The African Journal of Plant Science and Biotechnology ©2011 Global Science Books

Fungi and their Use in the Possible Control of Nematodes in Botswana Soils

Tabo Mubyana-John* • **Mareledi G. Wright**

Department of Biological Sciences, Univ. of Botswana, P/B 0022, Gaborone, Botswana

Corresponding author: * **mubyanat@mopipi.ub.bw**

ABSTRACT

A study assessing fungi isolated from three climatic regions of Botswana as a possible control for root knot nematodes (*Meloidogyne spp*.) affecting the tomato (*Lycopersicon esculentum* L. var. 'Money maker') crops was conducted. Out of 1,250 soil fungal isolates, only 232 soil fungi from the Okavango Delta, Bobirwa district and different saltpans in Botswana could be sub cultured, and screened for antagonism against *Meloidogyne* sp. Seven of the isolates i.e., *Trichoderma* sp., *Penicillium* sp., *Dendriphiopsis* sp., *Fusarium chlamydosporium*, *Cochliobolus sativus*, *Aspergillus fumigatus* and an unidentified sterile fungus showed potential to immobilize nematode juveniles on agar plates within 2-4 days. *C. sativus* and *Dendriphiopsis* sp. showed maximum paralysis on agar plates and eventually death of the *Meloidogyne* sp. juveniles. In greenhouse studies using tomato plants the fungi alleviated the effect of nematodes by increasing plant, shoot height and root weight as compared to the reference controls. *Cochliobolus sativus* and *Trichoderma* sp. showed maximum plant protection of tomato plants under greenhouse conditions. Fungi ability to produce cellulase and chitinase were some of the mechanisms studied. The results indicated that *Trichoderma* sp., *Penicillium* sp., *Dendriphiopsis* sp., *Fusarium chlamydosporium*, *Cochliobolus sativus*, and *Aspergillus fumigatus* are nematode antagonistic fungi indigenous to Botswana that can be used to control nematodes as they are better adapted in comparison to introduced fungi.

Keywords: Bobirwa, *Cochliobolus*, *Meloidogyne*, *Trichoderma*, Okavango, saltpan, tomato

INTRODUCTION

Plant parasitic nematodes can cause significant plant damage, ranging from negligible injury to total destruction of the plant material (Sharma *et al*. 1997). Economic losses of crops up to 40 to 50% or even more can occur due to the most damaging nematodes (Maqbool and Kerry 1997). As nematodes often do not provide clear symptoms, their economic effects tend to be underestimated by growers. Losses and damage due to nematodes is more extreme in the rain season, with members of the Solanaceae family such as potatoes and tomatoes being more susceptible. Botswana, being semi arid, grows commercial Solanaceous crops in greenhouses throughout the year. Physiological disorders caused by nematodes are commonly observed in these crops (Nilsson 1972). It was estimated that root knot nematodes of the *Meloidogyne* spp. caused 20 to 50% loss in tomato fields (Nilsson 1972). There are no recent documented figures on losses from root knot nematodes in Botswana; although there is evidence that these plant parasites are a nuisance to farmers.

So far Botswana has relied primarily on the use of the highly effective fumigant, methyl bromide, to control root knot nematodes (DMS 2003). However, methyl bromide due to its poisonous nature and its being implicated in the depletion of 5-10% of the ozone layer (MBTOC 1995; Bell *et al*. 1998; Sikora 2002) implies it can no longer be used (EPA 2003; Montzka *et al*. 2003; Mbao 2003; Saunders 2004). Antagonistic fungi are among the most important potential biological control agents of plant parasitic nematodes (Taylor and Sasser 1980). Important antagonistic fungi genera against parasitic nematodes include *Paecilomyces*, *Verticillium*, *Hirsutella*, *Nematophthora* and *Arthrobotrys* species (Sikora 2002). Some of these fungi are being produced and used effectively in the control of root knot nematodes worldwide. *Paecilomyces lilacinus* Strain 251 is the active ingredient of '*Pl* Plus', a biological control product that is currently marketed in South Africa. '*Pl* Plus' has been tested in Botswana and undocumented findings from farmers indicate that so far the results are very poor. In most cases, the fungi failed to colonise the roots and survive in the introduced soils (Koosaletse 2001). One of the major obstacles in using introduced alien soil microorganisms for biological control purposes is the difficulty of establishing efficacy of the organism in the existing soil ecosystem (Carlile *et al*. 2001).

Fungi modes of action in nematode control vary from formation of loops to biochemical effects, such as the release of extracellular nematicidal metabolites (Segers *et al*. 1996; Barron 2003; Abubakar *et al*. 2004) and enzymes, which degrade the host cuticle. Some fungi are cellulolytic or ligno-cellulolytic and attack nematodes as sources of nitrogen to supplement a primarily carbohydrate diet (Barron 2003). Others release chitinases, which are involved in host infection since they can be localized on infection structures (Chet *et al*. 1997; Manocha and Zhonghua 1997; Schickler *et al*. 1998). Botswana is a semi-arid country with very high day temperatures in the hot season (> 40°C) and very low in winter nights (< 0°C). The extreme temperatures are accompanied by very low soil moisture content (DMSR 2003). Thus it is unlikely that introduced fungi may survive in the harsh environment whereas the indigenous fungi would be more adapted to these conditions. With this background, the present study was undertaken to find out the nematophagous fungi indigenous to Botswana soils and to determine their mode of action to control root knot nematode in tomato plants (*Lycopersicon esculentum* L. var. 'Money maker') under greenhouse conditions.

Received: 16 April, 2010. Accepted: 16 November, 2010.

Original Research Paper

Fig. 1 Map of Botswana showing sampling sites. X1 Okavango Delta, X2 Tshane, X3 Kang, X4 Sekoma and X5 Matlhabaneng. Adapted from http://photonet/travel/africa/botswana/botswana-map.gif

MATERIALS AND METHODS

Site location and sampling

Virgin soils were obtained from the Boro route of the Okavango Delta (23°–24°E and 19°–20°S), saltpans of Kang (22°E and 23°S), Tshane (21°E and 24°S) and Sekoma (23°E and 24°S) and semi-arid region of Motlhabaneng in the Bobirwa District (28°E and 22°S) (**Fig. 1**). These soils represented the three different climatic regions of Botswana i.e., arid, semi arid and the inland delta (Batisani and Yarnal 2010; Kayombo *et al*. 2010). The samples were collected from the A_1 horizon using a hand spade and stored in separate ziplock sterile bags (Nasco-Pak, Whirl Park, USA) and transported to the laboratory where they were kept at 4°C until the isolations.

