) and *Fusarium verticillioides* (FCC 4150) isolated from maize were obtained from the culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Isolates of *Rhizopus*, *Penicillium*, *Trichoderma harzianum* and *Aspergillus niger* were isolated from the maize seed. Isolates of *Penicillium crustosum* (Thorn), *P. digitatum* (Pers.:Fr.) Sacc.) and *P. italicum* (Whemer) were obtained from the *Penicillium* laboratory in the Department of Microbiology and Plant Pathology at the University of Pretoria, Pretoria, South Africa. All the experiments were performed in the Seed Science laboratory of the Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa.

3.2 Standard germination test

The standard germination test was performed according to the between paper procedure of the International Seed Testing Association (ISTA, 2011). Four replicates of 100 seeds were used. The sheets were moistened with 150ml of distilled water. Each roll consisted of two sheets of germination paper, followed by a layer of white paper towel and a third sheet of germination paper on which 25 seeds were placed equidistant in a line with the embryo side down. The fourth sheet of germination paper was used to cover the seeds. They were then rolled up and sealed within plastic bags (Figure 3.1) and incubated at 25°C under 12h light and 12h dark.

First rating was carried out 4d after incubation, recording the number of germinated and ungerminated seeds. The second rating, which was carried out after 7d, entailed the counting of normal and abnormal seedlings and was classified according to ISTA (2011) as follows: “Normal seedlings must match one of the subsequent categories: Intact seedlings- seedlings which have all their vital structures well developed, complete, healthy and in proportion. Seedlings with slight defects:- seedlings that show certain slight defects of their vital structures, provided that they show satisfactory and balanced development when compared to other seedlings within the same test. Seedlings with secondary infection:- seedlings which match the categories described above, although they have been infected by pathogens from sources other than the parent seed. Abnormal seedlings show no potential of developing into a normal plant and comprises of the following categories: Damaged seedlings:- seedlings that have any of its vital structures missing or damaged that balanced developed cannot be expected. Deformed or unbalanced seedlings:-seedlings with weak development or physiological balances or where vital structures are deformed and out of proportion. Decayed seedlings:- seedlings with any of their vital structures so badly decayed or diseased as a result of primary infection that normal development is not permitted” (ISTA, 2011).

3.3 Pre-treatment, plating out and incubation of the seeds

Seeds were surface disinfested with NaOCl at different concentration levels (0.5%, 1% and 1.5%) and at different time intervals (1min, 3min, 5min and 10min). The seeds were then triple rinsed with sterile distilled water, then left to air dry in the laminar flow on sterile paper towels. Subsequent to air drying, the seeds were aseptically plated out onto potato dextrose agar (PDA), half-strength PDA, carnation leaf agar supplemented with potassium chloride and malachite green agar (Table 3.1)(5 seeds per Petri-dish) placed equidistant. The effect of incubation under 12h black-blue light and 12h darkness was compared against incubation of the seeds under normal laboratory conditions (average temperature and light period of 25°C and 12h, respectively). The seeds were incubated (Figure 3.2) at 25°C for 12h under black-blue light (365nm) and 12h darkness placed 25cm from the light source with the Petri-dishes placed next to each other (not stacked).

3.4 Morphological identification, incubation and storage of the fungal species

The *Fusarium* species (Figure 3.3) were morphologically identified according to Nelson *et al.* (1981; 1983), Mathur and Kongsdal (2003), Leslie and Summerell (2006). *Aspergillus niger*, *Penicillium crustosum* and *Penicillium digitatum* were identified according to Samson and Pitt (1990).

For the experiments carried out on the *Fusarium* species and saprophytes on eight nutrient media PDA, PDA + malachite green oxalate, ½ PDA, ½ PDA + malachite green oxalate, 1% malt agar, KCLA, MGA 2.5 and MGA 2.5 + sterile carnation leaf pieces (Table 3.1), the isolates were incubated on PDA as described above. Subsequent to incubation, agar blocks (5 x 5mm) were aseptically cut and placed in the centre of the Petri-dishes (90mm) containing the different media and incubated as before. Each isolate was replicated five times on each of the different media. After 4 and 7d of incubation, colony colour, radial diameter of colony growth, sizes of macroconidia and microconidia were measured and sporulation was compared between the different media.

Table 3.1: Media composition and preparation for the isolation of *Fusarium* species within maize seed

Media	Ingredients	Quantity
PDA	potato dextrose agar powder distilled water	39g made up to 1L
Corn meal agar	corn meal agar powder distilled water	17g made up to 1L
Malt extract agar	malt extract agar powder distilled water	10g made up to 1L
MGA 2.5	peptone agar-agar powder MgSO ₄ KH ₂ PO ₄ malachite green oxalate distilled water	15g 15g 1g 0.5g 2.5mg made up to 1L
PDA + malachite	potato dextrose agar powder malachite green oxalate distilled water	39g 2.5mg made up to 1L
½ PDA + malachite	potato dextrose agar powder malachite green oxalate distilled water	19.5g 2.5mg made up to 1L
MGA 2.5 + carnation leaf pieces	peptone agar-agar powder MgSO ₄ KH ₂ PO ₄ malachite green oxalate sterile carnation leaf pieces distilled water	15g 15g 1g 0.5g 2.5mg 3-4 pieces per Petri-dish made up to 1L
KCLA	agar-agar powder KCL sterile carnation leaf pieces distilled water	15g 8g 3-4 pieces per Petri-dish made up to 1L
KMLA	agar-agar powder KCL sterile maize leaf pieces distilled water	15g 8g 3-4 pieces per Petri-dish made up to 1L

3.5 Preparation of sterile carnation and maize leaf pieces

Fresh carnation leaves or maize leaves were rinsed under running tap water to remove any external impurities and pesticides that could be present. Carnation or maize leaves were then cut into pieces approximately 2-3cm in length and placed in 9cm glass Petri-dishes. The Petri-dishes were sealed in an autoclaveable plastic bag, then in aluminium foil. The Petri-dishes were autoclaved for 15min at 121°C and 120kPa. The sterile carnation or maize leaf pieces were allowed to cool and stored at 4°C until used in the carnation leaf agar or maize leaf agar (KMLA, Table 3.1) and the malachite green agar supplemented with sterile carnation leaf pieces.

3.6 Molecular identification of *Fusarium* species

Molecular identification of the *Fusarium* species were carried out on the 5' portions of translation Elongation Factor (EF) 1 α coding region. The region was amplified using forward primer EF-1 (5'- ATG GGT AAG GAR GAC AAG AC- 3') and reverse primer EF-2 (5'- GGA RGT ACC AGT SAT CAT GTT- 3'). Single spore cultures were prepared and 100mg of mycelium for each *Fusarium* species (7 isolates) were used. DNA was extracted using the Zymo Research Soil Microbe DNA kit (with 50 μ l of Sabax water used at step 10 instead of DNA Elution Buffer). Subsequent to DNA extraction, a gel was run with Big dye 3.1 at 140V for 30min to see if the DNA had been successfully extracted. For the PCR, a master mix: Sabax water (19.5 μ l), buffer (2.5 μ l), MgCl₂ (1.25 μ l), dNTP's (0.5 μ l), EF-1 (0.25 μ l), EF-2 (0.25 μ l), Taq (0.25 μ l) and 0.5 μ l DNA of each *Fusarium* species was used. PCR cycle: 95°C for 4min, 95°C for 30sec, 56°C for 30sec, 72°C for 30sec and 72°C for 7min. Post PCR clean-up was done using a Qiagen kit with the modification of step 9 where 50 μ l of Sabax water was used instead of 50 μ l of EB buffer. After post PCR clean-up, a gel was run again to see if the DNA was still present. The sequencing PCR (10 μ l reaction volume) cycle: Initial denaturing (96°C for 1min), denaturing (96°C for 10sec), annealing (50°C for 5 seconds), extension (60°C for 4min) and holding temperature at 4°C. The cycles underlined above were repeated 35 and 25 times respectively (O'Donnell and Cigelnik, 1997) in a Bio-rad MJ Mini Personal Thermal Cycler (model number: PTC-1148). Post sequencing clean-up was completed and the DNA was sent to the sequencing unit of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria for sequencing and blasting.

3.7 Plating out of maize seeds

Two hundred maize seeds from two seed lots (PAN 6Q-308D and PAN 6223B) were surface disinfested with 1% NaOCl (sodium hypochlorite) for 5min, triple rinsed with sterile distilled water then left to air dry in a laminar on sterile paper towels. The seed was then aseptically plated onto PDA, KCLA, MGA 2.5 and MGA 2.5 supplemented with sterile carnation leaf pieces. The seed were plated 5 seeds per 90mm Petri-dish (1 in the centre and 1 in each quadrant). The seeds were incubated for 7 days at 25°C for 12h under black-blue light (365nm) and 12h darkness placed 25cm from the light source with the Petri-dishes placed next to each other (not stacked). Results were recorded on day 4 and day 7 of incubation.

3.8 Preparation of seed for scanning electron microscopy

Ten asymptomatic maize seeds were surface disinfested with 1% NaOCl (sodium hypochlorite) for 5min, triple rinsed with sterile distilled water and were soaked in sterile distilled water for 6h at 25°C. Subsequent to soaking, the seeds were aseptically cut longitudinally into two halves. One half of each seed was aseptically plated onto PDA to confirm the presence and identity of *F. verticillioides* and incubated at 25°C for 12h under black-blue light (365nm) and 12h darkness for 7 days, while the other half of the seed was used for scanning electron microscopy.

The half-seed samples were fixed with 2.5% gluteraldehyde for 1h then rinsed with 0.075M phosphate buffer thrice for 15min each time. The seeds were then dehydrated through an ethanol series (50%, 70%, 90% and 3x 100%) for 15min in each concentration, critical-point dried with a Bio-Rad E3000 (Watford, England) critical point drier and coated with gold on a Emitech K550X (Ashford, England) gold sputter coater. All samples were then viewed with a JSM-5800LV (JEOL, Tokyo, Japan) scanning electron microscope at 5kV.

