

# Chlamyospore Production, Inoculation Methods and Pathogenicity of *Fusarium oxysporum* M12-4A, a Biocontrol for *Striga hermonthica*

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*Fusarium oxysporum* isolate M12-4A is currently being evaluated for the biological control of *Striga hermonthica*. Inoculum production, inoculum delivery to the target, chlamyospore germination, and weed growth suppression of this weed-pathogen system were investigated. Liquid fermentation systems using organic material were evaluated for the production of large numbers of chlamyospores. A 1% sorghum straw powder (< 1 mm) substrate, exposed to black light at 21°C for 21 days, yielded  $3.23 \times 10^8$  colony forming units (CFU) l<sup>-1</sup> medium. A two-stage fermentation system using 5% w/v straw substrate under black light at 30°C for 14 days yielded  $3.5 \times 10^8$  CFU l<sup>-1</sup> medium. In vitro variations in chlamyospore germination were governed by the presence of exogenous carbon, nitrogen, and sorghum root exudates. Ammonium–nitrogen compounds and urea, in combination with glucose had a stronger stimulatory effect on chlamyospore germ tube growth than did potassium nitrate. Maximal germ tube elongation occurred when chlamyospores were exposed to urea at a C/N ratio of 10. Some mineral solutions and sorghum root exudates inhibited chlamyospore germ tube elongation; however, arabic gum, a complex polysaccharide, stimulated chlamyospore germ tube elongation and the production of secondary chlamyospores. In field trials, chlamyospore powder harvested from small-scale fermenters reduced *S. hermonthica* emergence by 92%. Complete inhibition of *S. hermonthica* emergence occurred when the chlamyospore powder was added to the soil at sowing and when sorghum seeds coated with chlamyospores were sown. Effective biological control of *S. hermonthica* was achieved using a simple fermentation system with sorghum straw as the inoculum growth substrate. For inoculum delivery to the farmers' fields, sorghum seeds were coated with the inoculum using arabic gum as the adhesive. This simple delivery system permits a uniform inoculation of the field as well as the proper positioning of the inoculum in the immediate environment of sorghum roots, where *S. hermonthica* attaches to its host. To facilitate a broad usage of *F. oxysporum* M12-4A for the biocontrol of *S. hermonthica*, we propose an inoculum production strategy based on a cottage industry model that utilizes a liquid fermentation process and inexpensive locally-available substrates including sorghum straw and arabic gum.

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## INTRODUCTION

*Striga hermonthica* (Del.) Benth. is a parasitic angiosperm of a number of economically important crops within the Poaceae family including sorghum [*Sorghum bicolor* (L.) Moench], maize (*Zea mays* L.), millet [*Pennisetum americanum* (L.) K. Schum.] and rice (*Oryza sativa* (L.) (Stewart, 1990; Johnson *et al.*, 1997). *Striga* is very difficult to control and the use of a multiple integrated management approach for controlling *Striga* infestations has been commonly proposed (Carson, 1988; Parker, 1991; Thalouarn & Fer, 1993). Despite substantial research efforts, no effective means of controlling *Striga* have been achieved to date. Biological control is one option that has received attention recently. Numerous surveys for pathogens as possible biological control agents of *Striga* species demonstrate the growing interest for using alternative strategies to combat these noxious weeds (Abbasher *et al.*, 1995; Ciotola *et al.*, 1995; Kroschel *et al.*, 1996; Czerwenka-Wenkstetten *et al.*, 1997; Abbasher *et al.*, 1998; Nirenberg *et al.*, 1998; Marley *et al.*, 1999). Most of these studies have focused on soil microorganisms of the genus *Fusarium*. In glasshouse experiments, various *F. oxysporum* isolates have been found to be highly pathogenic against *S. hermonthica* (Kroschel *et al.*, 1996; Abbasher *et al.*, 1998).

*Fusarium* spp. are long-lived soil inhabitants that can survive extended periods in the absence of their host by colonizing crop debris and producing chlamydospores, dormant resting propagules (Nelson, 1981). Chlamydospores can withstand extreme environmental conditions (Nash *et al.*, 1961) and readily germinate when conditions are favourable (Schipper & Van Eck, 1981). Given the arid climate and sporadic rainfall characteristics of the Sub-Saharan region of Africa, *Striga* control using the bioherbicide approach would most likely be enhanced from soil applications of a dry-form inoculum consisting of drought-resistant fungal structures.

Soil carbon depletion, nutrient requirements (carbon, nitrogen and minerals), nutrient stress, and light quality are factors influencing chlamydospore production (Qureshi & Page, 1969; Huang *et al.*, 1983; Oritsejafor, 1986; Mondal *et al.*, 1995). Hebbbar *et al.* (1996, 1997) described a one-step liquid fermentation system using a low utilizable carbon substrate that produced large quantities of chlamydospores of a *F. oxysporum* isolate.

