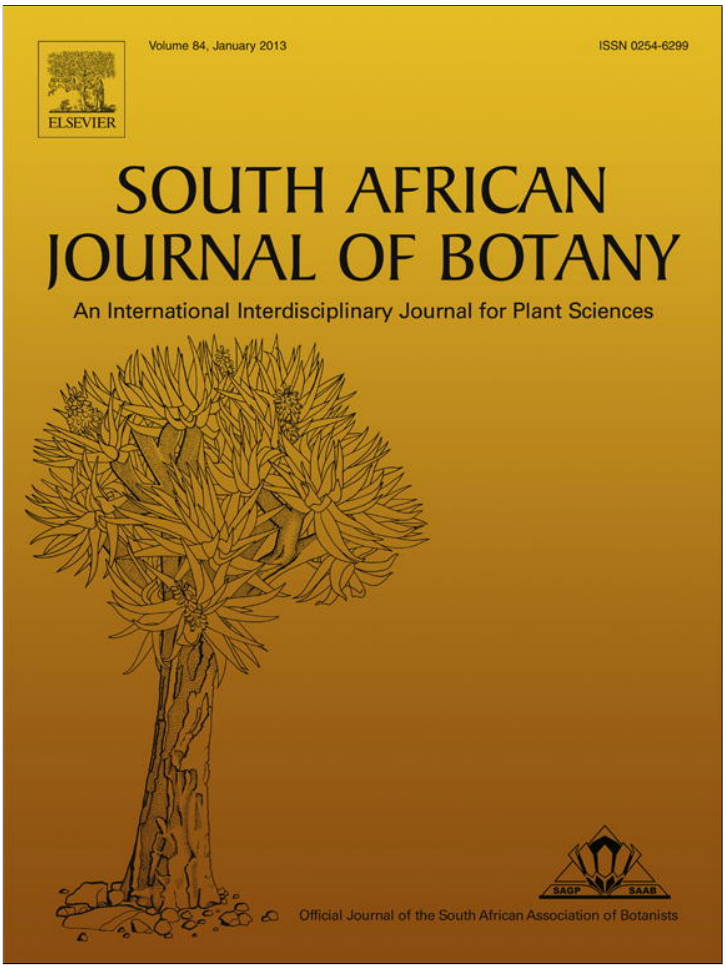

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A new semi-selective medium for *Fusarium graminearum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* in maize seed

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ABSTRACT

Zea mays L., known also as corn and maize, is the most important crop according to the amount of tonnes produced each year. 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. 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. Currently no method has been developed to test for *Fusarium* species in maize seed that has been validated and published by the International Seed Testing Association (ISTA). 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, 1/2 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 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% NaOCl) and for different time intervals (1 min, 3 min, 5 min and 10 min). The effect of black-blue light (365 nm) on sporulation of the fungi was also investigated. Surface disinfection of maize seeds with 1% NaOCl for 5 min provided consistent results. PDA, 1/2 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. The *Fusarium* species growing on these media formed underdeveloped morphological structures, thereby obscuring accurate identification. MGA 2.5 showed better hindering of the saprophytes in some instances. MGA 2.5 amended with sterile carnation leaf pieces was the most satisfactory medium in hindering the growth of the saprophytes while allowing adequate sporulation by the four *Fusarium* species to permit accurate identification. The media also resulted in higher *F. verticillioides* and lower saprophytic fungal isolation frequency when compared to the other media tested.

1. Introduction

Zea mays L., commonly known as corn in America, belongs to the family Poaceae (Farnham et al., 2003). According to the FAOSTAT (2010), the crop is the main crop according to the amount of tonnes produced each year with 11,597,000 tonnes produced in South Africa in 2008. 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 (Troxell, 1996). 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). 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).

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). *Fusarium* contamination is a key agricultural difficulty,

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although more notably, many species within the genus are proliferous producers of mycotoxins that are implicated for severe diseases in farm animals and humans (Nicolaisen et al., 2008).

Fusarium species are varied in their mycotoxin profiles and host-associations, and visibly identifying and quantifying 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 (Glenn, 2007; Nelson et al., 1981).

Distinguishing species from one another in the *Gibberella fujikuroi* species complex based on morphological characteristics is challenging, even for experts (Leslie and Summerell, 2006; Rossi et al., 2009; Summerell et al., 2003). DNA sequence-based identification and species-specific PCR assays are commonly used to accurately identify species inside the complex (Rahjoo et al., 2008).

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 (Bragulat et al., 2004; Castellá et al., 1997; Dhingra and Sinclair, 1995; Gamanya and Sibanda, 2001; Leslie and Summerell, 2006; Nelson et al., 1981, 1983; Tschanz et al., 1975).

The International Seed Testing Association (ISTA) currently does not have a method to test for any *Fusarium* species in maize seed (ISTA, 2011). The aim of the study was to evaluate eight different media and media combinations, with the exclusion of media containing pentachloronitrobenzene (PCNB) due to its carcinogenic properties (IARC, 1987), in order to develop a media suitable for the identification of *Fusarium* spp. in maize seed. ISTA will not validate any method that contains any compound that is considered to be carcinogenic. The work in this paper is as a result from preliminary experiments carried out on the eight different media.

