J. Crop Prot. 2014, 3 (3): 305-313

Evaluation of Fungal Isolates as Possible Biocontrol Agents Against *Striga hermonthica*

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Abstract: *Striga hermonthica* is a parasitic weed which largely constraints maize and sorghum production in Western Kenya. The weed mostly invades small scale farms and depending on severity, it may cause damage ranging from 10% to complete crop failure thereby aggravating the food insecurity in that region. This study aimed at evaluating fungal isolates as possible biocontrol agents against the weed. Fungi were isolated from diseased *Striga hermonthica* and their virulence efficacy against the weed tested in a greenhouse. All the fungal isolates tested caused infection and consequently death of the weed. *Fusarium incarnatum* had the highest infection rate of 92% followed by *Gibberella intricans* and *F. chlamydosporum* at 90% each. *Fusarium oxysporium* caused the highest mortality of 60% with *Gibberella intricans*, causing the least mortality of 36%. *F. oxysporium* was the most aggressive and potent fungal isolate against the weed hence a suitable candidate for exploitation as a mycoherbicide against the weed.

Keywords: *Striga hermonthica*, *Fusarium*, Biocontrol, Mycoherbicide.

Introduction

Striga hermonthica is a root parasitic weed which causes severe constrains in cereal crop production in the sub Saharan Africa by parasitizing the roots of the host crop. The parasite, attaches itself to the roots of its host from where it siphons nutrients and water intended for the plant to grow (Jamil *et al*., 2012; Ndambi *et al*., 2011). Several species of *Striga* have been identified worldwide with *S. hermonthica* being the most notorious and causing serious damage to the agro economic systems in Western Kenya (Gacheru *et al*., 2002). Agriculture is the mainstay economic activity in Western Kenya region with cereals being the main source of food and income. Infestation by *S. hermonthica* has become a real threat to maize and sorghum cultivation in the region. In the Lake Victoria Basin of Kenya alone, 0.24 million hectares of arable land, which is 15% of the total arable land in the region is infested. This causes yield losses between 10-100% and or monetary losses of upto US$ 41 million annually (Jamil *et al*., 2012). In many cases, the damage caused by the weed is so severe that a farmer can only harvest a paltry 0.5tons per hectare of maize instead of the potential 5tons or zero yields under heavy infestation (Olwenya, 2012).

The *S. hermonthica* menace is worsening due to continuous mono-cropping of maize and sorghum, little effort to control the weed and the increasing population which is

Handling Editor: Dr. Mohammad Mehrabadi

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Received: 6 August 2013, Accepted: 27 January 2014
Published online: 27 January 2014

increasing pressure on the already limited arable land. In return, this leads to a decline in the soil fertility, thus creating favourable conditions for the weed to flourish (Esilaba, 2006). This weed is reported to be spreading rapidly to high land areas previously thought to be immune to it (Gacheru *et al*., 2002). The currently recommended control strategies against the weed are ineffective due to the big disparity between the cost of the control strategies and the farmers' socioeconomic status (Atera *et al*., 2013). Hence alternative, affordable and easy to use control measures should be developed to alleviate the *Striga* menace. This project aimed at evaluating fungal isolates as possible biocontrol agents against *Striga hermonthica*.

Methods

Isolation of fungal species infecting Striga hermonthica

Diseased *S.hermonthica* plants showing necrosis, spots on the leaves and stem, wilting and other abnormalities were uprooted from farms in Kibos–Kisumu (GPS coordinates: latitude 0.03861, longitude 34.815965, elevation 1196) and Alupe–Busia (GPS coordinates: latitude 0.50372514, longitude 34.1214814, elevation1157 meters) research stations in Kenya, placed in brown paper bags and transported to the laboratory. The diseased parts were cut using a laboratory scalpel into pieces of about 4-7mm, surface-sterilized with 1% sodium hypochlorite (NAOCL) for 1½ minutes and rinsed 3 times in sterile distilled water. The cut plant parts were then dried with sterilized filter papers and some placed on Peptone PCNB Agar (PPA) while others on potato dextrose agar (PDA) half strength (Rahjoo *et al.,* 2008). For purification and subsequent morphological identification of the fungus which grew, the isolates were transferred on to potato-dextrose agar (PDA), Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar CLA (Kwasna and Bateman, 2007). All the cultures were incubated at 25 ºC for two to four weeks. Cultural characters were assessed by eye and by microscopic examination. Colony morphology and colour (reverse and front) were recorded from cultures grown on PDA. The morphology of macroconidia was assessed from cultures grown on CLA while the morphologies of microconidia, conidiogenous cells and chlamydospores were assessed from cultures grown on SNA. Morphological identifications of the *Fusarium* isolates were made using the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006). The non *Fusarium* isolates were identified using the criteria of Dugan (2006).