Isolation

Fungi were isolated using the dilution plate method on malt extract agar [MEA (Oxoid Ltd. Basingstoke, Hampshire, England)] and potato dextrose agar [PDA (Oxoid Ltd. Basingstoke, Hampshire, England)] amended with 0.3 g/l streptomycin sulphate to inhibit bacteria. The plates were incubated at 25°C for 4-7 days to allow for fungal colony development. The obtained colonies were purified by sub-culturing them on fresh MEA and PDA media for sporulation which was deemed essential for identification. Non sporulating fungi were induced by growing them on water agar supplemented with carnation leaves and incubating the plates under ultra-violet light (short wave length-340 nm) at 25°C until the fungi sporulated (Fisher *et al*. 1982; Kirk *et al*. 2001). When induction of sporulation was not successful, isolates were recorded as 'sterile mycelia and unidentifiable. Identification of fungi was based on visible macroscopic colonies and microscopic reproductive features (Carmichael *et al*. 1980; Sutton 1980; Hanlin 1992; Domsch *et al*. 1993; Klich 2002).

Isolation of root knot nematodes (*Meloidogyne* spp.)

Tomato roots with galls symptomatic of root knot were collected from a farm in Gaborone, Botswana (**Fig. 1**) placed in plastic bags and transported to the laboratory. Nematode juveniles of *Meloidogyne* sp. were isolated from the infected roots using modified standard methods (Hooper 1986; Ingham 1994). The galled tomato roots were washed thoroughly with tap water to remove excess soil, surface sterilized with 3% sodium hypochloride (NaOCl), and then cut into 3-5 cm segments with a sterile scalpel and then agitated in 1% NaOCl for 3 min to dislodge egg masses (Ingham 1994). The sections were then immersed in 10 ml sterile tap water in 90 mm sterile Petri dishes and incubated at 25°C in the dark for 24 hrs.

After incubation, nematode juveniles were harvested by removing the root segments out of the Petri dishes leaving a water suspension containing nematode juveniles. The segments were re-incubated in sterile water and harvesting was done until no further juveniles could be obtained. The isolated root knot nematodes were identified under a microscope (Zeiss, Germany GSZ) to the genus level with temporary wet mounts (Jepson 1987). The number of eggs and juveniles in the suspension was determined by dilution using counting chambers.

Screening of fungi for nematode antagonism

Fungal-nematode interaction studies were carried out using standard procedures (Rosenzweig and Pramer 1980; Tzean and Liou 1993). A mycelial disc was cut from the edge of a growing fungal colony using a sterile scalpel and transferred onto cornmeal agar in 90-mm Petri dishes. Two replicates for each fungal isolate were made. The inoculated agar plates were then incubated at 25°C in the dark. After 5 days of incubation, a few drops of the nematode suspension containing approximately 3×10^3 infective *Meloidogyne* stages (eggs and juveniles) were placed near the edges of the growing colony of each isolated fungus and reincubated at 25°C in the dark. The first microscopic observation was made 15 min after incubation, followed by sequential observations every 2 hrs for 4 days, from 0700–1900 hrs daily.

To evaluate the parasitic ability of the test fungi on nematodes, each plate was scored with a minus (–) for no visible effect or a series of pluses (+) for a positive visible effect. Visible effect in this case was the presence of dead or paralysed nematode juveniles on the plate. The percentage larval immobilization or paralysis was calculated by the following formula: (number of immobilized juveniles) × 100 / total number of inoculated juveniles. Only those fungi, which showed the ability to negatively affect nematodes, were further studied in green house experiments.

Fungal-nematode interactions on slide cultures

The materials required for slide cultures studies were sterilized by autoclaving at 121°C for 15 min. A sterile moist filter paper was aseptically lined on the base of a sterile 90-mm Petri dish followed by a cavity slide suspended on a V-shaped glass rod. A few drops of sterile molten cornmeal agar (CMA) were added onto the slide in a Petri dish and left to set. Using a sterile scalpel blade and needle, a germinating spore or hyphal tip of the test fungus was transferred onto the solidified agar and incubated at 25°C for 24-48 hrs to allow the fungus to grow. After incubation, using aseptic techniques, a drop of nematode suspension was applied to the centre of a sterile microscope cover slip. The cover slip carrying nematode inoculum was then inverted onto the cavity slide bearing the test fungus, ensuring that the nematode suspension drop hung from the cover slip onto the fungal colony. The control slide consisted of fungi with a drop of sterile tap water, without the nematodes. The plates containing the fungi were then covered and incubated at 25°C for 24-48 hrs to allow the test fungi to interact with the nematodes. The fungi-nematode interactions were monitored on the slides daily from 0700 hrs till 1900 hrs at 30-min intervals. A compound light microscope (Zeiss GSZ, Germany) mounted with an axio-camera was used to observe the interactions at ×40 and ×100 magnifications paying special attention to nematode juveniles and eggs behaviour.

Study of the fungal modes of action

1. Determination of fungal cellulase activity

Some nematode-trapping fungi have been reported to be cellulolytic or ligno-cellulolytic; others release chitinases which play a role in host infection (Chet *et al*. 1997; Manocha and Zhonghua 1997). Hence in this study, the antagonistic fungal isolates were assayed for the production of cellulose and chitin degrading enzymes.

Glucose is a product of cellulose activity and the amount of glucose produced in carboxymethylcellulose (CMC) medium is

assumed to be directly proportional to the amount of enzyme produced (Köning *et al*. 2002). A 1 cm^2 disc carrying fungal mycelia was cut from the edge of a growing colony using a sterile blade and transferred onto CMC agar (Köning *et al*. 2002). The inoculated plates were then incubated at 25°C for 5 days to establish fungal growth and exhaust all the stored sugars. One millilitre was then used to inoculate fresh CMC in 250 ml Erlenmeyer flasks. The inoculated media were then placed on an orbital shaker at 150 rpm at 25°C for 10 days. The samples were then centrifuged and the filtrate amended with 0.6% sterile (autoclaved at 121°C for 15 min) Tween 80 solution (Gashe 1992). The amount of glucose produced was determined using a spectrophotometer (Spectronic 20D+, ThermoSpectronic) by reading at 540 nm. Glucose was used as standard.

2. Determination of chitinolytic activity

Chitinolytic activity of the nematode antagonistic fungi was detected on agar plates following the method proposed by Chernin *et al*. (1998) and Tikhonov *et al.* (2002). Fungi discs of approximately 1 cm^2 were inoculated on to chitin agar (0.5 g chitin, 1.2 g $MgSO_4$, 0.5 g K_2HPO_4, 1.0 g KCl, 15.0 g bacteriological agar, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$ and 0.001 g $MnCl_2$ in 1 l distilled water (Hsu and Lockwood 1975). The medium contains chitin as the sole carbon source thus assumes only fungi capable of degrading chitin grow on it. For each test fungus, two Petri dishes were inoculated and incubated at 25°C for 7-10 days in the dark. Any zone of clearance developed around growing fungal colonies indicated the chitinolytic activity of the test fungi.