2. Materials and methods

2.1. Origin of maize seeds and fungal 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). *F. graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas (teleomorph *Gibberella subglutinans* (Edwards) P. E. Nelson, Toussoun & Marasas) and *F. verticillioides* (Sacc.) Nierenberg (= *F. moniliforme* Sheldon) (teleomorph *Gibberella moniliformis*, *G. fujikuroi* mating population A) were isolated from these seeds. Isolates of *F. graminearum* (FCC 4121), *F. subglutinans* (MRC 4145), *F. proliferatum* (Matsushima) Nirenberg (*Giberella intermedia*, *G. fujikuroi* mating population D) (FCC 4131) and *F. 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* sp., *Penicillium* sp., *Trichoderma* sp. and *Aspergillus* sp. were isolated from the maize seed. Isolates of *P. crustosum* (Thorn), *P. digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* (Whemer) were obtained from the *Penicillium* laboratory in the Department of Microbiology and Plant Pathology at the University of Pretoria, Pretoria, South Africa.

2.2. Morphological and molecular identification of Fusarium spp.

The *Fusarium* species were morphologically identified according to Nelson et al. (1981, 1983), Mathur and Kongsdal (2003) and Leslie and Summerell (2006). *A. niger*, *P. crustosum* and *P. digitatum* were identified according to Samson and Pitt (1990). Molecular identification of the *Fusarium* species were carried out on the 5′ portions of *translation* elongation factor (EF) 1α 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 100 mg of mycelium for each *Fusarium* species (7 isolates) were used. DNA was extracted using the Zymo research soil microbe DNA kit (with 50 μl of Sabax water used at step 10 instead of a DNA elution buffer). Subsequent to DNA extraction, a gel was run with BigDye 3.1 at 140 V for 30 min to see if the DNA had been successfully extracted. For the PCR, a master mix: Sabax water (19.5 μl), buffer (2.5 μl), $MgCl_2$ (1.25 μl), dNTP's (0.5 μl), EF-1 (0.25 μl), EF-2 (0.25 μl), Taq (0.25 μl) and 0.5 μl DNA of each *Fusarium* species was used. PCR cycle: 95 °C for 4 min, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s 72 °C for 7 min. Post PCR clean-up was done using a Qiagen kit with

Table 1
Media composition and preparation for the identification of *Fusarium* spp.

Media	Ingredients	Quantity
PDA (Merck, Darmstadt, Germany)	Potato dextrose agar powder	39 g
	Distilled water	Made up to 1 L
Corn meal agar (Biolab, New England)	Corn meal agar powder	17 g
	Distilled water	Made up to 1 L
Malt extract agar (1%) (Merck, Darmstadt, Germany)	Malt extract agar powder	10 g
	Distilled water	Made up to 1 L
MGA 2.5	Peptone	15 g
	Agar–agar powder	15 g
	$MgSO_4$	1 g
	KH_2PO_4	0.5 g
	Malachite green oxalate	2.5 mg
	Distilled water	Made up to 1 L
PDA + malachite	Potato dextrose agar powder	39 g
	Malachite green oxalate	2.5 mg
	Distilled water	Made up to 1 L
1/2 PDA(2%) + malachite	Potato dextrose agar powder	19.5 g
	Malachite green oxalate	2.5 mg
	Distilled water	Made up to 1 L
MGA 2.5 + carnation leaf pieces	Peptone	15 g
	Agar–agar powder	15 g
	$MgSO_4$	1 g
	KH_2PO_4	0.5 g
	Malachite green oxalate	2.5 mg
	Sterile carnation leaf pieces	3–4 pieces per Petri-dish
	Distilled water	Made up to 1 L
KCLA	Agar–agar powder	15 g
	KCL	8 g
	Sterile carnation leaf pieces	3–4 pieces per Petri-dish
	Distilled water	Made up to 1 L

Table 2
Results obtained from sequencing the amplified elongation factor (EF) 1α coding region of the *Fusarium* isolates used in the research.

Sample no.	Accession number	Species	E-value
Z1F	gb|EU220409.1|	*Gibberella moniliformis*	0.0
Z1R	emb|AM404138.1|	*Gibberella fujikuroi* var. *moniliformis*	0.0
Z2F	gb|EU220405.1|	*Fusarium proliferatum*	1×10^{-143}
Z2R	gb|AF291058.1|	*Fusarium proliferatum*	0.0
Z3F	gb|AF160294.1|	*Fusarium subglutinans*	1×10^{-153}
Z3R	gb|AF160294.1|	*Fusarium subglutinans*	0.0
Z4F	gb|DQ382170.1|	*Gibberella zeae*	0.0
Z4R	emb|AJ543576.1|FGR543576	*Fusarium graminearum*	0.0
Z5F	emb|AJ543589.1|FGR543589	*Fusarium graminearum*	0.0
Z5R	emb|AJ543576.1|FGR5435789	*Fusarium graminearum*	0.0
Z6F	gb|AF160294.1|	*Fusarium subglutinans*	1×10^{-153}
Z6R	gb|AF160294.1|	*Fusarium subglutinans*	1×10^{-153}
Z7F	emb|AM422699.1|	*Gibberella fujikuroi* var. *moniliformis*	0.0
Z7R	emb|AM422699.1|	*Gibberella fujikuroi* var. *moniliformis*	0.0