Molecular characterization of the isolated fungi

Molecular characterization of the isolated fungi was done to confirm their morphological identities. DNA was extracted from fungal isolates grown on PDA plates for 7 days. Their mycelia were harvested and resuspended in nuclease free water. Total DNA was extracted from the resuspended mycelia of each isolate (50-100 mg wet weight) using a ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo research, South Africa) according to the manufacturer's instructions.

PCR Amplification

The PCR amplifications were performed using the DreamTaq Green PCR Master mix (Thermo Scientific). The amplification reactions were carried out in volumes of 50 µL containing; 25 µL DreamTaq Green PCR Master mix, 1 µM of each primer i.e the forward primer and the reverse primer, 1µg of the template DNA and 23 µL of nuclease free water. The PCR reaction was carried out in a thermal cycler as follows: 1) 1 cycle of initial denaturation at 95 ^{o}C for 3 minutes; 2) 35 cycles of the following: denaturation at 95 ºC for 30 seconds, annealing at 57 ºC for 30 seconds and extension at 72 °C for 1 min; 3) final extension at 72 °C for 10 min, followed by cooling at 4 °C until recovery of the samples. Amplification products were

visualized in1.2% agarose gels stained with ethidium bromide. The gel was photographed under UV light at 254 nm (Kwasna and Bateman, 2007). The primers used in the PCR were; TEF primers (TEF1 5'-ATGGGTAAGGARGACAAGAC-3' and TEF2 5'-GGARGTACCAGTSATCATGTT-3') for the *Fusarium* species and ITS primers (ITS F 5 '-AACTCCCAAACCCCTGTGAACATA-3' and ITS R 5 '-TTTAACGGCGTGGCCGC-3') for the non *Fusarium* species. The PCR products were then sequenced and edited using the geneious program. The obtained DNA sequences were blasted using the NCBI BLAST (National Center for Biotechnology Information - Basic Local Alignment Search Tool) to reveal their identities. To reveal the relatedness of the isolates alignment of all the sequences was done using Clustalx 2.1 software and Phylogenetic analyses conducted using MEGA version 5.1 using the Neighbour Joining (NJ) method (Tamura *et al.,* 2011). In the NJ analysis, distances were calculated using the Kimura 2-Parameter model and bootstrap tests performed with 1,000 replications (Tamura *et al.,* 2004; Felsenstein, 1985)

Virulence efficacy of the fungal isolates on *Striga hermonthica*

The most frequently isolated species were tested for their efficacy as biocontrol agents against *S. hermonthica*. The inoculums were prepared by scraping the fungal hyphae into Armstrong medium and incubating it in a shaker at 25 ^{o}C at 100rpm for 5 days to produce spores. The spores were filtered through two layers of sterile cheesecloth into a 50ml falcon tube and centrifuged at 3500rpm for 10minutes, the supernatant was discarded and the pellet (conidia) washed twice with de-ionized autoclaved water. The spores were suspended in 350ml sterile distilled water. With the aid of a haemocytometer the conidia concentration was adjusted to 2×10^6 conidia per ml. Tween 20 surfactant (Polyoxyethylene 20-sorbitan monolaurate) was added to the conidial suspension before inoculation at the rate of 3 drops per liter (Booth, 1971).