Greenhouse experiments

A greenhouse experiment was conducted to test the effect of the isolated antagonistic fungi on root knot nematode in tomato plants grown in vermiculite. The fungal inoculum was grown on solid agar media and harvested as outlined by Katsantonis *et al*. (2003). Each fungal preparation was then diluted with 2 l sterile tap water to make the standard fungal inoculum. For each test fungus, the inoculum was divided equally among three 20 cm-diameter-pots containing sterile vermiculite and mixed in thoroughly. A standard colony plate count on PDA (Parkinson 1994) was used to estimate the fungal inoculum population in the growth medium (vermiculite).

The fungus-inoculated pots were left in the greenhouse (60–70% RH and 27°C day/16°C night temperatures, 12/12 hr light/day) for 10 days to allow the fungi to be established. After 10 days, 2-weeks-old tomato (*L. esculentum* L. var. 'Money maker') seedlings purchased from Sanitas Nurseries and Garden Centre (Pty) Ltd. in Gaborone, Botswana were transplanted into the pots. Three seedlings per pot and 3 replications per treatment were made. Long Ashton nutrient solution (Hewitt 1966) was added to the pots as nutrient solution at 20 ml/pot every 7 days.

Two weeks after transplanting, root knot nematodes were introduced to 24 pots (21 inoculated with fungi and 3 uninoculated). Approximately 3×10^3 mixture of eggs and *Meloidogyne* juveniles, collected from the roots over 24 hrs and then used within 2-3 days of collection were introduced into the four holes (2-3 cm holes) made around each individual tomato seedling, allowing the nematodes to migrate to the roots. The plants were grown in the greenhouse for 60 days before they were assessed for nematode damage, plant height, shoot fresh weight (FW) and dry weight (DW), and root FW and DW. Moisture content of the vermiculite was maintained at 75% field capacity during the growing period. The controls consisted of two groups i.e., tomato seedlings grown in vermiculite with neither fungi nor nematodes; while the other control consisted of plants grown in vermiculite inoculated with nematodes.

Statistics

Analysis of variance (ANOVA) was performed using SPSS 11.0. Duncan's multiple range test was used for mean separation based on fungi isolate and nematode inoculation. Plant parameters assessed were plant height, shoot and root fresh weight and dry weight (FW and DW).

RESULTS

Nematode and fungi isolates

In all, initially there were 1,250 fungi that grew on the isolation plates. Once isolated the fungi were purified by transferring to fresh agar medium. However, most of the fungi especially those from the saltpans and Bobirwa districts could not be grown upon transfer. Therefore out of all the fungi isolated, only 232 fungi could be transferred, purified and identified using the existing keys. These isolates belonged to 28 different genera. **Table 1** shows the most common and some of the rare fungal isolates and their origins. Of all the isolates, 43.05% originated from the Okavango delta region soils (along the Boro route), 29.15% from Motlhabaneng in the Bobirwa District and 27.80% were from the saltpans soil samples (Kang, Tshane and Sekoma). The most common were *Aspergillus* and *Penicillium* spp. isolates. There were many different species of *Fusarium* isolated and of these, only one could not be identified to species, and this species showed potential to immobilize nematodes. Other isolates, like *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* [Sacc. in Sorok.] Shoem.) and *Dendriphiopsis* sp. fell in between the most and least common species isolated. There were only few *Rhizopus* and *Trichoderma* species. Tentative identification was only done on the isolates which showed a potential in nematode control after screening.

Screening on agar plates

Screening of all the fungi for nematicidal effect on cornmeal agar (CMA) plates was based on their ability to immobilize or paralyse nematode juveniles *in vitro*. Seven

Table 1 Common and rare soil fungi isolated from the Okavango Delta region (OD), Bobirwa district (BD) and the saltpans (SP).

Fungi sp.	Okavango Delta	Bobirwa District	Saltpans
Common fungi			
Aspergillus parasiticus	✓	✓	✓
Aspergillus fumigatus	✓	✓	✓
Aspergillus wentii	✓		
Aspergillus sp.A	✓	✓	
Aspergillus sp.B	✓	✓	
Penicillium claviforme	✓		
Penicillium steckii	✓	✓	✓
Rare fungal species			
Penicillium sp.	✓		✓
Phoma medicaginis	✓		
Cochliobolus sativus	✓		
Dendriphiopsis sp.			✓
Fusarium sp.A	✓		
Rhizopus stolonifer	✓		✓
Trichoderma sp.	✓		
Total isolates from site (%)	538(43.05)	365 (29.15)	347 (27.9)
Total identified isolates from site	120	24	88

Table 2 Effect of fungal isolates on *Meloidogyne* sp. juvenile mobility on agar plates.

Treatments	Fungal source	Immobilization levels during incubation		
		Day 2	Day 3	Day 4
Control	-	-	-	
A. fumigatus	Bobirwa	-	++	++
C. sativus	Okavango	-	++	+++
Penicillium sp.	Okavango	-	+	++
Trichoderma sp.	Okavango	+	+	++
F. chlamydosporum	Saltpan	-	+	++
Dendriphiopsis sp.	Saltpan	+	++	+++
Sterile mycelia	Saltpan	+	+	+

Each result is was derived from five replicates
-, +, ++, +++ represent 0-25 %; 26-50 %; 51-75 %; 76-100 % immobilized *Meloidogyne* sp. juveniles respectively.

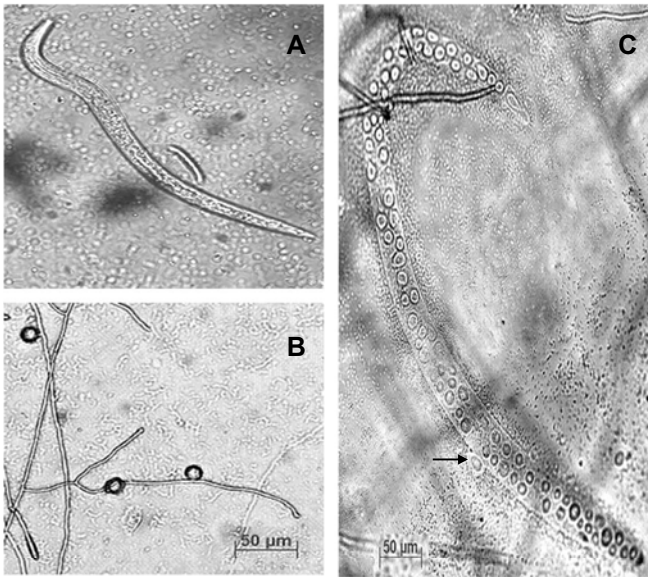

Fig. 2 (**A**) Juvenile body not infested with *Cochliobolus sativus*. (**B**) Spore-like structures from an older pure culture of *C. sativus*. (**C**) Nematode body containing many spore-like structures of *C. sativus*. Arrow shows a spore attached to the exterior of the juvenile (Bar = 50 µm in all cases).

fungal species showed the ability to control *Meloidogyne* sp. juveniles on agar plates (**Table 2**). *Cochliobolus sativus* and *Dendriphiopsis* sp. treatments gave the maximum nematode juvenile mortality (≥75% paralysis) by the 4th day after nematode inoculation. *Aspergillus fumigatus*, *Fusarium chlamydosporum*, *Penicillium* sp. and *Trichoderma* sp. had paralyzed about 51-75% *Meloidogyne* juveniles on agar plates by Day 4. Comparison of the percentage juvenile immobilization relative to time of exposure revealed that the maximum immobilization was observed after 4 days. Nematode immobilization was negligible in all the agar plates on Day 1. Exposure for 2 days gave the least immobilization of the nematodes. Paralysis of nematode juveniles was negligible in the control plates during the 4 days incubation period (**Table 2**).