Z1F-Z7R = sample numbers indicating forward primer (F) or reverse primer (R) sequencing. Z1F-Z4R = isolates obtained from the Forestry and Agricultural Biotechnology Institute, University of Pretoria, and Z5F-Z7R = isolates isolated in this research.

the modification of step 9 where 50 μl of Sabax water was used instead of 50 μl 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 μl reaction volume) cycle: initial denaturing (96 °C for 1 min), denaturing (96 °C for 10 s), annealing (50 °C for 5 s), extension (60 °C for 4 min) and holding temperature at 4 °C. The cycles above were repeated 35 and 25 times respectively (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.

2.3. Preparation of media

Media was prepared according to Table 1. Fresh carnation leaves were rinsed under running tap water to remove any external impurities and pesticides that could be present. The leaves were then cut into pieces approximately 2–3 cm in length and placed in 9 cm glass Petri-dishes. The Petri-dishes were sealed in an autoclaveable plastic bag, then in aluminium foil. The Petri-dishes were autoclaved for 15 min at 121 °C and 120 kPa. The sterile leaf pieces were allowed to cool and stored at 4 °C until their use in the carnation leaf agar medium and the malachite green agar medium supplemented with sterile carnation leaf pieces.

2.4. Incubation and plating of fungal isolates

Incubation under 12 h black-blue light and 12 h darkness was compared against incubation of the fungi under normal laboratory conditions (average temperature and light period of 25 °C and 12 h, respectively). The fungi were incubated at 25 °C for 12 h under black-blue light (365 nm) and 12 h darkness placed 25 cm from the light source with the Petri-dishes placed next to each other (not stacked). For the experiments carried out on the *Fusarium* species and saprophytes on the eight nutrient media, namely, potato dextrose agar (PDA), PDA + malachite green oxalate (PDA+), 1/2 strength PDA + malachite green oxalate (1/2 PDA+), 1% malt agar (MA),