Green house trials

Maize was grown in 5- litre plastic pots, in a screenhouse at Kibos–Kisumu research stations. The pots were filled with 5 kg of pure, filtered soil. Five replicates were used for each fungal isolate being tested. Prior to sowing, the pots were infested by mixing about 2000 to 3000 *S. hermonthica* seeds (in *Striga* seeds/ sand mixture) into the soil layer 5-10 cm below the surface. Prior to inoculation, all the non - *S. hermonthica* weeds growing in the pots were uprooted. Excess *S. hermonthica* were also uprooted so that each pot had only 10 emerged *S. hermonthica* plants. Eight weeks after planting, when the *S. hermonthica* plants were be approximately 5-15 cm tall, they were inoculated with the different fungal isolates. For each fungal isolate, 50 *S. hermonthica* plants were inoculated. The inoculum was sprayed throughout each *S. hermonthica* plant. Following inoculation, the *S. hermonthica* plants were assessed for disease symptoms at a 5 day interval for 3weeks. Plants with observed lesions and abnormal colour (maroon) changes on the leaves and stems were designated as infected. At the end of the experiment, the fungal isolates were then re-isolated from the infected *S hermonthica* onto PDA plates to confirm that they were the ones previously inoculated. The most infectious fungal isolate was determined by counting the infected plants and then expressing it in percent. The number of dead plants after 22 days was used to determine the mortality rate and therefore the most virulent isolate.

Results

Fungal species isolated

Ten fungal isolates were recovered from diseased *S. hermonthica* collected from Kibos and Alupe. *Fusarium* sp. was isolated from *S. hermonthica* collected from Alupe as well as from Kibos. *Hypocrea stellata* was isolated from *S. hermonthica* collected from Alupe, while *Colletotrichum gloeosporioide*s and *Artroderma otae* were isolated from *S. hermonthica* collected from Kibos. *Fusarium oxysporium* was the most

frequent fungal species isolated followed by *F. chlamydosporium* then *F. equiseti*. The least frequent species were *Colletotrichum gloeosporioide*s and *Hypocrea stellata* (Table 1).

Table 1 Frequency of isolation of the fungal species isolated from diseased *Striga hermonthica.*

Identity of the Fungus as in the Ncbi Blast	Origin (Area)	Origin (Part of Plant)	Frequency of Isolation
Fusarium equiseti	Kibos	Leaves	11
	Alupe	Leaves	9
Fusarium verticilloides	Kibos	Leaves	8
	Alupe	Leaves	10
Fusarium oxysporum	Kibos	Leaves	12
	Alupe	Leaves	14
	Kibos	Flowers	4
Fusarium incarnatum	Kibos	Leaves	8
	Alupe	Leaves	8
Fusarium chlamydosporium	Kibos	Leaves	10
	Alupe	Leaves	12
Gibberella moniliformis	Kibos	Stem	8
	Alupe	Stem	9
Gibberella intricans	Kibos	Stem	9
	Alupe	Stem	6
Colletotrichum gloeosporioide	Kibos	Stem	1
	Alupe		0
Hypocrea stellata	Kibos		0
	Alupe	Roots	1
Arthroderma otae	Kibos	Roots	2
	Alupe		0

Molecular characterization of the fungal isolates and their phylogenetic analysis

Isolates morphologically identified as *Fusarium* sp. and *Gibberella* sp. were positively amplified using TEF1 (20 base pairs) and TEF2 (21 base pairs) primers while the isolates morphologically identified as *Hypocrea stellata* and *Colletotrichum gloeosporioides*, were amplified using the ITSF (24 base pairs) and ITSR (17 base pairs) primers as shown in Fig. 1.

The blasted sequences of the extracted DNA showing positive bands in Fig. 1, agreed with the morphological identifications. Alignment of the sequences grouped the *Fusarium* sp. isolates into 3 clades. *Fusarium* sp. isolates demonstrated relatedness; however, they showed no relationship with *Arthroderma otae.* Close relationships were observed; amongst the different strains of *F.oxysporum* with the exception of the strain IBSD-GF13 which was in a different clade maybe because it was isolated from flowers while the other strains were isolated from leaves of *S. hermonthica*. Relatedness was also observed amongst the different strains of *F. Chlamydosporum.* Close relationships were observed between *F. equiseti* and its sexual state and between *F. verticilloides* and its sexual state. A relationship was also observed amongst the *Fusarium* sp. isolated from the leaves of *S hermonthica*, however no relationship was observed amongst the *Gibberella* sp. isolated from the stems (Fig. 2).