Microscopic screening

When viewed under the microscope, there were no direct interactions observed between the test fungal mycelia and nematode juveniles or eggs. However, for *C. sativus*, spherical spore-like structures were observed to have formed inside the bodies of *Meloidogyne* sp. juveniles (**Fig. 2A**) although none were observed germinating. Similar structures were also observed in older pure cultures of *C. sativus* (**Fig. 2C**). These were thought to be resting spores, although no documented data on the resting spores of *C. sativus* has been found in literature. Nevertheless, no such structures were observed in the control slides.

Although there were no microscopically visible physical interactions observed between nematode juveniles and eggs and the other six fungi, the fungi had shown positive nematode inhibition on agar plates. The inoculated *Meloidogyne* juveniles became immobilized after 2-3 days of incubation on the slides and no juveniles were immobilized in the controls.

Table 3 Glucose concentrations, as determined by spectrophotometric assay, obtained from the degradation of CMC by the tested fungal isolates.

Test fungus	Glucose concentration (µg/ml) Ψ
Aspergillus fumigatus	11.23 ± 1.6
Cochliobolus sativus	5.42 ± 0.5
Fusarium chlamydosporum	6.74 ± 0.7
Dendriphiopsis sp.	7.40 ± 1.0
Penicillium sp.	9.90 ± 1.4
Trichoderma sp.	7.11 ± 0.9
Sterile mycelia	6.45 ± 0.6
Paecilomyces sp.*	3.74 ± 0.2
Pestalotiopsis sp.**	2.04 ± 0.1

Ψ Values given are means of two replicates.
* Nematode control documented fungi (Mubyana 2002).
** Fungi without any effect on nematodes (Ye *et al.* 1993).

Cellulase activity

Table 3 shows the amount of glucose obtained from the breakdown of CMC by the seven antagonistic fungal isolates, after 10 days of incubation in the cellulose medium, as an indication of the cellulase activity of these fungi. Also tested were *Paecilomyces* sp., a nematophagous fungus from South Africa, and a non-nematode trapping soil fungus *Pestalotiopsis* sp. which were isolated from a previous study (Mubyana 2002). All the fungi tested induced cellulose production as shown in **Table 4**. These showed different glucose production levels ranging from 11.23 µg/ml in *Aspergillus fumigatus* to as low as 5.42 µg/ml in *C. sativus*. The commercial *Paecilomyces* species and a non-nematophagous fungus *Pestalotiopsis* sp. tested gave values of 3.74 and 2.04 µg/ml glucose, respectively i.e., lower production of cellulases as compared to the other fungi tested in this study.

Chitinolytic activity

All the seven fungal isolates tested grew well on the chitin agar; however, their growth was not measured as the interest was not on the amount of chitinases produced by each fungus, but their potential to degrade chitin. No visible clearance was observed around the fungal colonies growing in chitin agar.

Table 4 Effect of fungal isolates on the height of tomato plants infected with root knot nematodes (*Meloidogyne* sp.).

Plant treatment	Plant height Ψ		
	Plant height (cm) mean ± std dev	% change in plant height over Control-I	% change in plant height over Control-II
Non infested (Control-I)*	87.8 ± 6.7	-	36.4
Nematode infested (Control-II)**	64.4 ± 3.3	-26.7	-
A. fumigatus	74.4 ± 12.9	-15.3	15.6
C. sativus	93.5 ± 11.2	6.5	45.3
F. chlamydosporum	83.1 ± 9.7	-5.3	29.1
Dendriphiopsis sp.	88.7 ± 4.0	1.0	37.7
Penicillium sp.	84.9 ± 15.3	-3.3	31.9
Trichoderma sp.	89.7 ± 7.2	2.1	39.3
Sterile mycelia	82.7 ± 9.2	-5.9	28.4

Ψ Values given are means of 9 replicates.
* Plants not infested with nematodes and grown in soil without fungi.
** Plants infested with nematodes and grown in soil without fungi.
Figures having a negative sign indicate % decrease in plant height.

Effects of fungi inoculation on the growth of tomatoes inoculated with *Meloidogyne*

Table 4 shows the height of tomato plants inoculated with the different nematode antagonistic fungi. The results showed that plants subjected to the various fungal treatments had significantly ($P \leq 0.05$) increased heights when compared to the nematode infected control plants without fungal inoculation (**Table 4**). Inoculation of tomato plants with root knot nematodes alone significantly reduced the average height of tomato plants by 26.7% relative to the uninoculated control. The plants treated with *C. sativus* and *Trichoderma* sp. had the highest (93.5 cm) and second highest (89.7 cm) heights respectively, which neither differed from each other nor from the non-infected control ($P \leq 0.05$). The results also indicate that *C. sativus* inoculation resulted in 45.3% increase in plant height over the plants inoculated with nematodes only whilst there was a 6.5% increase over the uninoculated plants. Minimal increase (15.6%) in plant height was achieved where *A. fumigatus* was used (**Table 4**).

Table 5 shows the effects of treatments with fungal isolates on the shoot FW and DW of *Meloidogyne* sp. infested tomato plants after harvest. Inoculation of tomato plants with nematodes lowered the shoot FWs by 23.0% as compared to the uninoculated controls. Pre-treatment with fungi alleviated the nematode effects; however, the effect was not statistically significant on dry shoot weight ($P \leq 0.05$). A higher dry shoot weight (1.09 g) was obtained from treatment with *C. sativus* and the least (0.44 g) from the uninoculated control.