3.9 Statistical analysis

The radial diameter of the *Fusarium* species colony growth and size of the microconidia and macroconidia were statistically analysed using analysis of variance. The means were separated using least significant differences (LSD) ($P \leq 0.05$).



Figure 3.1: Maize seeds placed in an upright position in rolled moist germination paper within plastic bags used for the ISTA between paper standard germination test for maize



Figure 3.2: Incubation of Petri-dishes (90mm) under blue-black light (365nm)

CHAPTER 4

RESULTS

4.1 Standard germination test

The standard germination percentage was 98.75% and 89% for the Pannar cultivar and the maize seed obtained from Amandhla, respectively.

4.2 Pre-treatment, plating out and incubation of the seed

Surface disinfestation with 1% NaOCl for a time period of 5min gave the most consistent results. Results ranged from complete over growth of Petri dishes for all time periods at the lower concentration (0.5%), for the 1% concentration at 1 and 3min. At the 10min time period at 1% and the higher concentration of 1.5% there was no growth. Incubation of the seeds under black-blue light allowed the fungi to sporulate better allowing more accurate identification, when compared under a compound microscope to the fungi growing from the seed that was incubated under laboratory conditions.

4.3 Morphological identification of the *Fusarium* spp.

4.3.1 *Fusarium graminearum*

Fusarium graminearum was identified by the absence of microconidia formation (Figure 4.1a), shape and size of macroconidia, colony colour when grown on potato dextrose agar and carnation leaf agar supplemented with potassium chloride (Figure 4.2a and d), and was confirmed by the formation of chlamydospores within the macroconidia (Figure 4.1b) when placed in sterile water.

4.3.2 *Fusarium proliferatum*

Fusarium proliferatum was identified by the formation of microconidia in long chains arising from mono and polyphialides (Figure 4.1c), colony colour when grown on potato dextrose agar and carnation leaf agar supplemented with potassium chloride (Figure 4.3a and d) and the absence of chlamydospore formation.

4.3.3 *Fusarium subglutinans*

Fusarium subglutinans was identified by the absence of microconidia formation in long chains, production of microconidia in aerial false heads arising from mono and polyphialides (Figure 4.1d), colony colour when grown on potato dextrose agar and carnation leaf agar supplemented with potassium chloride (Figure 4.4a and d) and the absence of chlamydospore formation.

4.3.4 *Fusarium verticillioides*

Fusarium verticillioides was identified by the formation of microconidia in long chains arising from monophialides (Figure 4.1e), colony colour when grown on potato dextrose agar and carnation leaf agar supplemented with potassium chloride (Figure 4.5a and d) and the absence of chlamydospore formation.

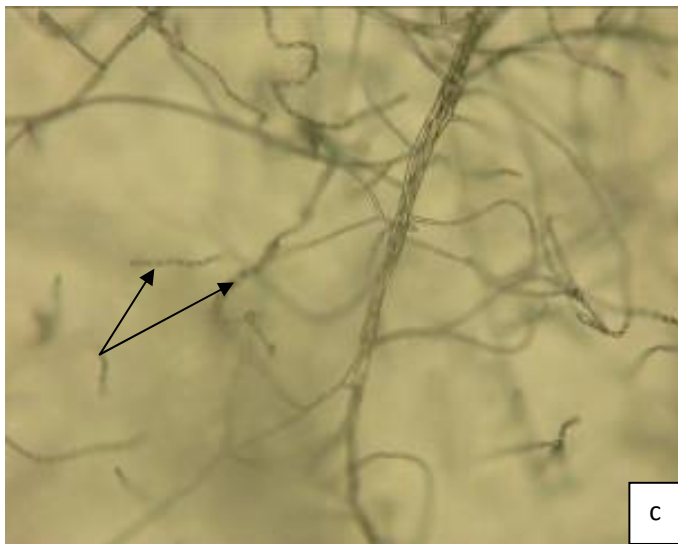


Figure 4.1: Morphological characteristics used to identify *Fusarium* species. a: long chains of microconidia arising from mono and polyphialides of *Fusarium proliferatum* (100x); b: numerous aerial false heads containing microconidia from mono and polyphialides of *Fusarium subglutinans* (100x); c: very long chains and false heads of microconidia arising from monophialides of *Fusarium verticillioides* (100x); d: absence of microconidia formation of *Fusarium graminearum* (100x) and e: chlamydospore formation within the macroconidia confirming the identification of *F. graminearum* (400x).

4.4 Molecular identification of the *Fusarium* species

The results obtained from amplification and sequencing (Table 4.1) of the 5' portions of the translation elongation factor 1 α coding region were accurate and confirmed that the *Fusarium* species were correctly identified using their morphological characteristics

Table 4.1: Results obtained from sequencing the amplified Elongation Factor (EF) 1 α coding region of the *Fusarium* isolates.

Sample no.	Accession number	Species	E-value
Z1F	gblEU220409.1	<i>Gibberella moniliformis</i>	0.0
Z1R	emblAM404138.1	<i>Gibberella fujikuroi</i> var. <i>moniliformis</i>	0.0
Z2F	gblEU220405.1	<i>Fusarium proliferatum</i>	1x 10 ⁻¹⁴³
Z2R	gblAF291058.1	<i>Fusarium proliferatum</i>	0.0
Z3F	gblAF160294.1	<i>Fusarium subglutinans</i>	1x 10 ⁻¹⁵³
Z3R	gblAF160294.1	<i>Fusarium subglutinans</i>	0.0
Z4F	gblDQ382170.1	<i>Gibberella zeae</i>	0.0
Z4R	emblAJ543576.1 FGR543576	<i>Fusarium graminearum</i>	0.0
Z5F	emblAJ543589.1 FGR543589	<i>Fusarium graminearum</i>	0.0
Z5R	emblAJ543576.1 FGR5435789	<i>Fusarium graminearum</i>	0.0
Z6F	gblAF160294.1	<i>Fusarium subglutinans</i>	1x 10 ⁻¹⁵³
Z6R	gblAF160294.1	<i>Fusarium subglutinans</i>	1x 10 ⁻¹⁵³
Z7F	emblAM422699.1	<i>Gibberella fujikuroi</i> var. <i>moniliformis</i>	0.0
Z7R	emblAM422699.1	<i>Gibberella fujikuroi</i> var. <i>moniliformis</i>	0.0

Z1F-Z7R= sample numbers indicating forward primer (F) or reverse primer (R) sequencing. Z1F-Z4R= Isolates obtained from FABI, Z5F-Z7R= Isolates personally isolated

4.5 Incubation, growth and sporulation of the fungal species

All the fungal species stored at 4°C in sterile distilled water remained viable for the duration of the experiments. Sporulation of the fungi was improved when incubated under 12h black-blue light and 12h darkness when compared under a compound microscope to the fungi incubated under normal laboratory conditions.

The radial colony growth of *Fusarium graminearum*, *Fusarium subglutinans*, *Fusarium proliferatum* and *Fusarium verticillioides* on potato dextrose agar (PDA), half-strength PDA (½ PDA), malt agar (MA), corn meal agar (CMA), PDA + malachite green oxalate (PDA +), ½ PDA + malachite green oxalate (½ PDA +), carnation leaf agar supplemented with potassium chloride (KCLA), malachite green agar (MGA 2.5), and MGA 2.5 + sterile carnation leaf pieces (MGA 2.5 +) is reported in Table 4.2.

Growth of *F. graminearum* (Figure 4.2) was the poorest on media that contained malachite green oxalate (MGA 2.5, ½ PDA + malachite (½PDA +) and PDA + malachite (PDA +)) and there was very little production of sporodochia containing macroconidia. The fungus developed and sporulated well on PDA, MA, CMA, KCLA and MGA + carnation leaf pieces. The fungus produced a red coloured pigment in 1% MA, KCLA and MGA 2.5 amended with sterile carnation leaf pieces, particularly adjacent to the carnation leaf pieces. In PDA + malachite green oxalate, ½ PDA + malachite green agar and MGA 2.5, *F. graminearum*'s radial colony growth and development of macroconidia were most restricted, whereas the fungus had a slimy white colony colour growth on CMA with well-defined macroconidia.

F. proliferatum (Figure 4.3) formed long chains of microconidia that arose from monophialides and polyphialides on PDA, 1% MA, CMA, KCLA and MGA + carnation leaves. Poor growth and sporulation occurred on ½ PDA +, PDA + and MGA 2.5 where the conidial chains were not as long and abundant as they were on PDA, 1% MA, CMA, KCLA and MGA + carnation leaves. The fungus produced a cream coloured pigment in CMA, KCLA and MGA 2.5 amended with sterile carnation leaf pieces with sufficient sporulation to allow for accurate identification. Radial growth and sporulation of the fungus was restricted in MGA 2.5, PDA + and ½ PDA + where sporulation of the fungus was insufficient to permit accurate identification.

F. subglutinans (Figure 4.4) produced abundant aerial false heads containing microconidia that arose from monophialides and polyphialides on PDA, 1% MA, CMA, KCLA and MGA + sterile carnation leaves. Radial colony development was also enhanced on the media mentioned when compared to the media that contained malachite green oxalate, with the exception of MGA + carnation leaf pieces where there was abundant sporulation to allow accurate identification, particularly adjacent to the carnation leaf pieces. The fungus produced a cream coloured pigment in MGA + carnation leaf pieces, KCLA and corn agar with sufficient sporulation to allow for accurate identification. Radial colony growth and sporulation of the fungus was restricted in PDA +, ½ PDA + and MGA 2.5 where there was dense growth of the fungus and poor sporulation not permitting accurate identification.