Infection and mortality rates of the fungal isolates on *Striga hermonthica*

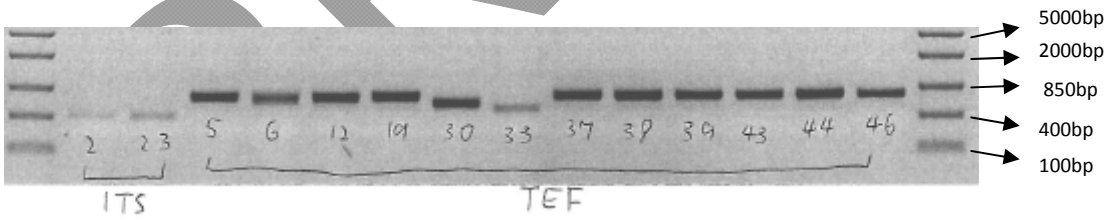
All the fungal isolates tested for their efficacy against *S. hermonthica* caused infections on the weed producing different symptoms as shown in Table 2. The number of weeds with observed lesions on the leaves and stems increased progressively with time. *Fusarium oxysporum* had the highest number of weeds with observed lesions after 5 days followed by *F. verticilloides* then *F. chlamydosporum*. After 20 days, *F. incarnatum* had the highest number of plants with observed lesions while *F. oxysporum* had the least. *Fusarium oxysporum* had the highest number of dead weeds followed by *F. equiseti* then *F. verticilloides* (Fig. 3). All the fungal isolates demonstrated significant infection rates (significance level less than 0.001 at p = 0.05) with *F. incarnatum* having the highest infection rate of 92%, followed by *Gibberella intricans* and *F. chlamydosporum* at 90% each. Consequently the fungal isolates lead to mortality of the weed (significance level less than 0.001 at p = 0.05). *Fusarium oxysporium* strains had the highest mortality

rates of 60% and 58% followed by *F. equiseti* at 46%. *Gibberella intricans*, on the other hand, had the least mortality rate at 36% (Fig. 4). Levene's test for equality of variances revealed that the means of infection and mortality rates of the fungal isolates were not significantly different (Significance level was 0.056 at p = 0.05). Infected *S. hermothica* plants which survived were emaciated and developed fewer flowers and consequently seeds than usual.

Discussion

Fusarium sp. was the most abundant fungal species isolated from *S. hermonthica*. All the *Fusarium* spp. were isolated from the leaves and flowers of *S. hermonthica* with *Colletotrichum gloeosporioide* being isolated from the stems and *Hypocrea stellata* and *Arthroderma otae* being isolated from the roots. Molecular characterization of the fungal isolates revealed that the amplified genomic DNA of the fungal isolates ranged between 100bp to 850bp, indicating their potential in genetic modification in producing more virulent strains for use as biocontrol agents. Phylogenetic analysis of the isolates revealed that the *Fusarium* spp. were related to each other, however, no relatedness was observed in terms of virulence.

In the screen house, all the fungal isolates tested showed high virulence efficacy on the weed revealing a wide variety of choice in developing a biological control against the weed. All the fungal isolates demonstrated infection rates greater than 75% indicating that the isolates tested could easily colonize the weed. However, it was only the two strains *Fusarium oxysporum* which had mortality rates greater than 50%, one having 60% and the other having 58% agreeing with the findings of Elzein *et al*., (2008). This demonstrated the suitability of *F. oxysporum* as a boicontrol agent against the weed. The less than 50% mortality rate exhibited by most of the isolates can be attributed to the time of inoculation of the weed with the fungal isolates. The fungal isolates were inoculated when the *S. hermonthica* were 8 weeks old, probably a higher mortality rate could have been achieved with early inoculation, because perhaps young *S. hermonthica* plants are more susceptible. Infection of *S. hermonthica* with the fungal isolates reduced the growth vigour and consequently the biomass of the weed. The infected weeds demonstrated a great reduction in the number of flowers and seeds produced agreeing with the findings of Yonli *et al*., (2010). Thus, the use of *Fusarium* spp. as biocontrol agents against *S. hermonthica* can limit the increase of the weed's soil seed bank.

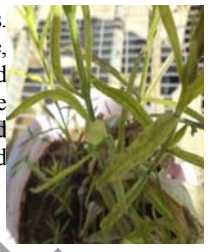
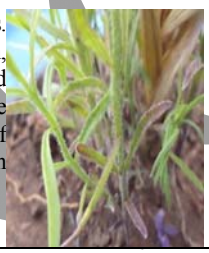


KEY:

2- *Hypocrea stellata* **23**- *Colletotrichum gloeosporioides* **5**- *Gibberella monoliformis*
6- *Fusarium equiseti* **12**- *Fusarium incarnatum* **19**- *Fusarium oxysporum*
30- *Gibberella intricans* **33**- *Arthroderma otae* **37**- *Fusarium oxysporum*
38- *Fusarium oxysporum* **39**- *Fusarium oxysporum* **43**- *Fusarium chlamydosporum*
44- *Fusarium chlamydosporum* **46**- *Fusarium verticilloides*

Figure 1 Micrograph showing amplified DNA bands.