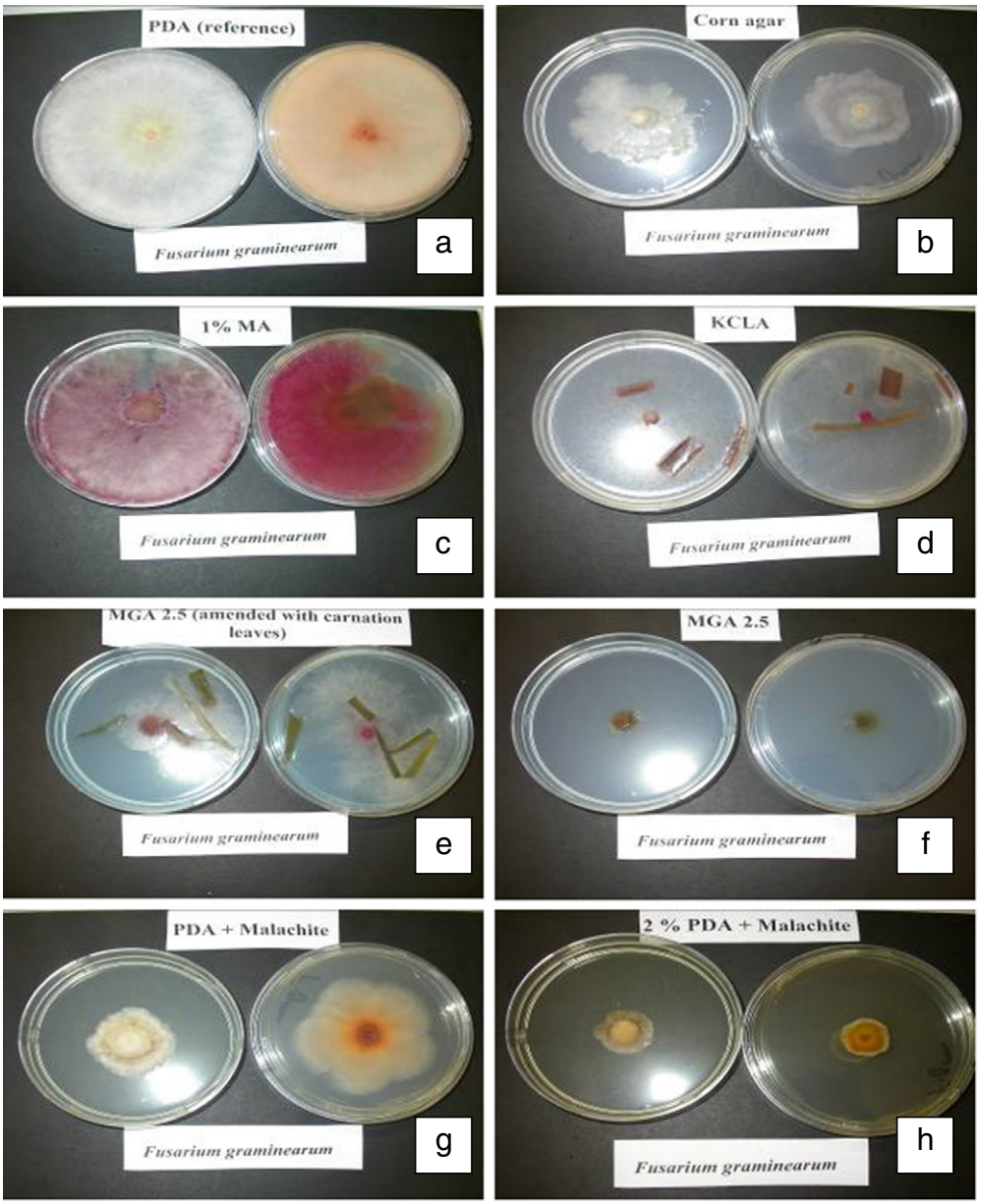

Fig. 1. (a–h). Colony colour and growth of *Fusarium graminearum* after 7 d of incubation on different media (see Materials and methods section for abbreviations).

carnation leaf agar supplemented with potassium chloride (KCLA), corn meal agar (CMA), MGA 2.5 and MGA 2.5 + sterile carnation leaf pieces (MGA +), the isolates were initially incubated on PDA as described above before being plated out. Subsequent to incubation, agar blocks (5 × 5 mm) were aseptically cut and placed in the centre of the Petri-dishes (90 mm) containing the different media and incubated as before. Each isolate was replicated five times on each of the different media. After 4 and 7 d of incubation, colony colour, radial diameter (measured from the edge of the agar blocks to the edge of the colony), sizes of macroconidia and microconidia were measured and sporulation was compared among the different media.

2.5. Plating of maize seeds

Two hundred (four replicates of 50) maize seeds from two maize cultivars (PAN 6Q-308D and PAN 6223B) were surface disinfested with 0.5, 1 or 1.5% NaOCl (sodium hypochlorite) for 1, 3, 5 or 10 min, triple rinsed with sterile distilled water then left to air dry in a laminar on sterile paper towels. The best treatment, namely 1% NaOCl for 5 min (results not given), was used to sterilise two hundred maize seeds from each of the two maize cultivars. The seeds were then aseptically plated onto PDA, KCLA, MGA 2.5 and MGA 2.5 supplemented with sterile carnation leaf pieces. The seeds were plated 5 seeds per 90 mm Petri-dish (1 in the centre and 1 in each quadrant) with four replicates of 50 seeds per cultivar. The seeds were incubated for 7 d at 25 °C for 12 h under black-blue light (365 nm) and 12 h darkness placed 25 cm from the light source with the Petri-dishes placed next to each other (not stacked). Growth, sporulation and results were observed and recorded on day 4 and day 7 of incubation.

3. Results

The molecular identification method of O'Donnell and Cigelnik (1997) used in this study was precise (Table 2) and confirmed that the identification of the *Fusarium* species, using their morphological characteristics, was accurate.

All the fungal species stored at 4 °C in sterile distilled water remained viable for the duration of the experiments. Sporulation of