Inoculating tomato plants with *Meloidogyne* sp. significantly lowered root FW (29.4% decrease) as compared to the uninoculated controls (**Fig. 3**). In contrast, plants pretreated with *C. sativus*, *Dendriphiopsis* sp. and *Trichoderma* sp. showed a significant increase in root FW. Root weight values from these treatments presented 36.6, 25.2 and 33.2% increments, respectively in comparison to the uninoculated control (**Fig. 3**). However, pre-treatments of soil with the other fungal isolates; *A. fumigatus*, *F. chlamydosporum*, *Penicillium* sp. and sterile mycelia did not show much improvement in root FW as values obtained were not significantly different from the nematode-inoculated control value (**Fig. 3**). The positive effects of fungal inoculation were more pronounced in root DW than on shoots (**Fig. 3**). *Cochliobolus sativus*, *Dendriphiopsis* sp. and *Trichoderma* sp. treatments differed significantly from the rest of the treatments, showing highly increased root DW of 145.9, 104.5 and 115.0%, respectively as compared to the nematode inoculated control (**Fig. 3**). Root DW for plants treated with *A. fumigatus*, *Penicillium* sp. and sterile mycelia showed decreases of 22.3, 26.0 and 27.4%, respectively as compared to the uninoculated control. The severity of the nematode infection was visible in nematode inoculated control (0.44 g), which was 39.3% less than the value obtained for the uninoculated control.

Table 5 Effect of fungal isolates on shoot fresh and dry weight of tomato plants inoculated with *Meloidogyne* sp.

Test fungus	Shoot fresh weight (g)	Shoot dry weight (g)
Control-I*	37.2 b	2.5 a
Control- II**	29.4 a	2.4 a
Aspergillus fumigatus	34.1 ab	2.4 a
Cochliobolus sativus	41.0 b	2.9 b
Fusarium chlamydosporum	36.3 ab	2.7 ab
Dendriphiopsis sp.	38.2 b	2.7 ab
Penicillium sp.	38.5 b	2.5 a
Trichoderma sp.	38.3 b	2.9 b
Sterile mycelia	38.1 b	2.4 a

Ψ Values given are means of 9 replicates.
* Plants not infested with nematodes and grown in soil without fungi.
** Plants infested with nematodes and grown in soil without fungi.
Means in the same column followed by the same letter are not significantly different from each other in the Duncan's multiple range test ($P < 0.05$).

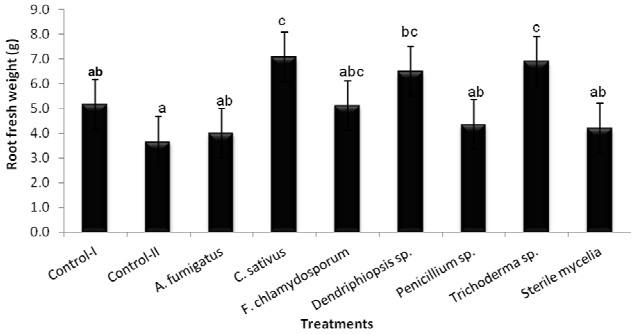

Fig. 3 Effect of fungal isolates on root fresh weight of plants infested with *Meloidogyne* sp. Bars labelled with the same letter are not significantly different ($P \leq 0.05$) from each other. Values of root fresh weight are means of 9 replicates.

DISCUSSION

Although 1,250 fungi were isolated from the soil samples collected in three regions of Botswana, most could not be recultured on fresh media and studied further. A factor that was associated with lack of unidentified micronutrients, growth factors or vitamins (Wollum II 1982; Warren *et al.* 2002). Cultivating fungi from extreme environment is challenging due to their slow growth and specific nutrient requirements (Pointing 2000). Even among those that grew on transfer, not all could be induced to sporulate, thus those were recorded as 'sterile mycelia and unidentifiable. Unknown fungi made up a large portion of all the isolates. The different identifiable soil fungi belonged to about 28 genera. Of these the high fungal population of 43.05% along the Boro route was attributed to the high grass biomass coupled with alternating burning and flooding, which provides a high nutrient substrate in the subsoil for the fungal growth (Mubyana-John *et al*. 2007). In contrast, the fewer fungi (29%) from Motlhabaneng in Bobirwa District, could be positive due to less rainfall, bare and elevated soil temperatures and the area being subjected to wind, erosion and nutrient losses (Thomas *et al*. 1999).

In this study, 27.80% of the total isolated fungal population originated from the saltpans soils. This region of Botswana is dominated by saline soils (Thomas and Shaw 1991; Owen 2005; Lebogang *et al*. 2009), which impose a great challenge to the soil inhabiting organisms, including fungi. The high salt contents in the soils limit the density and diversity of soil fungi as only those which are salt-tolerant will be favoured. Since these isolated fungi are well adapted to the saline environment, it would not be easy to utilize them as substrates in less saline soils.

The most frequently isolated genera were *Aspergillus* and *Penicillium* (**Table 1**) from the 3 sampled regions. The two genera were not only high in density but also presented a wide species variety. From the soil samples tested in this study, *Rhizopus stolonifer* and *Trichoderma* sp. had the least number of representative species, contributing a very small percentage of the total fungal population isolated respectively (**Table 1**).

Antagonistic effects of the isolates against *Meloidogyne*

The seven fungal isolates (**Table 2**) that immobilized nematode juveniles *in vitro* were *Aspergillus fumigatus*, *Cochliobolus sativus*, *Dendriphiopsis* sp., *Fusarium chlamydosporium*, *Penicillium* sp., *Trichoderma* sp. and an unidentified white sterile mycelial fungus. These fungi immobilized nematode juveniles to varying degrees ranging from 26 to 100% immobilization after the 4 day incubation period depending on the species involved. Nematode juvenile immobilization was treated as a sign for nematode antagonism as it leads to reduction in the inoculation density or patho-

gen activity (Khan *et al*. 2001).

After 2 days of incubation *Trichoderma* sp. had already immobilized about 26-50% nematode juveniles on agar plates (**Table 2**). This is in accordance with Schicklerl *et al*. (1998) and Meyer *et al*. (2001) who isolated substances from *Trichoderma* that significantly inhibited mobility of *M. incognita* juveniles *in vitro*. Several mechanisms involved in *Trichoderma* antagonism include production of antibiotics, competition for nutrients, mycoparasitism and excretion of lytic enzymes such as chitinases, β-1,3 glucanases and proteases (El-Katany *et al*. 2000; Bailey and Tähtiharju 2003). In this study the *Trichoderma* species isolated showed the ability to produce chitinase and degrade cellulose *in vitro* (**Table 3**).

Dendriphiopsis sp. immobilized 26-50% of nematode juveniles on agar plates after 2 days of inoculation (**Table 2**). Although no documented data on the interaction of *Dendriphiopsis* with nematode juveniles were found in literature, it is suggested that this fungus probably released extracellular substrates which acted on the juveniles and resulted in paralysis.