Table 2 Table of Symptomatology as portrayed by *Striga hermonthica* infected with the fungal isolates.

Isolate name	Symptoms	Illustrations	Isolate name	Symptoms	Illustrations
Gibberella monoliformis	Caused lesions on both the stem and leaves of the weed. The leaves turned dark maroon, followed by blackening of the stems and eventual drying and death of the weed.		*Fusarium incarnatum*	Caused lesions on the leaves of the weed. The leaves became twisted and developed an ashy burned appearance at the tip. The weed then dried and died.	
Gibberella intricans	Caused the leaves to turn pale, then maroon and then twisted. The stems darkened from the ground up the plant this was followed by drying and eventual death of the weed.		*Fusarium equiseti*	Caused lesions on the leaves. The leaves became pale, rough textured and developed spots. Some leaves also curled and became twisted. The weed then dried and died.	
Fusarium oxysporum	Caused lesions on the leaves and the stem. The leaves turned pale then maroon with curling and twisting. Some leaves developed spots which perforated them. The stem darkened from the ground up the plant, followed by drying then death of the weed.		*Fusarium chlamydosporum*	Caused lesions on the leaves and on the stem. The leaves turned brownish, developed whitish spots, curled, became twisted and then dried. The weed dried from the tip down the stem and eventually died.	
Fusarium verticilloides	Caused lesions on the leaves. The leaves turned maroon, developed whitish spots and an ashy burned appearance at the tip and on the edges of the leaves. The plant then dried and died.				

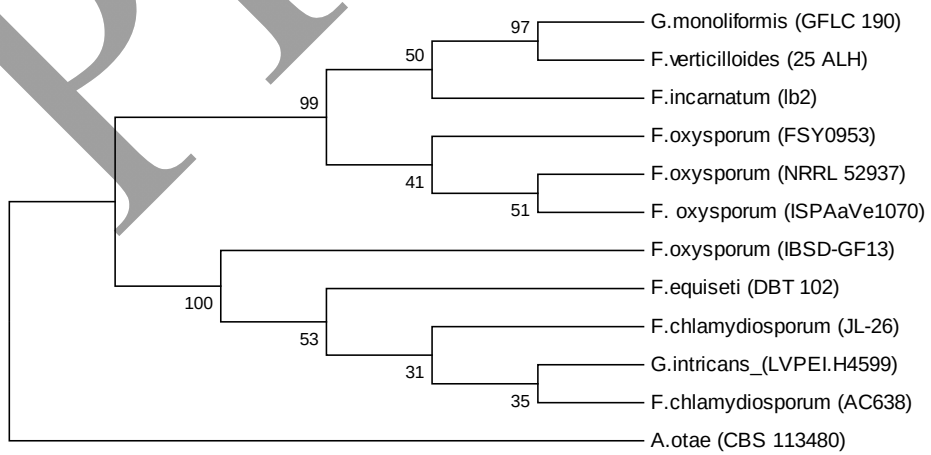


Figure 2 Phylogenetic analysis of the isolated fungal species.