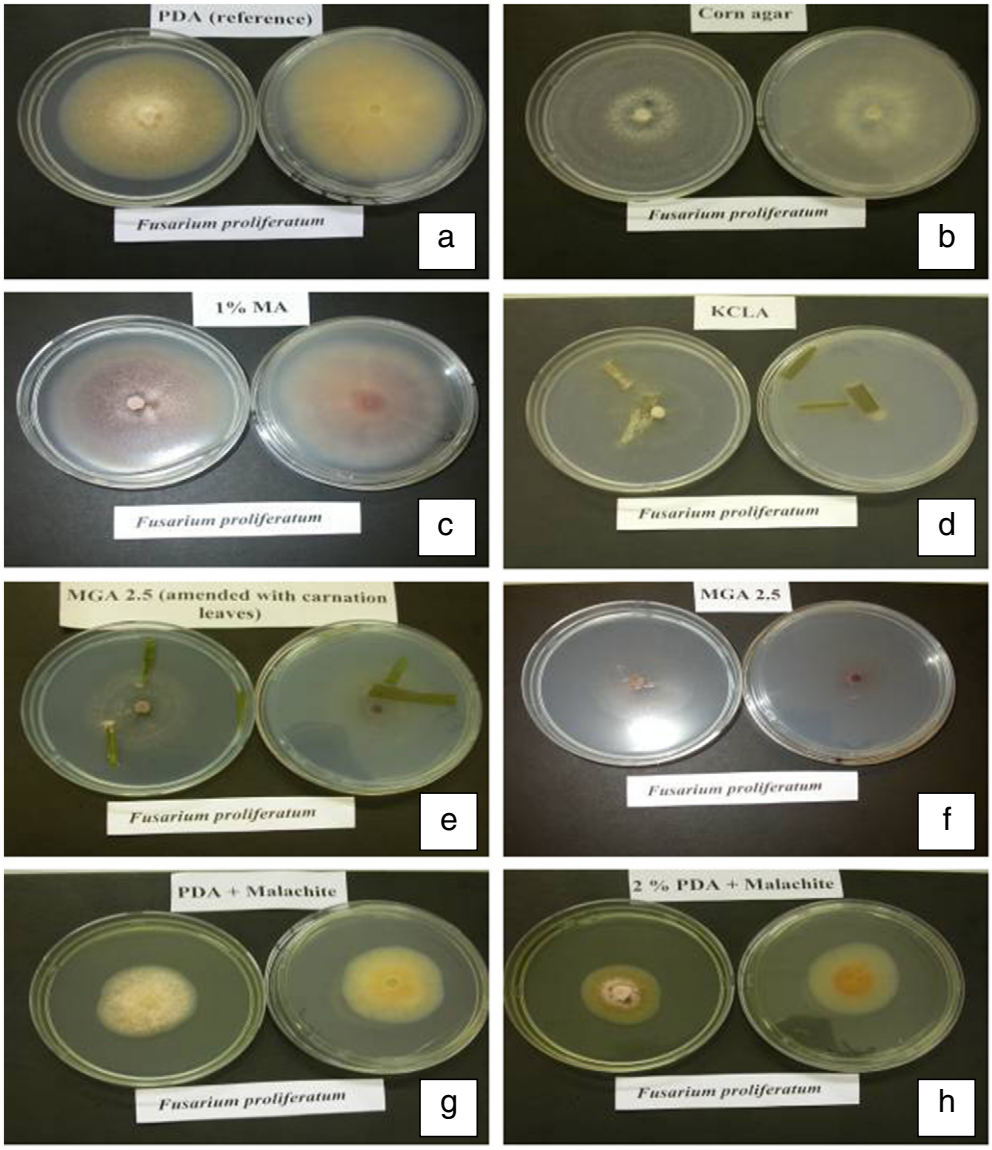

Fig. 2. (a–h). Colony colour and growth of *Fusarium proliferatum* after 7 d of incubation on different media (see Materials and methods section for abbreviations).

the fungi was improved when incubated under 12 h black-blue light and 12 h darkness when morphologically compared under a compound microscope to the fungi incubated under normal laboratory conditions.

Growth of *F. graminearum* was the poorest on media that contained malachite green oxalate (MGA 2.5), 1/2 PDA + malachite (1/2 PDA +) and PDA + malachite (PDA +) and with few sporodochia containing macroconidia. The fungus developed and sporulated well on PDA, MA, CMA, KCLA and MGA + carnation leaf pieces (Fig. 1). The fungus produced a red colour pigment in MA, KCLA and MGA 2.5 amended with sterile carnation leaf pieces, particularly adjacent to the carnation leaf pieces. In PDA + malachite green oxalate, 1/2 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 (Fig. 2) 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 1/2 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 colour 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 1/2 PDA + where sporulation of the fungus was insufficient to permit accurate identification.

F. subglutinans (Fig. 3) produced abundant aerial false heads containing microconidia that arose from monophialides and polyphialides on PDA, 1% MA, CMA, KCLA and MGA + carnation leaf pieces. 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 colour pigment in MGA + carnation leaf pieces, KCLA and corn meal agar with sufficient sporulation to allow for accurate identification. The radial colony growth and sporulation of the fungus was restricted in PDA +, 1/2 PDA + and MGA 2.5 where there was dense growth of the fungus and poor sporulation not permitting accurate identification.

F. verticillioides produced a cream colour pigment in CMA, KCLA and MGA amended with sterile carnation leaf pieces (Fig. 4). There