F. verticillioides (Figure 4.5) produced a cream coloured pigment in CMA, KCLA and MGA amended with sterile carnation leaf pieces. There was adequate radial colony growth and sporulation of the fungus in these media to allow for accurate identification. *F. verticillioides*'s radial colony growth and sporulation was limited in PDA +, ½ PDA + and MGA 2.5 where there was little colony diameter growth and sporulation and dense growth of the fungus which obscured accurate identification. The fungus grew and sporulated abundantly on PDA, CMA, 1% MA, KCLA and MGA + sterile carnation leaf pieces, whereas the fungus had reduced growth and sporulation on ½ PDA + malachite, PDA + malachite and MGA 2.5 where the production of microconidia in long chains was suppressed.

The radial colony growth of all the *Fusarium* species tested on the different media was significantly different to the control (PDA) with the exception of KCLA whose radial colony growth was significantly higher than the control. Radial colony growth of the *Fusarium* species was limited in media that contained malachite green oxalate (PDA +, MGA and ½ PDA +), with the exclusion of MGA supplemented with sterile carnation leaves which was in some cases statistically significant from the control in radial colony growth (Table 4.2) and (Figures 4.2, 4.3, 4.4, 4.5).

Media that contained malachite green oxalate (PDA +, MGA, MGA + and ½ PDA +) were most successful in limiting the radial colony growth of *Aspergillus niger*, *Penicillium crustosum* and *P. digitatum* with no statistical significant difference in radial colony growth among the media

(Figure 4.6). There was little or no significant difference among PDA, KCLA, CMA and MA in limiting the growth of *A. niger*, *P. crustosum* and *P. digitatum*.

MGA, MGA + and ½ PDA + limited the growth of *Rhizopus* significantly more than PDA, KCLA, MA and CMA where there was little or no significant difference among PDA, KCLA, MA and CMA when compared with each other (Figure 4.7).

MGA, MGA +, PDA + and ½ PDA + limited the growth of *Trichoderma* more significantly than PDA, KCLA, MA and CMA where there was little or no significant difference among PDA, KCLA, MA and CMA when compared with each other (Figure 4.7).

4.6 Plating out of maize seeds

The *Fusarium* species and fungi within other genera were identified on the maize seed from which the fungi grew and sporulated. From the experiments carried out on the two maize seed lots, MGA 2.5 supplemented with sterile carnation leaf pieces allowed more frequent *Fusarium* isolation from the maize seed when compared to the other media tested (Figure 4.9 and Figure 4.10). *Fusarium* spp. were more frequently isolated from maize seeds on MGA 2.5 supplemented with sterile carnation leaves with a 61% *Fusarium verticillioides* infection level followed by MGA 2.5 on which maize seed had an infection level of 59% (Figure 4.9). PDA and KCLA had seeds with a *F. verticillioides* infection level of 38.5% for both media tested. PDA and KCLA also notably had a higher percentage saprophytic growth from the seeds when compared to MGA 2.5 and MGA 2.5 supplemented with sterile carnation leaf pieces. There was no significant difference between MGA 2.5 supplemented with sterile carnation leaf pieces and MGA 2.5, although MGA 2.5 supplemented with sterile carnation leaf pieces permitted better sporulation of the *Fusarium* species. The maize seeds on the media that did not contain malachite green oxalate germinated and lifted the lid of the Petri-dishes (Figure 4.10 and Figure 4.11) causing desiccation of the media as well as creating cross contamination. This was not the case with MGA 2.5 and MGA 2.5 supplemented with sterile carnation leaf pieces, where the excessive germination and growth of the maize seeds were hindered.

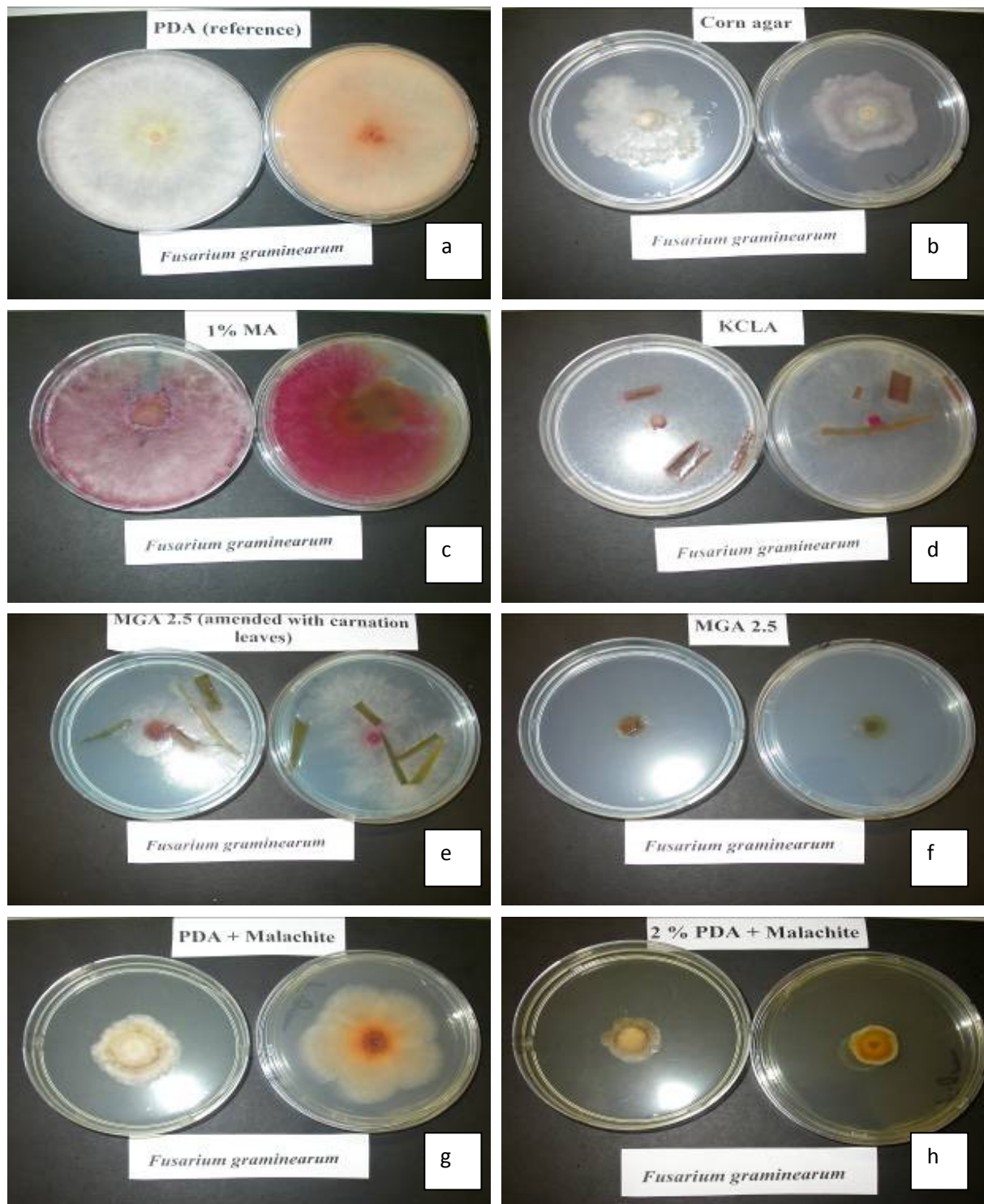


Figure 4.2: Colony colour and growth of *Fusarium graminearum* after 7d of incubation on the different media (see materials and methods for abbreviations).

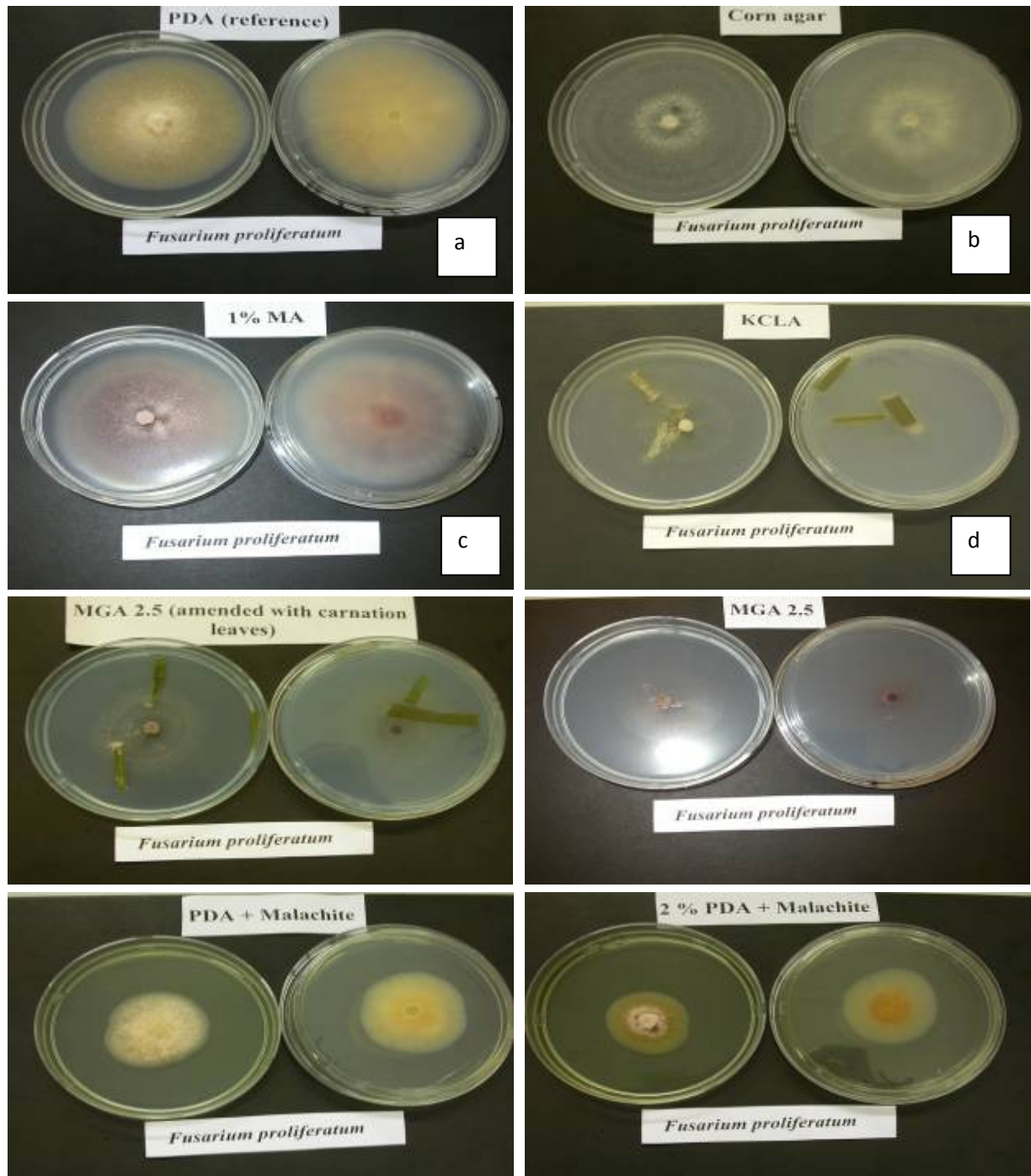


Figure 4.3: Colony colour and growth of *Fusarium proliferatum* after 7d of incubation on the different media (see materials and methods for abbreviations).