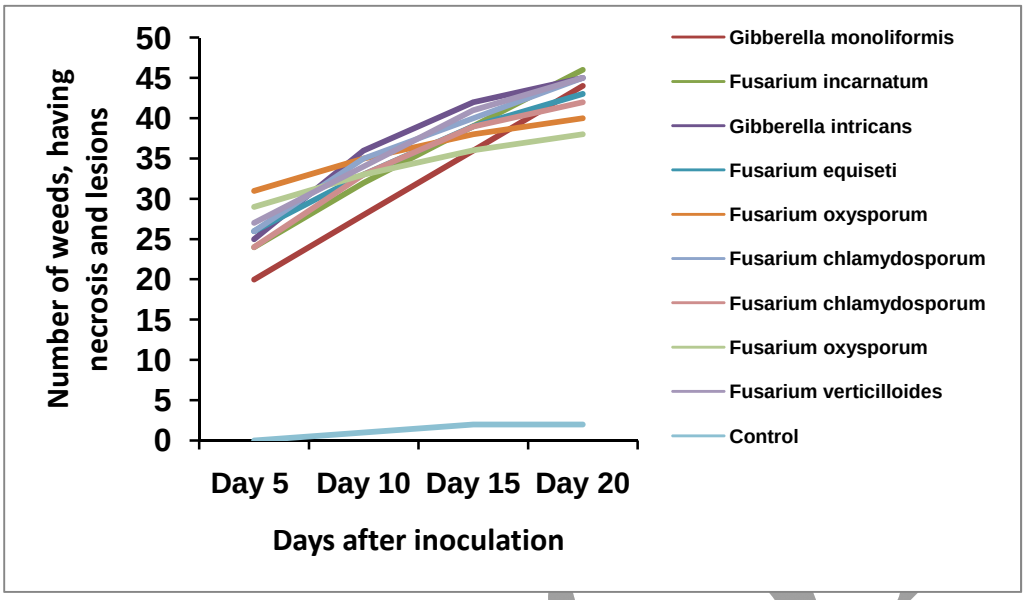


Figure 3 Progressive infection of *Striga hermonthica* after inoculation with the fungal isolates.

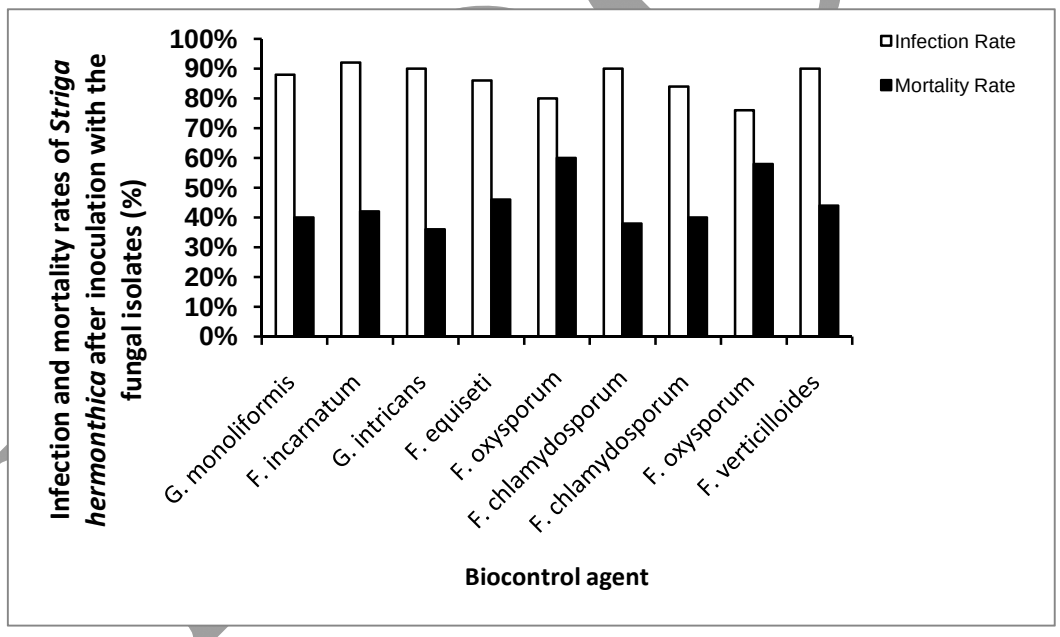


Figure 4 Infection and mortality rates of *Striga hermonthica* 22 days after inoculation with fungal isolates (in %).

Conclusion

Though all the fungal isolates tested against the weed could cause infection and consequently kill the weed, *Fusarium oxysporum* was the most aggressive recording the highest mortality rate of more than 50% within the shortest time. The others took longer which is not advantageous in the development of a biological control agent. Further research should be done on the fungal isolates tested as mycoherbicides so as to elucidate their maximum potential, safety and to find out how best they can be used and exploited as biocontrol agents against the weed on a commercial scale.

Acknowledgement

The author expresses deep and sincere gratitude to the International Maize and Wheat Improvement Centre (CIMMYT) and International Centre for Tropical Agriculture (CIAT) for jointly funding this research work. A mentioned also goes to the University of Nairobi for providing the technical support in piecing this work together.

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