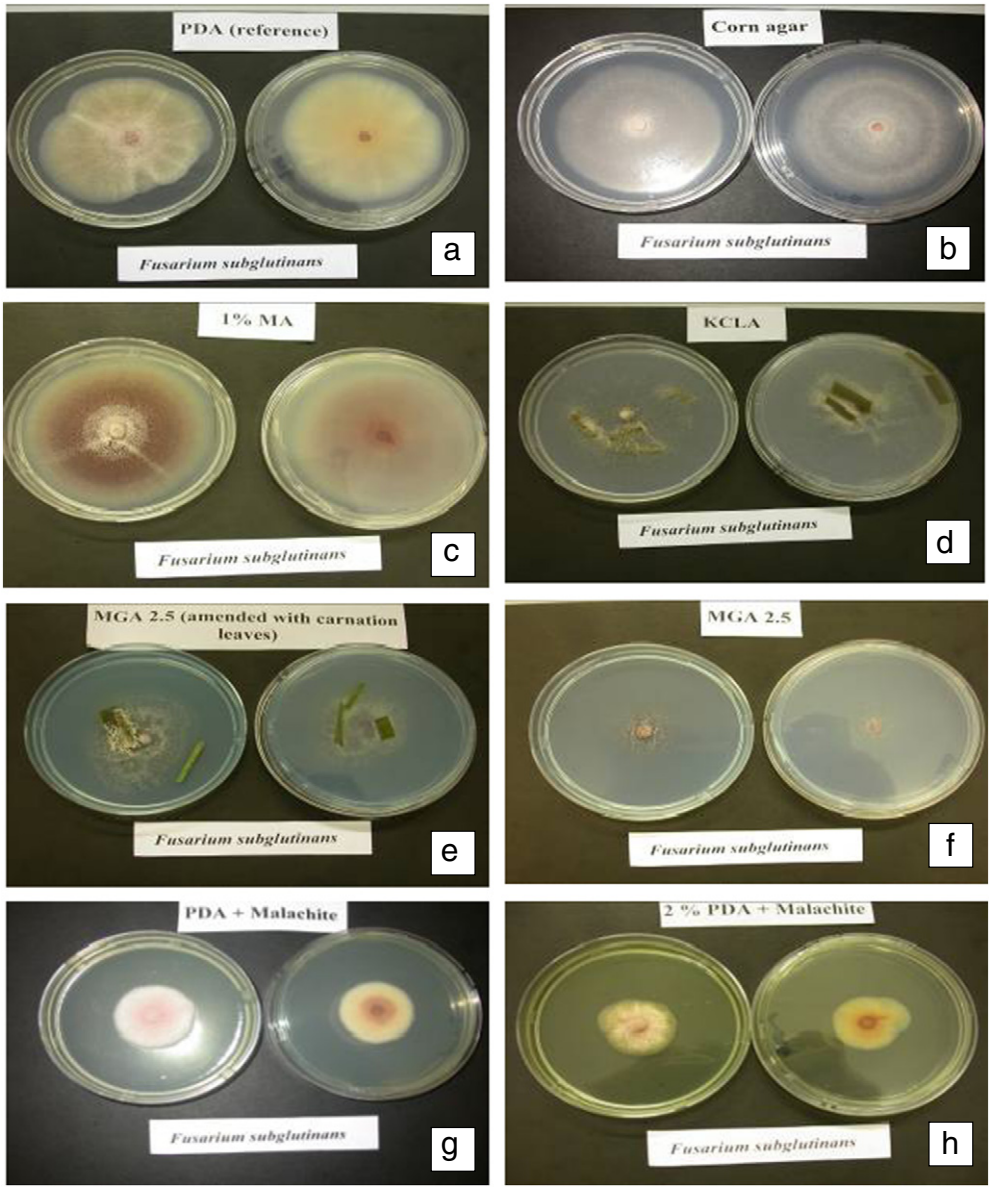

Fig. 3. (a–h). Colony colour and growth of *Fusarium subglutinans* after 7 d of incubation on different media (see Materials and methods section for abbreviations).