Aspergillus fumigatus and *C. sativus* had immobilized 51-75% juveniles by day 3 (**Table 2**). Secondary metabolite production by these fungi might have been induced upon host recognition. Thus it took time before sensing the presence of *Meloidogyne* sp. juveniles and then producing the toxic substances. Different species of *Aspergillus* are known to produce toxins and antibiotics like malformin, nadacidine, gliotoxin, viridian, and penicillin (Khan *et al*. 2001), paraherquamides (mycotoxins) with potent antihelmintic and antinematodal properties (Williams 2002).

Within the 4 days of incubation, *C. sativus* had immobilized more than 75% of *Meloidogyne* sp. juveniles. It was not clear whether the spore-like structures of *C. sativus* observed inside the nematode juveniles were ingested by the juveniles or injected by the antagonistic fungus into the bodies of the *Meloidogyne* sp. Juveniles and however, some samples showed spores attached to the exterior of the host juveniles (**Fig. 2C**). No structures were observed on the control slides, suggesting that they formed as a response to the presence of *Meloidogyne* sp. Although no literature on the resting spores of *C. sativus* could be found, Glockling and Shimazu (1997) reported resting spores of *Harposporium cycloides* forming inside nematode bodies but never germinating.

Penicillium sp. immobilized 26-50% of the inoculated nematode juveniles by the third day after inoculation and increased to 51-75% by day 4, although no physical immobilizing structures were observed. The ability of *Penicillium* sp. to antagonise root knot nematodes has been attributed to production of mycotoxins and antibiotics (Laich *et al.* 2002; Gotlieb *et al*. 2003). The immobilization of juveniles may be attributed to extracellular metabolites such as chitinase and cellulolytic ability of the fungus. Similarly, *F. chlamydosporium* immobilized *Meloidogyne* sp. juveniles also increased with incubation period indicating immobilization of the root knot nematodes was directly proportional to the incubation time.

Cellulolytic activities of the isolated putative nematode antagonistic fungi

All the fungal isolates tested in this study were able to degrade carboxymethylcellulose (CMC), but with varying degrees/capacities (**Table 3**). *Aspergillus fumigatus* presented the highest level of cellulase followed by *Penicillium* sp., *Aspergillus*, *Penicillium* and *Trichoderma* have been reported to be the main sources of cellulase and hemicellulase enzyme production in soil (Bailey and Tähtiharju 2003).

The amount of cellulases produced by a particular fungus did not necessarily determine its ability to antagonize nematodes as indicated by *Paecilomyces* sp., a well documented nematode antagonistic fungus (Khan *et al*. 2004), which yielded very low cellulolytic ability, whereas *A. fumigatus* produced a higher amount of glucose than *Dendriphiopsis* sp. a much more aggressive nematode antagoniser. Nevertheless, the mere production of cellulase enzymes may somehow be an indication of nematode antagonism.

Chitinolytic activities of the antagonistic fungal isolates

All the tested fungi grew on chitin agar but the growth was not intense as compared to on a nutrient-rich MEA. Chitinases are highly associated with the fungal infection process and many nematophagous and entomopathogenic fungi have been reported to produce them (Khan *et al*. 2004; Park *et al*. 2004). Although *Cochliobolus sativus*, *Dendriphiopsis* sp. and *Penicillium* sp. grew on the chitin medium in this study, no literature could be found on their ability to grow on chitin agar. However, their growth was interpreted as chitinolytic activity. Chitin induced nematode suppression has also been documented by Schickler *et al*. (1998) and El-Katatny *et al*. (2000). The production of chitinases has been identified as the key mode of action for *Paecilomyces lilacinus* Strain 251 against root knot nematodes (Khan *et al*. 2001). Therefore, the ability of the fungal isolates to degrade chitin *in vitro* suggests that chitinase ability may contribute to nematode suppression in soil.

Effect of fungal isolates on the growth of infested tomato plants in the green

Inoculation of tomato plants with root knot nematodes significantly reduced the average plant height by 26.7% when compared to the uninoculated controls (**Table 4**). Preinoculation of the tomato seedlings alleviated the effect of the nematodes as the average plant height of nematode-infested plants treated with the various test fungi were significantly higher than those of the nematode-inoculated controls. Giving increments ranging from 15.6 to 45.3% depending on the fungal isolate involved (**Table 4**). Not only did the tested fungal isolates improve plant height among nematode-inoculated tomato plants; *Cochliobolus sativus* also increased the plant height of uninoculated controls by 6.5% (**Table 4**). *Trichoderma* spp. apparently exhibited plant growth promoting activities (El-Katatny *et al*. 2000), which may have increased plant heights as compared to the uninoculated controls. It has also been reported that pre-plant treatments with *Trichoderma* is effective in suppressing nematode reproduction and inhibit both penetration and egg-hatch (Khan *et al.* 2001; Meyer *et al.* 2001).

Nematode infection of tomato plants significantly reduced shoots FW (**Table 5**). More than 23.0% decrease in shoot weight was recorded as compared to the uninoculated control. Abubakar *et al.* (2004) showed a reduction in plant height and weight as a result of root knot nematode inoculation.

Fungal inoculation effects were more visible in root weight where the *C. sativus*, *Dendriphiopsis* and *Trichoderma* spp. inoculation significantly alleviated the negative effect of the nematodes on the roots (**Figs. 2, 3**). Infection by *Meloidogyne* spp. most likely led to inefficiency of the root system hence stunted growth.

Overall the antagonistic properties of the isolated fungi gave rise to improved growth of *Meloidogyne* sp. infected tomato plants, in terms of height, shoot and root weight. It is therefore suggested that the isolated nematode antagonistic fungi have the ability to enhance plant growth of nematode inoculated plants. The differences in the efficacy of these isolates to improve plant growth might be explained by differences in their effectiveness to infect and suppress the root knot nematode parasite and their ability to grow in the soil and colonize the rhizosphere. In this study, nematode inhibition *in vitro* was not directly proportional to the suppression of nematodes in the root system of the tomato plants. The percentage immobilization capacities of the fungal isolates on *Meloidogyne* sp. juveniles in agar might not always correspond to the alleviation in the tomato plants.

The sterile unidentified mycelia showed low immobilization of nematode juveniles compared to the other fungi (**Table 2**), thus may not be recommended for intensive future investigation activity (**Table 3**).

In this study, *C. sativus* demonstrated parasitism through infectious spores (**Fig. 2**), suggesting that the production of spores within the bodies of juveniles killed the nematodes and lowered the nematode inoculum density. Not only did infection with these spore-like structures reduce infective nematode juveniles, these structures could ensure a continuous survival of *C. sativus* under harsh environmental conditions. The fungus *C. sativus* is known to survive as dormant spores in soil (MacNish 2005). Some researchers have documented *C. sativus* as the causal agent of common root rot diseases in crops (MacNish 2005). However, no incidence of disease was observed in this study.