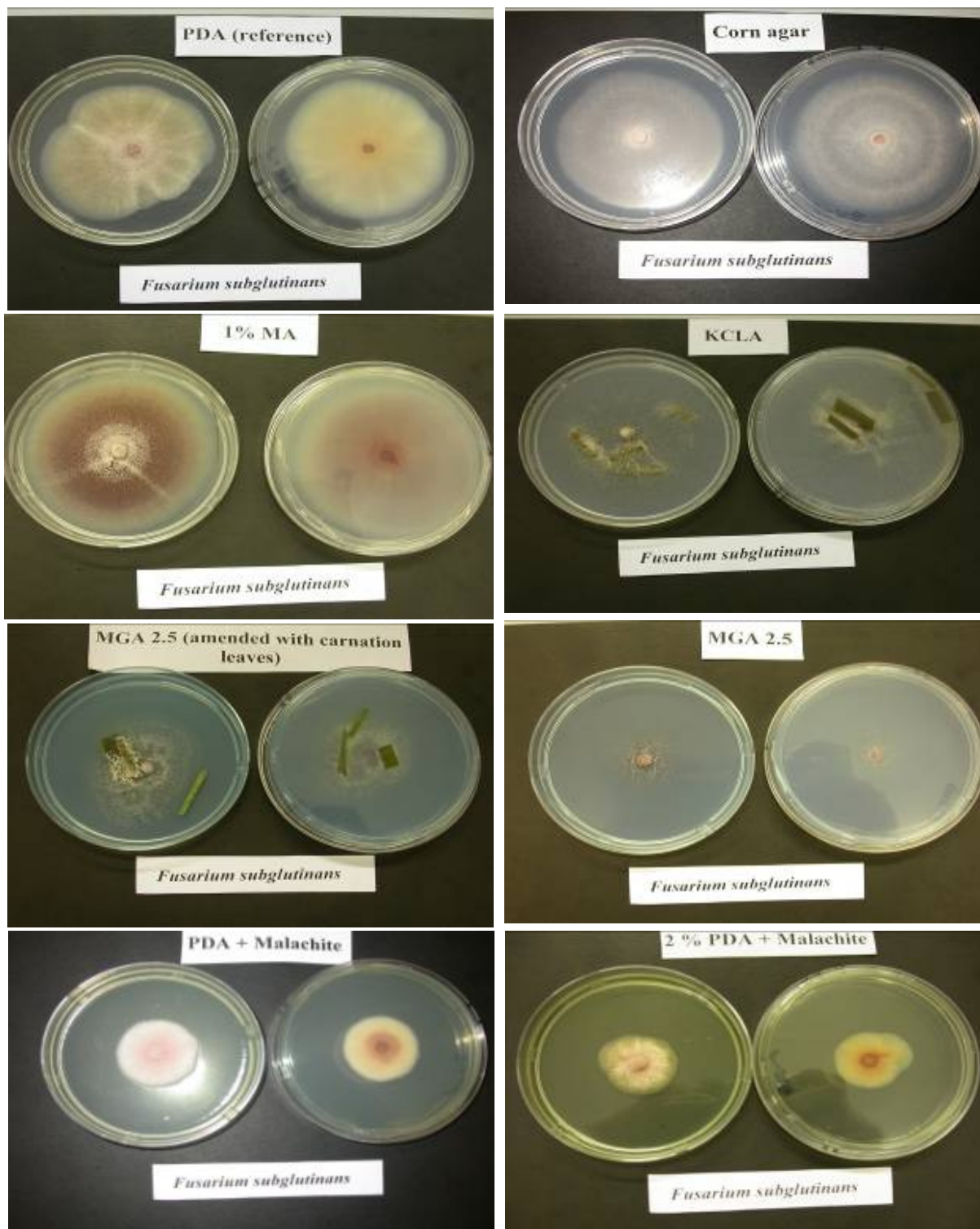


Figure 4.4: Colony colour and growth of *Fusarium subglutinans* after 7d of incubation on the different media (see materials and methods for abbreviations).

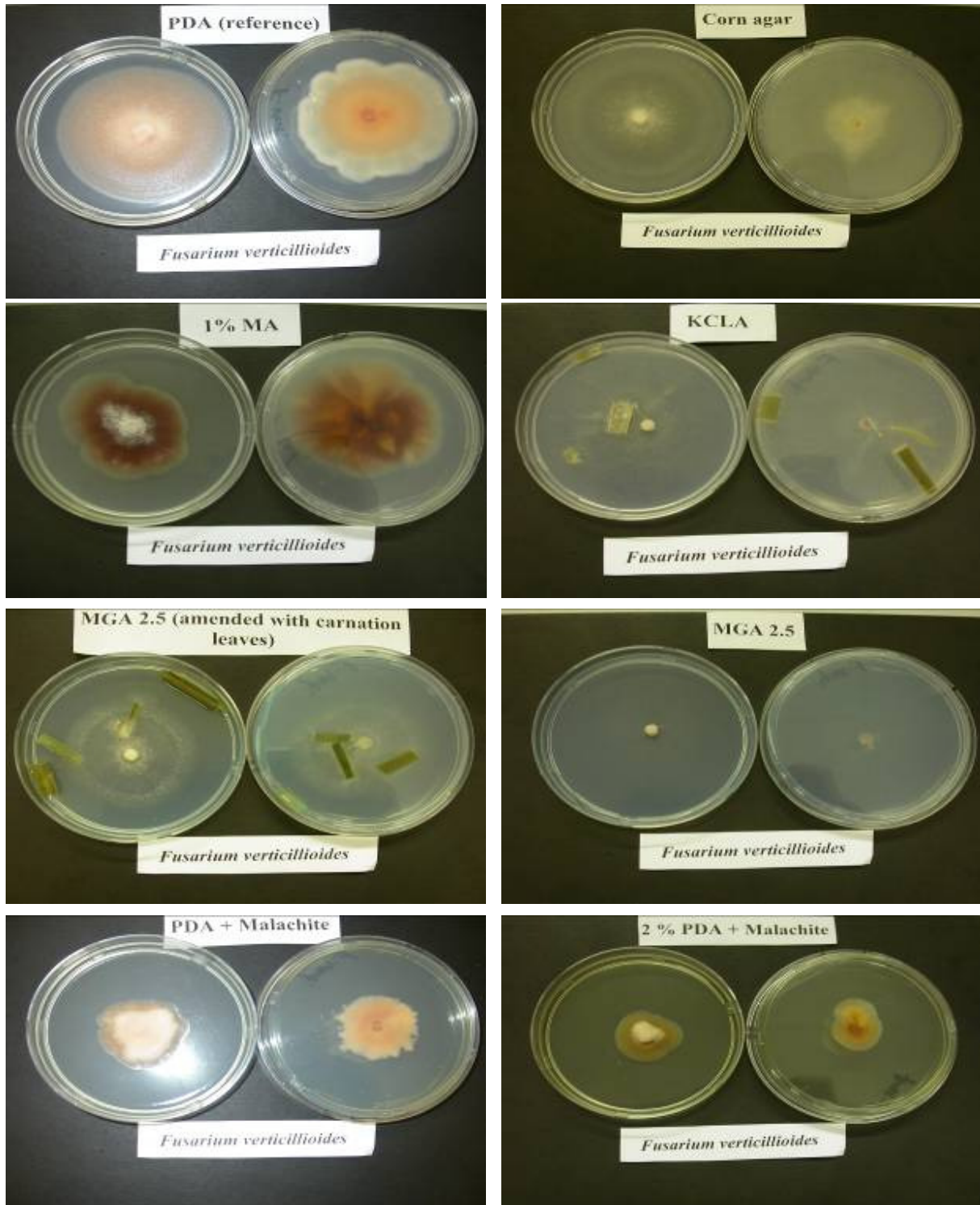


Figure 4.5: Colony colour and growth of *Fusarium verticillioides* after 7d of incubation on the different media (see materials and methods for abbreviations).