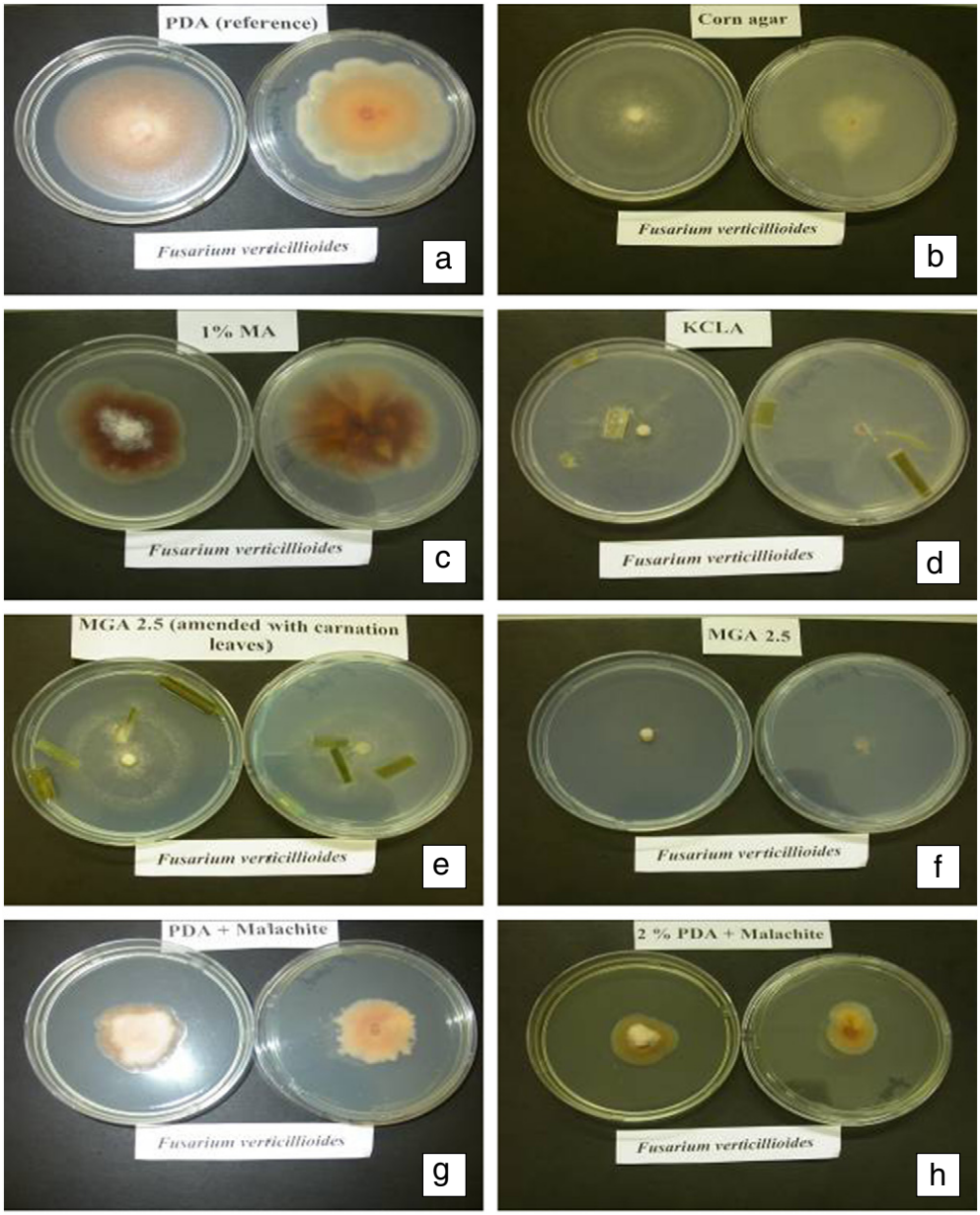

Fig. 4. (a–h). Colony colour and growth of *Fusarium verticillioides* after 7 d of incubation on different media (see Materials and methods section for abbreviations).

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+, 1/2 PDA+ and MGA 2.5 where there was little colony diameter growth and sporulation but 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 1/2 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. The radial colony growth of the *Fusarium* species was limited in media that contained malachite green oxalate (PDA+, MGA and 1/2 PDA+), with the exclusion of MGA supplemented with sterile carnation leaves that was in some cases statistically significant from the control in radial colony growth (Figs. 1, 2, 3, and 4).

The media that contained malachite green oxalate (PDA+)+, MGA, MGA+ and 1/2 PDA+) were most successful in limiting the radial colony growth of *A. niger*, *P. crustosum* and *P. digitatum* with no statistical significant difference in the radial colony growth among the media. There was little or no significant difference among PDA, KCLA, CMA and MA against limiting the growth of *A. niger*, *P. crustosum* and *P. digitatum* (Table 3).

MGA, MGA+ and 1/2 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 (Table 3). MGA, MGA+, PDA+ and 1/2 PDA+ limited the growth of *Trichoderma* more significantly than PDA, KCLA, MA and CA where there was little or no significant difference among PDA, KCLA, MA and CMA when compared to each other (Table 3).

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 (Fig. 5). *Fusarium* spp. were more frequently

Table 3
Radial colony diameter (in cm) of *Aspergillus niger*, *Penicillium crustosum*, *Penicillium digitatum*, *Rhizopus* and *Trichoderma* after 7 d of incubation at 25 °C under black-blue light growing on the different media (see Materials and methods section for abbreviations) and percentage fungal species isolation on the four media tested carried out on maize seed lot PAN 6223B.

Fungi	Media									
	PDA	MA	CA	1/2 PDA +	PDA +	KCLA	MGA 2.5	MGA 2.5 +	LSD	SED
Aspergillus niger	7.94[a]e	7.3d	6.96c	3.86a	4.1ab	7.84e	3.84a	4.22b	0.322	0.157
Penicillium crustosum	5.54c	5.36e	4.28b	2.64a	2.78a	5.2c	2.98a	2.82a	0.827	0.404
Penicillium digitatum	6.08e	5.38d	3.88c	2.68a	3.16b	5.14d	2.98a	2.96a	0.383	0.187
Rhizopus	8 g	8 g	5.72e	0a	5.1d	7.18f	2.5e	1.8b	0.359	0.175
Trichoderma	5.92 g	5.2f	3.92d	1a	2.3b	4.64e	2.78c	2.88c	0.357	0.174
PAN 6223B										
Aspergillus niger	16.5[b]c					11b	7.5ab	6a	4.104	1.291
Aspergillus flavus	14b					4.5a	3.5a	5.5a	2.248	0.707
Penicillium	21c					19.5cb	12a	15b	3.371	1.061
Chaetomium	8b					3.5a	4.5a	4a	1.124	0.354

[a] Each value is mean of five replicates. Means within a ROW not followed by the same letter are significantly different ($P \leq 0.05$).
[b] Each value is percentage fungal species isolation on the four media tested. Means within a ROW not followed by the same letter are significantly different.

isolated on MGA 2.5 supplemented with sterile carnation leaves with 61% *F. verticillioides* infection followed by MGA 2.5 which had an infection level of 59%. PDA and KCLA had a *F. verticillioides* infection level of 38.5% for both media tested. PDA and KCLA also had a notably higher percentage saprophytic growth from the seeds when compared to MGA 2.5 and MGA 2.5 supplemented with sterile carnation leaf pieces (Table 3 and Fig. 5). 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 thereby allowing more accurate identification. The maize seeds on the media that did not contain malachite green oxalate germinated and lifted the lids of the Petri-dishes. This was not the case with MGA 2.5 and MGA 2.5 supplemented with sterile carnation leaf pieces, where excessive germination and growth of the maize seeds were inhibited.