To our knowledge there is little or no literature on the antagonistic activity of *Dendriphiopsis* spp. against plant parasitic nematodes. This suggests that this fungus produced nematodal metabolites which immobilized *Meloidogyne* sp. juveniles, and lowered the nematode population. This fungus originated from the saltpan soils and showed a potential in controlling root knot nematodes *in vitro*, it might be an option to biological control of *Meloidogyne* infection in the saline areas of Botswana.

CONCLUSION

This study indicates that *Cochliobolus sativus*, *Trichoderma* sp., *Penicillium* sp., from the Okavango Delta; *Fusarium chlamydosporium*, and *Dendriphiopsis* sp from the Salt Pans; and *Aspergillus fumigatus* from Bobirwa district soils are potential indigenous nematode antagonistic fungi which can be used to control nematodes in Botswana soils as they are adapted to these soils. Of the different isolates, *C. sativus* exhibited the strongest immobilization of nematode juveniles, while the sterile mycelia showed the least *in vitro* indicating its least potential as a biological control agent. The study also suggests that *C. sativus* invades nematode juveniles through the infectious spore-like structures.

ACKNOWLEDGEMENTS

The study was supported by the W. K. Kellogg Foundation (WKKF) Africa Program through The Academy for Educational Development (AED) Botswana.

REFERENCES

Abubakar U, Adamu T, Manga SB (2004) Control of *Meloidogyne incognita* (kopoid and white) chitwood (root-knot nematode) of *Lycopersicon esculentum* (tomato) using cowdung and urine. *African Journal of Biotechnology* **3 (8)**, 379-381

Bailey MJ, Tähtiharju J (2003) Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. *Applied Microbiology and Biotechnology* **62 (2-3)**, 156-162

Barron GL (2003) Predatory fungi, wood decay, and the carbon cycle. *Biodiversity* **4**, 3-9

Batisani N, Yarnal B (2010) Rainfall variability and trends in semi-arid Botswana: Implications for climate change adaptation policy. *Applied Geography* **30**, 483-489

Bell A, Boye J, Muck O (1998) Methyl Bromide Substitution in Agriculture: *Objectives and Activities of the Federal Republic of Germany Concerning the Support to Article 5 Countries of the Montreal Protocol*. Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ) GmbH

Carlile MJ, Watkinson SC, Gooday GH (2001) *The Fungi* (2nd Edn), Academic Press, London, Great Britain, 588 pp

Carmichael JW, Kendrick WB, Conners IL, Sigler L (1980) *Genera of Hyphomycetes*, University of Alberta Press, Alberta, Canada, 386 pp

Chernin LS, Winson MK, Thompson JM, Haran S, Bycroft BW, Chet I, Williams P, Stewart GSAB (1998) Chitinolytic activity in *Chromobacterium violacem*: Substrate analysis and regulation by quorum sensing. *Journal of Bacteriology* **180 (7)**, 4435-4441

Chet I, Inbar J, Hadar Y (1997) Fungal Antagonists and Mycoparasites. In: Wicklow DT, Söderström B (Eds) *The Mycota IV. Environmental and Microbial Relationships*, Springer-Verlag, Berlin, 184 pp

Department of Meteorological Services Report (2003) Republic of Botswana

Domsch KH, Gams W, Anderson T (1993) *Compendium of Soil Fungi* (Vol 1), IHW-Verlag, Eching, Germany, 860 pp

El-Katatny MH, Somitsch W, Robra KH, El-Katatny MS, Gübitz GM (2000) Production of chitinase and β-1,3-glucanase by *Trichoderma harzianum* for control of the phytopathogenic fungus *Sclerotium rolfsii*. *Food Technology and Biotechnology* **38 (3)**, 173-180

EPA (2003) The phaseout of methyl bromide. Available online: www.epa.gov/ozone/mbr

Fisher NL, Burgess LW, Toussoun TA, Nelson PE (1982) Carnation leaves as a substrate and for preserving cultures of *Fusarium* species. *Phytopathology* **72**, 151-153

Gashe AB (1992) Cellulase production and activity by *Trichoderma* sp. *Journal of Applied Bacteriology* **73**, 79-82

Glockling SL, Shimazu M (1997) Culturing of three species of endoparasitic fungi infecting nematodes. *Mycological Research* **10 (1)**, 55-60

Gotlieb DY, Oka B, Ben-Daniel H, Cohen Y (2003) Dry mycelium of *Penicillium chrysogenum* protects cucumber and tomato plants against root knot nematode *Meloidogyne javanica*. *Phytoparasitica* **31 (3)**, 217-225

Hanlin RT (1992) *Illustrated Genera of Ascomycetes*, The American Phytopathological Society, Minnesota, USA, 262 pp

Hewitt EJ (1966) *Sand and Water Culture Methods Used in the Study of Plant Nutrition*, Technical Communication No. 22. Commonwealth Bureau, London, 56 pp

Hooper DJ (1986) Extraction of Nematodes from Plant Material. In: Southey JF (Ed) *Laboratory Methods for Work with Plant and Soil Nematodes*, Her Majesty's Stationary Office, London, 65 pp

Hsu SC, Lockwood JL (1975) Powdered chitin agar as a selective medium for enumeration of *Actinomycetes* in water and soil. *Applied Microbiology* **29 (3)**, 422-426

Ingham RE (1994) Nematodes. In: Weaver RW, Scott A, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum A (Eds) *Methods of Soil Analysis Part 2: Chemical and Microbiological Properties* (3rd Edn), American Society of Agronomy, Soil Science of America Publishers INC, Madison, Wisconsin, USA, 1121 pp

Jepson SA (1987) *Identification of Root-knot Nematodes (Meloidogyne Species)*, C.A.B. International, Wallingford, 265 pp

Katsantonis D, Hillocks RJ, Gowen S (2003) Comparative effect of root knot nematode on severity of Verticillium and Fusarium wilt in cotton. *Phytoparasitica* **31 (2)**, 733-739

Kayombo B, Pule-Meulenburg F, Mmolawa KB, Mzuku M, Tsheko R, Batisani N, Patrick C, Tapela M, Gaboutloeloe G, Mubyana-John T, Taylor JE, Machacha S, Njogu RM, Motsamai T (2010) Characterization of desert margins program sites in Botswana, 1. Soil factors. *Botswana Journal of Agriculture and Applied Sciences* **6 (2)**, 62-70

Khan A, Williams KL, Nevalainen HKM (2004) Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. *Biological Control* **31**, 346-352

Khan HU, Ahmad R, Ahmed W, Khan SM, Khan MA (2001) Evaluation of the combined effects of *Paecilomyces lilacinus* and *Trichoderma harzianum* against root knot disease of tomato. *Online Journal of Biological Sciences* **1 (3)**, 139-142

Kirk PM, Cannon PF, David JC, Staplers JA (2001) *Ainsworth and Bisby's Dictionary of the Fungi*, C.A.B. International, Wallingford, UK, 655 pp