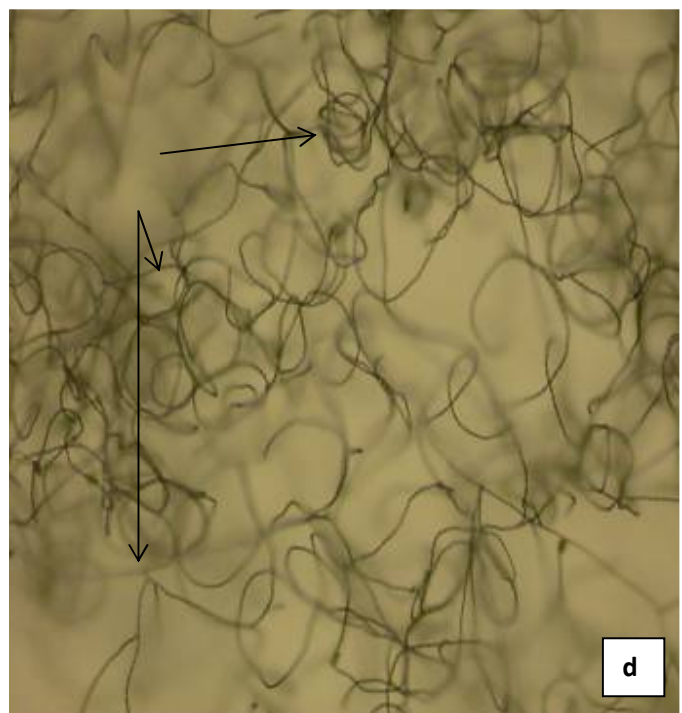


Figure 4.6: Morphological characteristics as observed on MGA 2.5 + sterile carnation leaves of *Fusarium graminearum* (a), *F. proliferatum* (b), *F. subglutinans* (c) and *F. verticillioides* (d) under 100x, 100x, 40x and 40x magnification, respectively. **A:** Macroconidia formation and absence of microconidia by *F. graminearum*, **B:** Formation of long chains of microconidia arising from both monophialides and polyphialides by *F. proliferatum*. **C:** Formation of microconidia in aerial false heads and the absence of microconidia in chains by *F. subglutinans*. **D:** Microconidia formed in long chains arising from monophialides produced by *F. verticillioides*.

Table 4.2: Radial measurement of *Fusarium* species colony growth (4 isolates obtained from the Forestry and Agricultural Biotechnology Institute collection and 3 isolates isolated from the maize seed) in cm.

	<i>F. graminearum</i> gblDQ382170.11		<i>F. proliferatum</i> gblEU220405.11		<i>F. subglutinans</i> gblAF160294.11		<i>F. verticillioides</i> gblEU220409.11		<i>F. graminearum</i> emblAJ543589.1IFGR543589		<i>F. subglutinans</i> gblAF160294.11		<i>F. verticillioides</i> emblAM422699.11	
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
PDA	7.42* e**	8.00 e	4.90 c	7.32 d	5.44 e	6.44 e	5.86 e	7.10 f	7.15d	8.00f	5.24d	6.14c	5.95d	7.3e
MA	4.42 b	7.86 e	4.82 c	7.42 d	5.66 e	7.70 g	5.10 d	6.26 d	4.30c	6.75e	5.72e	8.00f	5.45c	7.81f
CA	4.94 c	5.18 c	5.34 d	8.00 e	5.12 d	7.12 f	5.76 e	7.90 g	3.05b	4.80c	4.86c	7.88e	5.40c	7.90f
½ PDA +	1.98 a	2.24 a	2.18 a	3.96 a	2.08 a	3.04 a	2.56 a	2.76 a	1.67a	1.90a	2.08a	2.48a	2.30a	3.00a
PDA+	2.02 a	3.52 b	3.22 b	3.74 a	2.48 b	3.96 b	3.46 b	3.62 b	1.65a	3.00b	2.42b	2.94b	2.65a	4.65b
KCLA	7.28 e	8.00 e	6.90 e	8.00 e	6.80 g	8.00 g	7.10 f	8.00 g	8.00e	8.00f	6.92f	8.00f	7.20e	8.00b
MGA	2.04 a	2.34 a	2.96 b	4.64 b	2.86 c	4.94 c	4.46 c	5.60 c	4.10c	5.38d	2.60b	6.80e	4.24b	6.10c
MGA +	5.44 d	6.90 d	3.24 b	5.26 c	6.04 f	5.86 d	4.82 d	6.76 e	7.28d	8.00f	5.50e	6.48d	5.58cd	6.44d

*Each value is mean of five replicates.** Means within a column not followed by the same letter are significantly different ($P \leq 0.05$) PDA = Potato Dextrose Agar; MA = 1 % Malt Agar; CMA = Corn Meal Agar; Malachite= Malachite Green Oxalate; MGA 2.5= Malachite Green Agar; PDA + = Potato Dextrose Agar + malachite; ½ PDA + = 2% PDA + malachite; MGA + = MGA 2.5 + carnation leaves

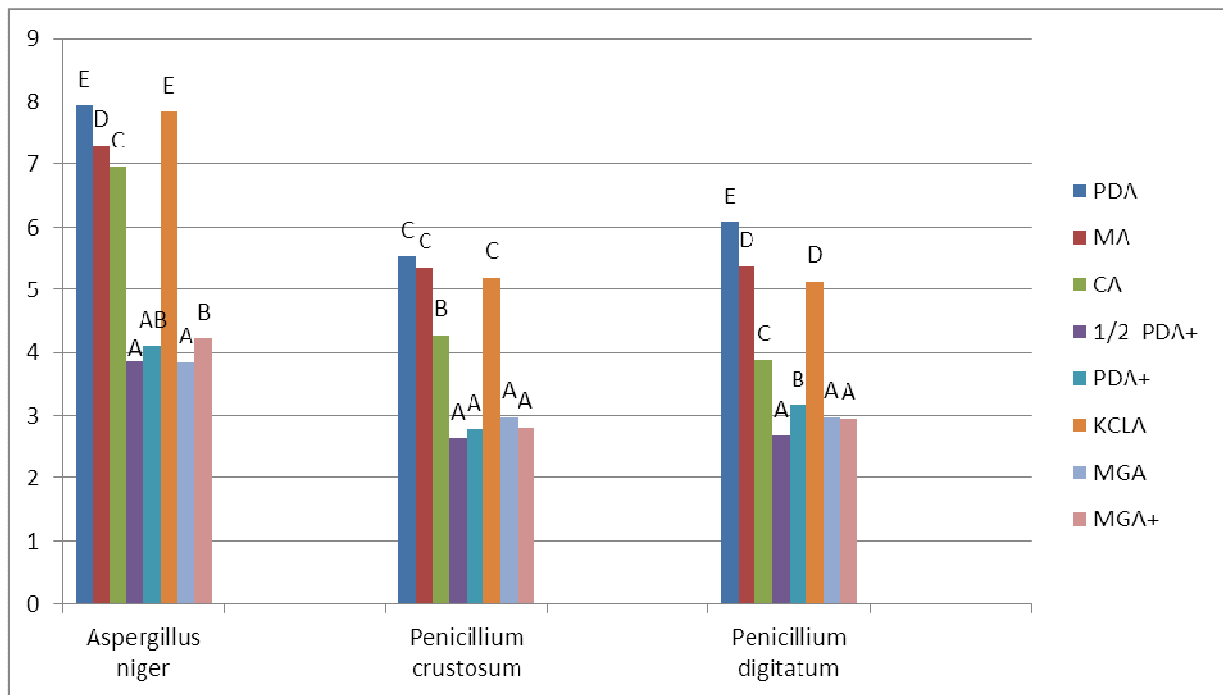


Figure 4.7: Radial growth (mean of five replicates) in cm of *Aspergillus niger*, *Penicillium crustosum* and *P. digitatum* after 7d of incubation at 25°C under black-blue light growing on the different media (see material and methods for abbreviations). Columns within a fungal species not headed by the SAME LETTER are significantly different ($P < 0.05$).

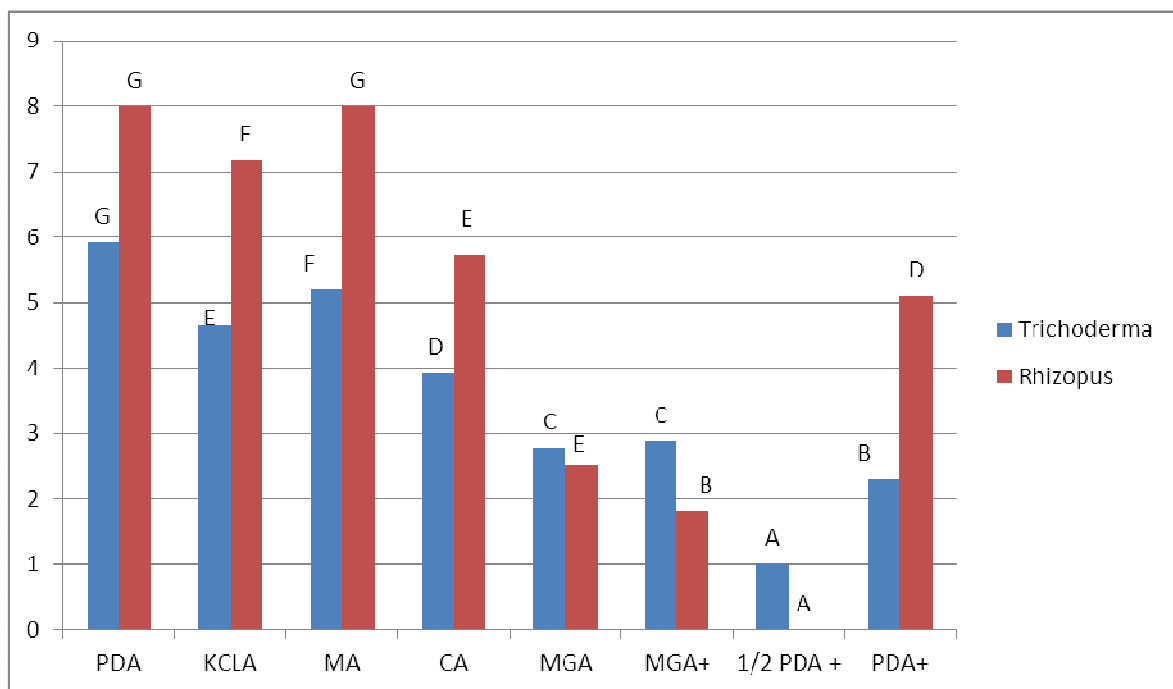


Figure 4.8: Radial colony growth (mean of five replicates) in cm of *Rhizopus* and *Trichoderma* after 7d of incubation at 25°C under black-blue light growing on different media (see material and methods for abbreviations). Columns within a fungal species not headed by the SAME LETTER are significantly different ($P < 0.05$).