F. verticillioides had a higher isolation percentage on MGA 2.5 + (Fig. 5) when compared to the other media and less saprophytic fungi (Fig. 5; Table 3) were isolated on this medium. MGA 2.5 and MGA 2.5 + were significantly different to the other media tested in hampering the growth of the saprophytes. MGA 2.5 + had a higher significant *F. verticillioides* isolation difference when statistically compared to MGA (Fig. 5).

4. Discussion

The use of light during the incubation of the fungi permitted better sporulation of the *Fusarium* species when compared to incubation without the use of light and resulted in a more accurate identification. The results confirmed the results of Snyder and Hansen (1941) who illustrated the effect of light on taxonomic characters in *Fusarium*.

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

The results revealed that malachite green agar (MGA 2.5), potato dextrose agar (PDA) + malachite green oxalate, 1/2 PDA + malachite was most inhibitory to the growth of the saprophytes which is in agreement with Castellá 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).

The radial colony growth of the *Fusarium* species was restricted on PDA + malachite, 1/2 PDA + malachite and MGA 2.5 when compared with PDA, KCLA, MA, CA and MGA 2.5 + sterile carnation leaf pieces. This confirms the results found by Castellá et al. (1997) who reported that the mean percentage of reduction in colony diameters of *Fusarium* spp. strains were higher on MGA 2.5. 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 (Castellá et al., 1997; Dhingra and Sinclair, 1995; Nelson et al., 1981,

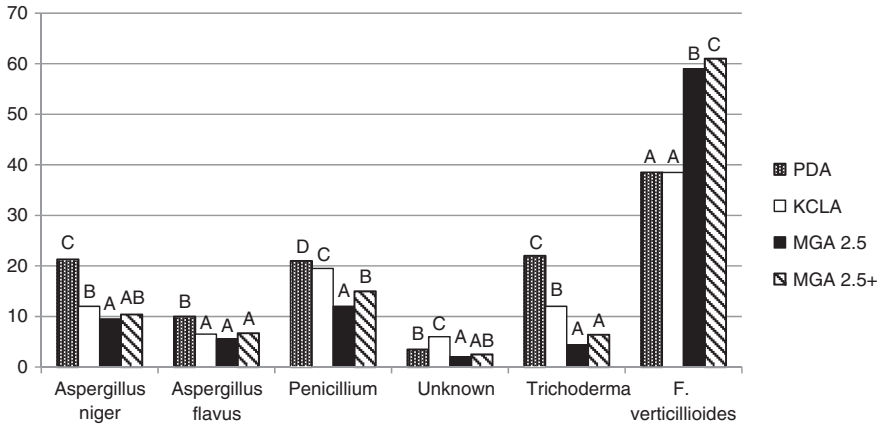

Fig. 5. Percentage fungal isolation on the four different media carried out on seed lot PAN 6Q-308D using four replicates of 50 seeds. Columns within a fungal species not headed by the *same letter* are significantly different ($P \leq 0.05$).

1983). Alborch et al. (2010) further reported that MGA 2.5 was a potent selective medium for the detection of *Fusarium* in infected maize kernels using the direct plating technique and could be used as an alternative to Nash and Snyder medium which contains pentachloronitrobenzene (PCNB), which is reported to be carcinogenic (IARC, 1987).

Although the 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 supplemented 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.

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, thereby causing desiccation of the media and the possibility of cross contamination between the Petri-dishes. 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).

Distinguishing species from one another in the *G. fujikuroi* species complex based on morphological characteristics is challenging, even for the experts (Leslie and Summerell, 2006; Rossi et al., 2009; Summerell et al., 2003). 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.

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. MGA 2.5 showed, in some instances, better hindering of the saprophytes when compared to MGA 2.5 +. *F. verticillioides* had a higher isolation percentage on MGA 2.5 + when compared to MGA 2.5 and this could be contributed to the better sporulation of the fungal species on MGA 2.5 +. The *Fusarium* species sporulated more on MGA 2.5 + which allowed more accurate identification. *F. verticillioides* formed longer chains of microconidia on MGA 2.5 + than on MGA 2.5 only.

Although the medium showed a high potential in the isolation of *Fusarium* spp., hindering the growth of the 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. Comparative studies using the method will be done with five to eight laboratories to validate the new method. Once the method has been validated and approved by the Seed Health Committee it will become part of the International Rules of ISTA to be used worldwide in the trading of maize seed for planting. Although further tests are required under different conditions, the results from the experiments carried out proves that this medium is a potent *Fusarium* selective medium. The suitability of this selective medium could also be used for other plant parts such as stems and roots and also for other crops infected with *Fusarium* spp.

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