Klich MA (2002) *Identification of Common Aspergillus Species*, Centraalbureau voor Schimmelculture, Utrecht, The Netherlands, 116 pp

Köning J, Grasser R, Pikor H, Vogel K (2002) Determination of xylanase, β-glucanase, and cellulose activity. *Journal of Analytical Bioanalytical Chemistry* **374**, 80-87

Koosaletse P (2001) The influence of solarisation and other fumigants on fungal populations in the rhizosphere and endorhizosphere of tomato plants (*Lycopersicum esculentum*). Undergraduate Research Project Report, University of Botswana, 56 pp

Laich F, Fierro F, Martín JF (2002) Production of penicillin by fungi growing on food products: Identification of a complete penicillin gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. *Applied Environmental Microbiology* **68 (3)**, 1211-1219

Lebogang L, Taylor JE, Mubyana-John T (2009) A preliminary study of the fungi associated with saltpans in Botswana and their anti-microbial properties. *Bioremediation, Biodiversity and Bioavailability* **3 (2)**, 61-71

MacNish G (2005) Rhizoctonia bare patch and Rhizoctonia root rot of cereals. Farmnote 34/1994, Department of Agriculture and Food, Western Australia, 60 pp

Manocha MS, Zhonghua Z (1997) Immunocytochemical and cytochemical Localization of chitinase and chitin in the infected hosts of a biotrophic mycoparasite *Piptocephalis virginiana*. *Mycologia* **89**, 185-194

Maqbool MA, Kerry B (1997) Plant nematode problems and their control in the near east region. FAO Plant Production and Protection, paper-144. Food and Agriculture Organisation (FAO) of the United Nations, Rome

Mbao (2003) Methyl Bromide Alternatives Outreach. Available online: www.mbao.org/

MBTOC (1995) Alternatives to Methyl Bromide: Experts from U.N. Methyl

Bromide Technical Options Committee Assessment Report PANNA, 12 pp
Meyer SLF, Roberts DP, Chitwood DJ, Carta LK, Lumsden RD, Mao W (2001) Application of *Burkholderia cepacia* and *Trichoderma virens*, alone and in combinations, against *Meloidogyne incognita* on bell pepper. *Nematropica* **31**, 75-86
Montzka SA, Butler JH, Hall BD, Mondeel DJ, Elkins JW (2003) A decline in tropospheric bromine. *Geophysical Research Letters* **30**, 1826
Mubyana T (2002) A demonstration study on effects of methyl bromide and its alternatives on soil fungi at New Pass (Lobatse), Hortulus (Gaborone) and Legola (Francistown) Botswana: Botswana's contribution towards protecting the ozone layer. UNIDO Project MP/BOT/98>061, 45 pp
Mubyana-John T, Wutor VC, Yeboah SO, Ringrose S (2007) Fire and its influence on microbial community structure and soil biochemical properties in the Okavango Delta Botswana. *Science Research Essays* **2 (2)**, 47-54
Nilsson CI (1972) *A Report to the Government of Botswana on Plant Diseases*. Food and Agriculture Organization (FAO) of the United Nations, Rome
Owen J (2005) Lost African Lake Spawned Fish Diversity "Beyond Belief". National Geographic News, May 4
Park JO, Hargreaves JR, McConville EJ, Stirling GR, Ghisalberti EL, Sivasithamparam K (2004) Production of leucinostatins and nematicidal activity of Australian isolates of *Paecilomyces lilacinus* (Thom) Samson. *Letters in Applied Microbiology* **38**, 271-276
Parkinson D (1994) Filamentous fungi. In: Weaver RW, Scott A, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum A (Eds) *Methods of Soil Analysis Part 2: Chemical and Microbiological Properties* (3rd Edn), American Society of Agronomy, Soil Science of America Publishers Inc., Madison, Wisconsin, USA, 1121 pp
Pointing SB (2000) Lignocellulolytic enzymes assays. In: Hyde KD, Pointing SB (Eds) *Marine Mycology – A Practical Approach*. *Fungal Diversity Research Series 1*, Fungal Diversity Press, Hong Kong, pp 137-157
Rosenzweig WD, Pramer D (1980) Influence of cadmium, zinc and lead on growth, trap formation, and collagenase activity of nematode trapping fungi. *Applied and Environmental Microbiology* **40 (4)**, 694-696
Saunders P (2004) Methyl bromide ban. Institute of Science in Society (ISIS), Report, 29/1
Schickler H, Haran S, Oppenheim A, Chet I (1998) Induction of the *Trichoderma harzianum* chitinolytic system is triggered by the chitin monomer *N*-acetylglucosamine. *Mycological Research* **102**, 1224-1226
Segers R, Butt TM, Kerry BR, Beckett A, Peberty JF (1996) The role of the Proteinase VCP1 produced by the nematophagous fungus *Verticillium chlamydosporium* in the infection process of nematode eggs. *Mycological Research* **100**, 421-448
Sharma SB, Price NS, Bridge J (1997) The past, present and future of plant nematology in international agricultural research centers. *Nematological Abstracts* **66 (3)**, 121-142
Sikora RA (2002) Strategies for biological system management of nematodes in horticultural crops: Fumigate, confuse or ignore them. *Mededelingen* **67**, 5-18
Sutton BC (1980) *The Coelomycetes*, Commonwealth Mycological Institute, Kew, UK, 696 pp
Taylor AL, Sasser JN (1980) *Biology, Identification and Control of Root Knot Nematodes*, International Meloidogyne Project. North Carolina State University Graphics, USA, 111 pp
Thomas DSG, Shaw PA (1991) *The Kalahari Environment*, Cambridge University Press, UK, 284 pp
Thomas AD, Walsh RPD, Shakesby RA (1999) Nutrient losses in eroded sediment after fire in eucalyptus and pine forests in the wet Mediterranean environment of northern Portugal. *Catena* **36**, 283-302
Tzean SS, Liou JY (1993) Nematophagous resupinate basidiomycetous fungi. *Phytopathology* **83**, 1015-1020
Tikhonov VE, Lopez-Llorca LV, Salinas J, Janson HB (2002) Purification and characterization of chitinases from nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. *Fungal Genetics and Biology* **35**, 67-78
Warren A, Day JG, Brown S (2002) Cultivation of algae and protozoa. In: Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stetzenbach LD (Eds) *Manual of Environmental Microbiology* (2nd Edn), ASM Press, USA, pp 71-83
Williams RM (2002) Total synthesis and biosynthesis of the paraherquamides: An intriguing story of the biological diels–alder construction. *Chemical and Pharmaceutical Bulletin* **50**, 711-740
Wollum II AG (1982) Cultural methods for soil microorganisms. In: Page AL, Baker DE, Keeney DR (Eds) *Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties*, American Society of Agronomy, Inc. and Soil Science Society of America, Inc. Madison, USA, pp 781-802