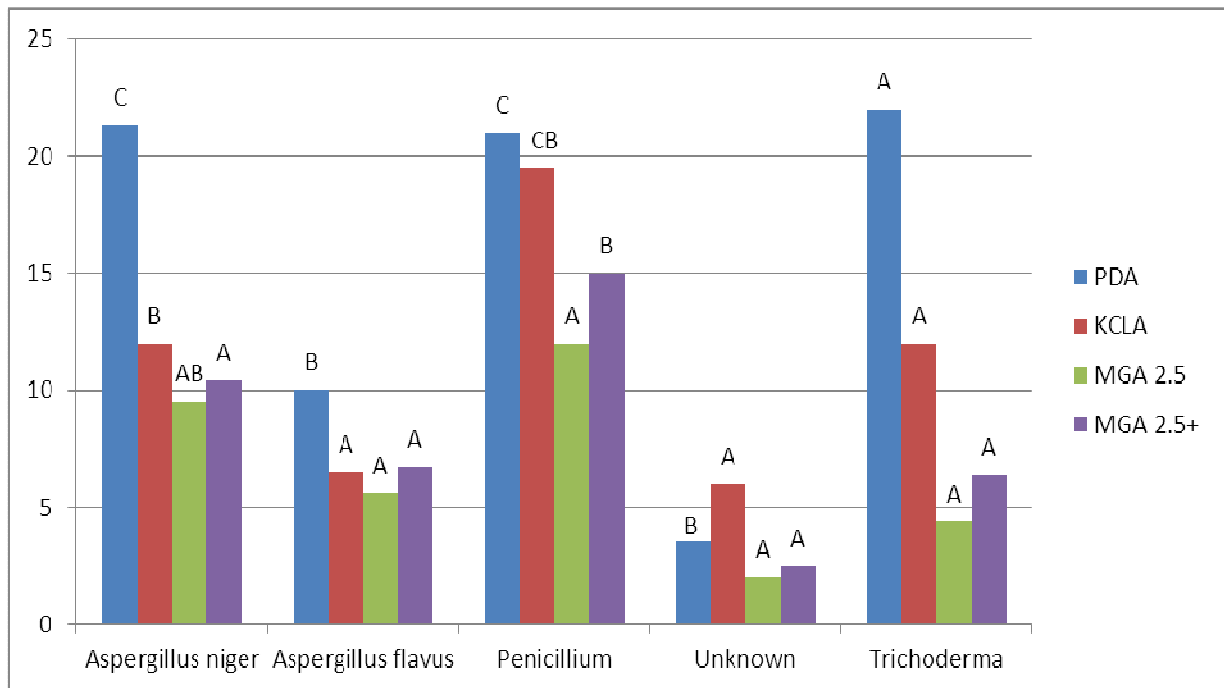


Figure 4.9: Percentage fungal isolation on the four different media carried out on maize seed lot PAN 6223B. Columns within a fungal species not headed by the SAME LETTER are significantly different ($P < 0.05$).

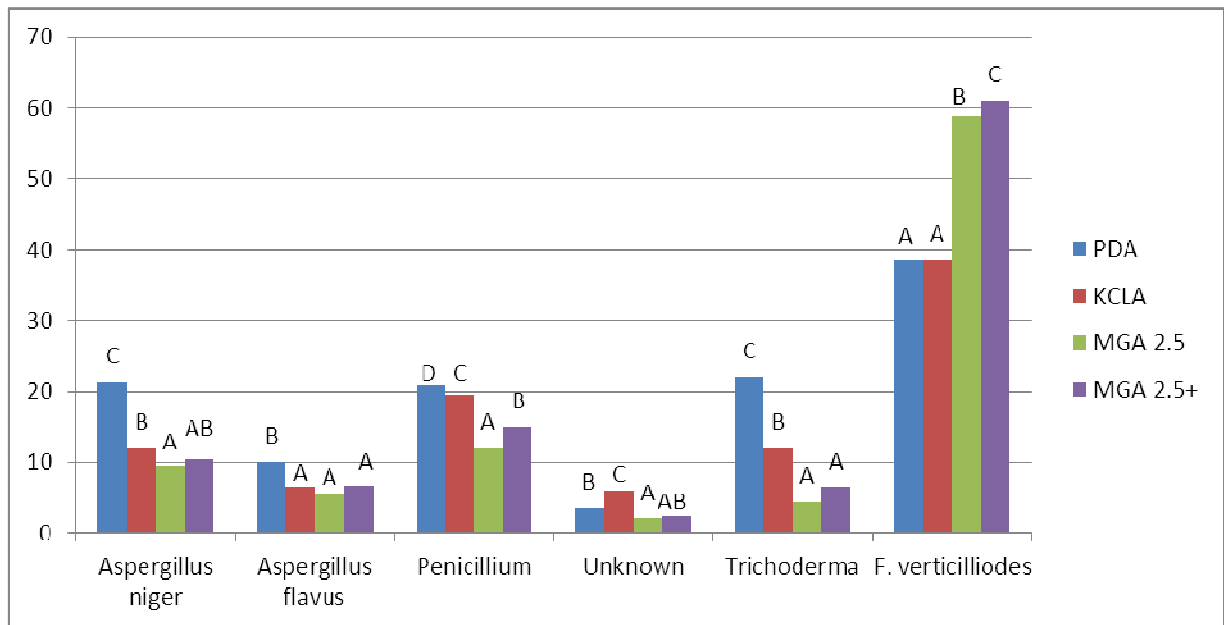


Figure 4.10: Percentage fungal isolation on the four different media carried out on maize seed lot PAN 6Q-308D. Columns within a fungal species not headed by the SAME LETTER are significantly different ($P < 0.05$).

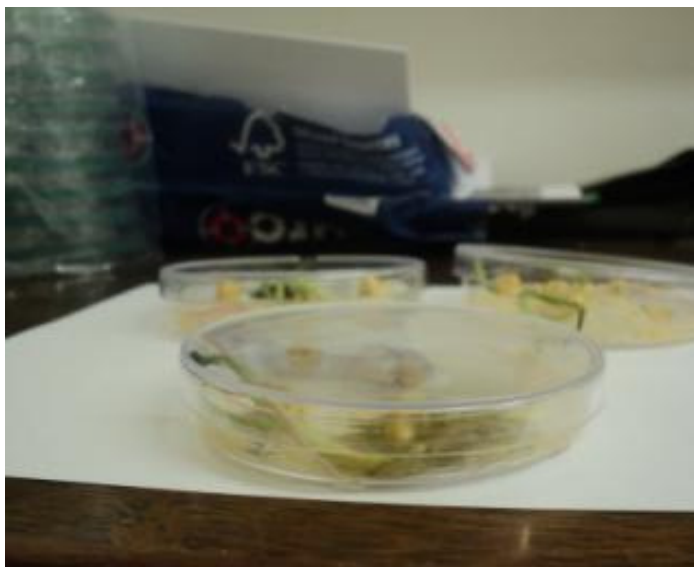


Figure 4.11: Lifting up of the Petri-dish's lids by the germinating maize seedlings



Figure 4.12: Germination of maize seeds on MGA 2.5 supplemented with sterile carnation leaf pieces (left) and PDA (right)

Micrographs of maize seeds using scanning electron microscopy showed that *Fusarium verticillioides* was only associated with tissue of the upper pedicel (Figure 4.12). No fungal hyphae were observed within the vascular tissue of the seed (Figure 4.12C) or within the endosperm or embryo tissue of the kernel (Figure 4.12G and H). Hyphae observed within the upper pedicel of the seed were sparse and did not have any indication of thickening of the cell walls indicative of a survival structure (Figure 4.12E and F). Hyphae in this area were viable as *F. verticillioides* was isolated from all the seed halves plated onto PDA.

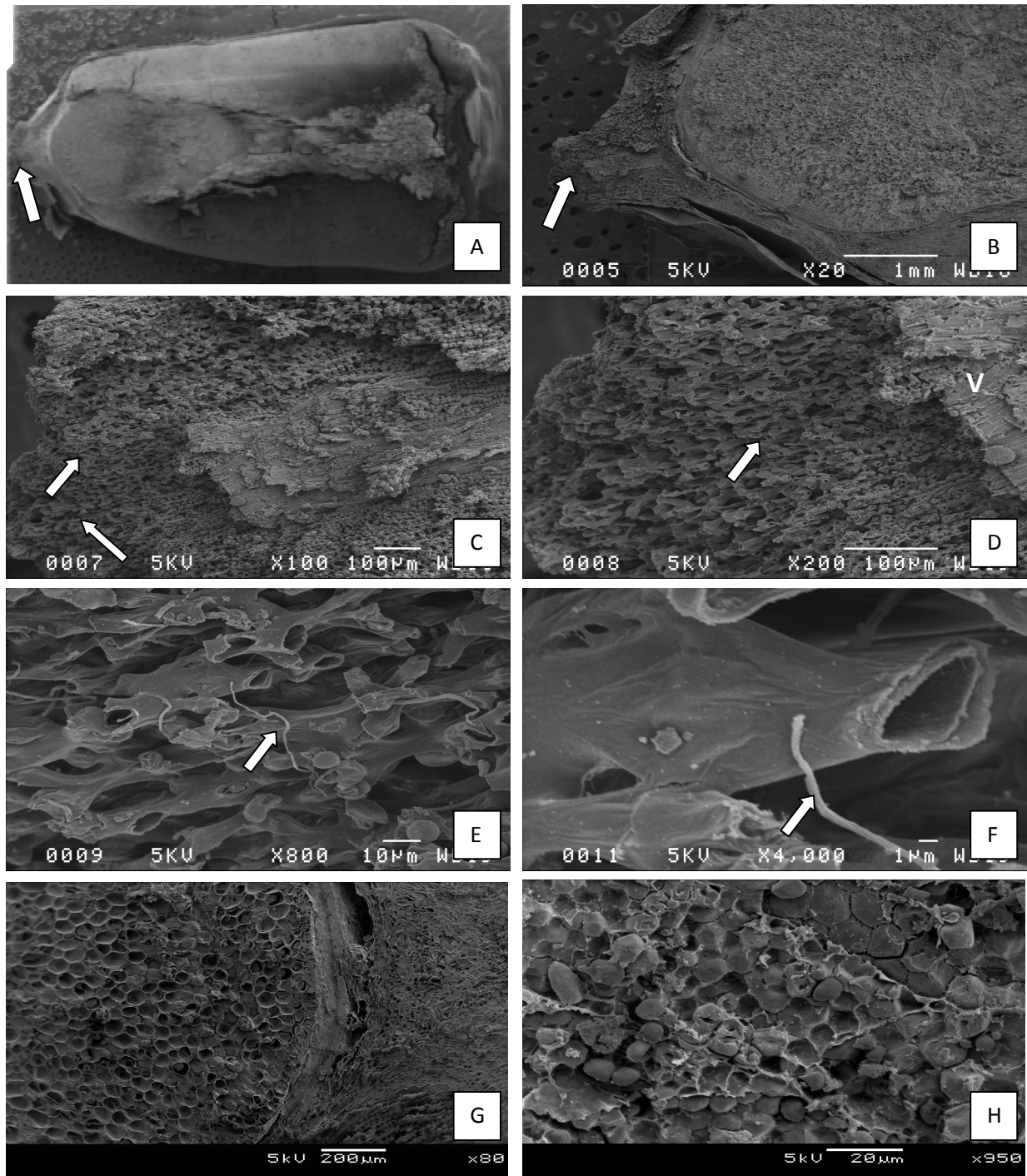


Figure 4.13: Scanning electron micrographs showing the location of *Fusarium verticillioides* within maize seed. A: longitudinal section showing the location of the fungus at the upper pedicel (arrow) (5x). B, C and D: magnification of figure A showing the location of the fungus below the vascular tissue (V); E: higher magnification of figure D showing location of fungus at arrow and F: higher magnification of hyphal tip. G and H: scanning electron micrographs of the endosperm and embryo showing absence of fungal hyphae penetration.

CHAPTER 5

DISCUSSION

The standard germination test carried out on the seed obtained from Pannar (98.75% germination) and Amandhla (89% germination) revealed that the seed obtained from Pannar had a higher germination percentage when compared to the seed acquired from Amandhla. This indicates that the seed from Pannar should have a higher emergence percentage out in the field (ISTA, 2011; Govender *et al.*, 2008) and that both seed lot samples are acceptable as the adequate percentage germination of maize is 70% according to the South African Plant Protection Act (1976).

Surface disinfestation with 1% NaOCl for a time period of 5min gave the most consistent results compared to the other treatments, supporting the findings of Cuero *et al.* (1986), and destroyed the majority of the saprophytic microflora present on the maize seed surface as described by Sauer and Burroughs (1986). The use of light during the incubation of the fungi permitted better sporulation (Devi and Singh, 1994; Rossi *et al.*, 2009) of the *Fusarium* species when compared to incubation without the use of light and resulted in more accurate identification. The results confirmed the results of Snyder and Hansen (1941) who illustrated the effect of light on taxonomic characteristics in *Fusarium*.

Distinguishing species from one another in the *G. fujikuroi* species complex based on morphological characteristics is challenging, even for experts (Summerell *et al.*, 2003; Leslie and Summerell, 2006; Rossi *et al.*, 2009). DNA sequence-based identification and species-specific PCR assays are commonly used to accurately identify species inside the complex (Rahjoo *et al.*, 2008). Accordingly, species-specific PCR and sequence analysis was used to confirm the identification of the *Fusarium* species. The molecular identification method of O'Donnell and Cigelnik (1997) used in this study was precise and confirmed that the identification of the *Fusarium* species, using their morphological characteristics, was accurate. The molecular method of O'Donnell and Cigelnik (1997) could be used in other experiments where correct identification of other *Fusarium* species is required and not only those included in this study.

From these experiments it was found that carnation leaf agar amended with potassium chloride (KCLA) favoured sporulation over mycelial growth and stimulated the development of well-defined morphological characteristics which allowed more accurate identification of the *Fusarium* species. However, the medium also allowed *Aspergillus niger*, *Penicillium crustosum*, *P. digitatum*, *Trichoderma harzianum* and *Rhizopus stolonifer* to grow profusely which will obscure accurate and correct identification of the *Fusarium* species emerging from maize seed.

The results revealed that malachite green agar (MGA 2.5), potato dextrose agar (PDA) + malachite green oxalate, ½ PDA + malachite was most inhibitory to the growth of the saprophytes which is in agreement with Castella *et al.* (1997) who pointed out that MGA 2.5 only allowed the development of *Fusarium* spp. colonies and not of *Aspergillus flavus* and *Penicillium aurantiogriseum* (Dierckx).

Radial colony growth of the *Fusarium* species was restricted on PDA + malachite, ½ PDA + malachite and MGA 2.5 when compared with PDA, KCLA, MA, CMA and MGA 2.5 +sterile carnation leaf pieces. This confirms the results found by Castella *et al.* (1997) who reported that the mean percentage of reduction in colony diameters of *Fusarium* spp. strains was higher on MGA 2.5. However, PDA, malt agar (MA) and corn meal agar (CMA) allowed the profuse growth and sporulation of the *Fusarium* species and saprophytes due to the high nutrient content as well as the absence of a non-inhibitory substance within the media (Nelson *et al.*, 1981; 1983; Dhingra and Sinclair, 1995; Castella *et al.*, 1997). Alborch *et al.* (2010) further reported that MGA 2.5 is a potent selective medium for the detection of *Fusarium* in infected maize kernels using the direct plating technique and can be used as an alternative to Nash and Snyder medium that contains pentachloronitrobenzene (PCNB), which is reported to be carcinogenic (IARC, 1987).

Although radial colony growth of the *Fusarium* species was restricted on media containing malachite green oxalate, it was possible to observe microconidia and macroconidia.

MGA 2.5 with sterile carnation leaf pieces was the most successful medium in limiting the growth of the saprophytes while allowing sufficient sporulation of the *Fusarium* species for accurate identification. The medium also allowed the most frequent isolation of the *Fusarium* species when compared to the other media tested.

Media that contained malachite green oxalate also slowed down the growth of the germinating maize seedlings which lifted the lid of the Petri-dishes containing other media and preliminary studies also showed that, although not as effective, sterile carnation leaf pieces could be substituted with sterile maize leaf pieces as found by Nelson *et al.* (1981; 1983).

The presence of the fungus in the tissue associated with the upper pedicel part of the kernel confirms an earlier report on the isolation incidence of *Fusarium* from the pedicel part of the kernel by Zummo and Scott (1990). These findings also correlate with an earlier report by Bacon *et al.* (1992) who did scanning electron microscopy experiments on asymptomatic maize kernels associated with equine leukoencephalomalacia.

The hyphae observed within the seed were intercellular as reported by Oren *et al.* (2000) who found that the asymptomatic infection of *F. verticillioides* is characterized by intercellular growth of a limited number of fungal hyphae.

The fungus does not produce overwintering structures such as chlamydospores (Nelson *et al.*, 1981; Nelson *et al.*, 1983; Leslie and Summerell, 2006), although it has been reported to survive in maize debris in soil as thickened hyphae (Nyvall and Kommedahl, 1968). No thickening of the hyphae was observed within the maize seed which was indicative of a survival structure which confirmed the results obtained by Bacon *et al.* (1992).

5.1 General conclusion

MGA 2.5 amended with sterile carnation leaf pieces was the most successful medium for the delay of saprophytic growth while allowing adequate sporulation of the *Fusarium* species to permit accurate identification. Although the medium showed a very high potential in the isolation of *Fusarium* spp., hindering the growth of non-target fungi and preventing excessive germination by the maize seedlings of the media tested, further tests are required in laboratories around the world to test the effectiveness of the medium within different laboratories, by analysts with differing levels of experience in identifying *Fusarium* spp. and on different seed lots with varying infection levels.

A test plan has been developed for submission to the International Seed Testing Association Seed Health Committee for approval (Appendix 1). Comparative studies using the method will be done with five to eight laboratories to verify the new method. If it is found that the random addition of carnation leaves to the media affects the reproducibility and repeatability of the test, further studies using powdered carnation leaves or leaves placed directly adjacent to individual seeds will be evaluated. If the method is found suitable by all the laboratories involved and similar repeatable results are obtained the method will undergo the ISTA validation procedure. Once the method has been validated and approved by the Seed Health Committee it will be voted on by the ISTA membership to become part of the International Rules of ISTA used worldwide in the trading of maize seed for planting.

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APPENDIX 1: ISTA TEST PLAN

ISTA test plan for the detection of *Fusarium graminearum* and *F. verticillioides* in maize seed lots

June 2012

1. ORGANIZATION AND DESIGN

1.1. Test Organizer

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1.2. Pathogens

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch)
and *Fusarium verticillioides* (Sacc.)Nierenberg (= *F. moniliforme* Sheldon)
(teleomorph *Gibberella moniliformis*, *G. fujikuroi* mating population A)

1.3. Crop

Zea mays (maize)

1.4. Participating laboratories and contact persons

Laboratory 1

Laboratory 2

Laboratory 3

Laboratory 4

Laboratory 5

Laboratory 6

Laboratory 7

2. INTRODUCTION AND SCOPE OF THE METHOD

2.1 Background

Fusarium graminearum and *F. verticillioides* are important seed-borne fungal pathogens that infect maize with symptoms ranging from symptomless infections to severe ear rot (Logrieco *et al.*, 2007; Fandohan *et al.*, 2003; Nelson *et al.*, 1981). *Fusarium* contamination is a key agricultural difficulty, although more notably many species within the genus are prolific producers of mycotoxins that are implicated for severe diseases in farm animals as well as in humans (Nicolaisen *et al.*, 2008).

This method is based on the developmental work of information described in the literature. The protocol presented in the current test plan is based on Nelson *et al.* (1981) and Castellà *et al.* (1997). The semi-selective medium malachite green agar (MGA2.5) supplemented with sterile carnation leaf pieces has been evaluated through comparative tests carried out at the University of Pretoria. The protocol was developed for the detection of *Fusarium graminearum* and *F. verticillioides* in untreated or disinfected seed lots where the disinfection process leaves no chemical residue, *i.e.* hot water, heat. This method is not intended for the testing of chemically treated seed. This method is to be carried out by laboratories experienced with the testing of seed for seed-borne pathogens and equipped with the needed equipment and supplies necessary to conform to the requirements of the method.

2.2 Scope

In this comparative test, 24 seed samples coming from 4 seed lots with varying infection levels will be used to evaluate the proposed method for *Fusarium graminearum* and *F. verticillioides* recovery on malachite green agar supplemented with sterile carnation leaf pieces.

2.3 Objective

The objective of this comparative test is to provide an ISTA internationally accepted method for the detection of *Fusarium graminearum* and *F. verticillioides* in *Zea mays* seed lots. The suitability has been tested using untreated or seed treated by physical processes such as hot water or heat treatment. There is no data to support the use of this method for the testing of treated seed.

2.4 Sensitivity of the proposed method

In cases where reading the results is hampered by the overgrowth of saprophytes, this test will need to be repeated after the seed lot is subject to a non chemical seed treatment, such as hot water or heat treatment. The description of the surface sterilization procedure is described in the method under section 4.6.

3. STATISTICAL ANALYSIS

The data will be analyzed with the help of the ISTA Statistical Committee using existing tools (e.g. Seedcalc8) or tools developed specifically for this study.

4. MATERIALS AND METHODS

4.1 Seed lots and seed subsamples

In this comparative test, three infected seed lots, each one with a different infection level and one seed lot not previously giving a positive result will be used. Seeds lots are naturally infected and will be selected according to their infection level by the test organizing laboratory which will use the proposed described method.

Each participating laboratory will test 4 subsamples of 100 seeds for each of the highly infected and healthy seed lots and 8 subsamples of 100 seeds for each of the low and medium infected seed lots shown as follows:

- i. One highly infected seed lot pretested at +/- 5% infection level (4 subsamples of 100 seeds)

- ii. One medium infected seed lot pretested at +/- 2% infection level (8 subsamples of 100 seeds)
- iii. One low infected seed lot pretested at +/- 1% infection level (8 subsamples of 100 seeds)
- iv. One seed lot pretested at a non detectable infestation level (4 subsamples of 100 seeds)

All subsamples will be coded randomly and their correspondence to seed lots will be known only to the test coordinator.

4.2 Positive control (Reference culture)

Each participating laboratory will use its own *Fusarium graminearum* and *F. verticillioides* reference strain on proposed selective medium as a reference culture.

4.3 Materials needed to perform the test

- *Fusarium graminearum* and *F. verticillioides* reference strains
- Vessel for sterilizing seed in NaOCl
- Fresh, mature carnation plants
- NaOCl (1% active ingredient)
- Sterilized forceps
- Autoclaved blotter papers for the drying of seeds
- Laminar flow hood
- Distilled or de-ionized H₂O
- Chemicals needed for medium preparation
- Magnetic stirrer
- Autoclave
- Autoclavable plastic bags
- Suitable equipment to cool down medium to 50°C before pouring over sterile carnation leaf pieces
- Aluminium foil
- Incubator capable of operating at 25°C
- Black-blue light (365 nm)
- Compound microscope capable of magnifying at x100

4.4. Sterile carnation leaf preparation

Rinse fresh carnation leaves under running tap water to remove any external impurities and pesticides that could be present. Cut the carnation leaves in pieces approximately 2-3 cm in

length and place in a 90 mm glass Petri-dish. Seal the Petri-dish in an autoclaveable plastic bag, then in aluminium foil. Autoclave the Petri-dish (can be done in the same cycle as the MGA 2.5) for 15 min at 121°C and 120 kPa. Subsequent to cooling, the sterile carnation leaves can be stored at 4°C until needed in the medium.

4.5. Medium preparation

In this comparative test malachite green agar supplemented with sterile carnation leaf pieces will be used to plate seed sub-samples. A minimum of 960 Petri dishes of approximately 90 mm will be needed, to include plate/plates for the reference culture. This quantity corresponds to approximately 20 liters of medium.

Ingredients for malachite green agar with sterile carnation leaf pieces preparation:

Peptone (<i>e.g.</i> Sigma or equivalent)	15.0 g
Agar	15.0 g
MgSO ₄	1.0 g
KH ₂ PO ₄	0.5 g
Malachite green oxalate	2.5 mg
Sterile carnation leaf pieces	3-4 pieces per Petri-dish
Distilled H ₂ O	up to 1000 ml

- 4.5.1. In an appropriate flask add peptone, agar, MgSO₄, KH₂PO₄ and malachite green oxalate. Fill up the flask to 1000 ml with distilled H₂O and dissolve with a magnetic stirrer.
- 4.5.2. Seal the flask and sterilize by autoclaving at 121°C with 15 p.s.i. for 30 min. Cool down to 50°C.
- 4.5.3. Place 3-4 pieces of sterile carnation leaves in the Petri-dish.
- 4.5.4. Pour cooled MGA 2.5 in the Petri-dish containing sterile carnation leaves approximately 20 ml into each Petri dish. Pour from 1 liter of medium approximately 50 Petri dishes.
- 4.5.5. If possible leave the plates at room temperature for 72 h and check for contamination before use. This will also allow some excess moisture to evaporate.
- 4.5.6. Prepared plates can be stored at 4-7°C for up to 4 weeks.

4.6. Seed plating

- 4.6.1. Place each maize seed subsample in a perforated sterilization vessel and label the vessel.
- 4.6.2. Dip the vessel in 1% NaOCl solution (1% active ingredient) for 5 min.
- 4.6.3. Shake a few times and rinse in sterile distilled H₂O for 60 sec. Repeat 3 times.
- 4.6.4. Dry the seeds on labeled sterile blotter papers under a sterile laminar flow hood.
- 4.6.5. Use sterile forceps to aseptically place a maximum of 5 seeds per medium plate and separate seeds. 1 seed in each quadrant and 1 in the center.
- 4.6.6. Label the plates with the corresponding code and date.
- 4.6.7. Transfer a pure culture of *Fusarium graminearum* and *F. verticillioides* onto two plates of the same semi selective medium for each fungus.
- 4.6.8. Incubate all the plates at 25 °C for 12 h under black-blue light (365 nm) and 12 h darkness placed 25 – 30 cm from the light source with the Petri-dishes placed next to each other (not stacked).

4.6 Evaluation of plating

- 4.6.1. Count the number of suspect *Fusarium graminearum* and *F. verticillioides* colonies developed in each plate at 4 and 7 days. Final results are recorded after 7 days. The 4 day counting is performed in case of highly developed saprophytic growth in plates.
- 4.6.2. *Fusarium graminearum* on MGA 2.5 plus sterile carnation leaf pieces medium develops a red colour pigment particularly adjacent to the carnation leaf pieces with the absence of microconidia production. Confirming the identification of the fungus can be done by aseptically placing a 5 mm x 5 mm agar block from the suspected colony in sterile water within a Petri-dish and observing chlamydospore production within the macroconidia after 3 to 5 days.
- 4.6.3. *Fusarium verticillioides* on MGA 2.5 plus sterile carnation leaf pieces medium develops a cream colour pigment. The fungus produces microconidia in long chains that only arise from monophialides as well as aerial false heads.
- 4.6.4. Use a compound microscope with x100 magnification to identify suspect colonies.
- 4.6.5. Make sure the reference culture has grown properly onto the semi-selective medium.
- 4.6.6. Compare suspect colonies to the reference culture.
- 4.6.7. Record zero if there are no suspect colonies on the plate.

Fill in the data record sheet provided with the test plan for each seed sub-sample. For the reference culture, indicate in the “Comments/Observations” column if the fungus has developed a proper growth pattern onto the semi-selective medium.

5. PARTICIPANT SCHEDULE AND INSTRUCTIONS

5.1. General schedule

- 5.1.1. Receive comparative test program
- 5.1.2. Accept obligations in participating in this comparative test
- 5.1.3. Receive the seed sub-samples
- 5.1.4. Secure all ingredients, equipment needed
- 5.1.5. Perform the test
- 5.1.6. Send results and comments to ISTA technical coordinator

5.2 Expected time of work for each participating laboratory

The time needed to perform the test depends on the experience of participating laboratories. An estimation is given in the table below.

Time needed for 24 seed sub-samples

Day	Activity	Time needed
0	Preparation of: medium, NaOCl solution, other materials	6h
1	Seed plating on medium	4 h
4,7	Evaluation of plates Recording of results on data record sheet	2 h each evaluation day 1 h each evaluation day

5.3 Critical points

- 5.3.1. Storage of seed samples at 5-7 °C.
- 5.3.2. Good quality of medium ingredients for medium preparation.
- 5.3.3. Cold storage of medium before use.

5.3.4. Elimination of cross contamination during seed plating: Materials of use should be sterilized with alcohol and flamed between seed sub-samples.

5.3.5. Utilization of sterile techniques for all aspects of the procedure.

5.3.6. Proper evaluation of plates for the validation of a negative result.

5.4 Safety precautions

Principles of Good Laboratory Practice and aseptic technique should be followed during the whole testing procedure. Disposal of waste materials in an appropriate way and in accordance with local health, environment and safety regulations is considered essential. Handle all chemicals according to required country health and safety risk assessments.

6. References

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