An isolate of *F. oxysporum* (hereinafter referred to as M12-4A) isolated in Mali, West Africa from *S. hermonthica*-diseased stem tissues reduced *S. hermonthica* emergence and inhibited seed germination under controlled conditions (Ciotola *et al.*, 1995; Ciotola *et al.*, 1996a). Isolate M12-4A inoculum was produced on solid substrates; sorghum glumes and straw pieces, and the 5-day old inoculum was comprised primarily of mycelia and microconidia, with few macroconidia and no chlamydospores (Diarra *et al.*, 1996b). This 5-day old inoculum was efficacious in reducing *S. hermonthica* emergence under field conditions when incorporated into the soil at the time of sorghum or millet sowing (Ciotola *et al.*, 1996b; Diarra *et al.*, 1996a). However, variability in the performance of the fungus was observed in the field (unpublished data) which was likely due to variable soil moisture conditions (i.e. erratic rainfall during the rainy season) reducing the survival and colonizing ability of the mycelia, the microconidia, and the macroconidia.

*Fusarium* infection of a host plant is accomplished either by germinating conidia or by direct hyphal penetration (Nelson, 1981; Ciotola *et al.*, 1996a). Various factors including nitrogen amendments and host root exudates affect the survival and pathogenicity of *Fusarium* chlamydospores. Flavonoids and other host root exudates stimulate chlamydospore germination (Schroth *et al.*, 1963; Ruan *et al.*, 1995; Mondal *et al.*, 1996). The impact of non-host exudation on phytopathogenic species has received far less attention but might, nonetheless, play an important role in soil fungistasis (Schroth & Hendrix, 1962; Schroth & Hildebrand, 1964). Sorghum is a host of the parasitic *S. hermonthica* but not a host of

*F. oxysporum* M12-4A. *In vitro* testing of sorghum root extract could provide valuable information on the interactions occurring in the rhizosphere, the location where *Fusarium* inoculum is typically incorporated. The objectives of the research reported in this study were: (1) to evaluate a liquid fermentation system for inoculum production; (2) to assess chlamyospore germination potential when subjected to various nitrogen-based compounds, polysaccharides, and sorghum root exudates; and (3) to measure the efficacy of the formulated fungus to suppress *S. hermonthica* under natural conditions.

## MATERIALS AND METHODS

### Stock Cultures

*F. oxysporum* M12-4A was isolated from diseased *S. hermonthica* stems, and stock cultures were maintained on dry sterile soil at 20°C. Starter cultures were grown on potato dextrose agar (PDA, Difco) for 5 days or in potato dextrose broth (PDB, Difco) shake flasks for 3 days.

### Parameter Definition for Optimal Liquid Fermentation of Chlamyospore

*Preliminary trials.* Several natural substrates (corncoobs, sunflower and sorghum ground straw, cotton seed embryo flour (Pharmamedia), Brewer's yeast, corn steep liquor, wheat bran, and corn meal) were evaluated for their ability to stimulate *Fusarium* M12-4A chlamyospore development in shake flasks. The best chlamyospore yielding substrate was ground sorghum straw (1% w/v) suspended in water (data not shown). Chlamyospore production was greatest at 21°C compared with 28 or 32°C.

*Inoculum production.* *F. oxysporum* M12-4A was produced in a system modified after Hebbar *et al.* (1996, 1997). A 20 l carboy filled with 15 l of distilled H<sub>2</sub>O and sorghum ground powder material was used for the fermentation of *Fusarium* M12-4A inoculum. The standard fermentation process included 1% (w/v) ground sorghum straw (< 1 mm), 5.4±0.2 pH, 21°C, and near-ultraviolet (NUV) light [F15T8-BLB (black light) Sylvania Co.] for 21 days. In separate experiments, the effect of: (a) light regime; fluorescent/incandescent light [400 μEm<sup>-2</sup>s<sup>-1</sup> PAR (Photosynthetically Active Radiation)], NUV, or darkness; and (b) substrate type; straw powder sizes of < 1 mm, < 500 μm, or sorghum glumes (< 500 μm) were evaluated.

A two-stage fermentation process was designed to compare the effect of different straw powder (< 1 mm) concentrations [1, 3, and 5% (w/v)] on the number of infective units produced. The first phase in the liquid broth with forced aeration stimulated fungal growth for 10 days. The second phase (solid state) involved removing the liquid from the fermentation vessels, re-closing the vessel, and leaving the remaining moist sludge stationary for 4 days before harvest.

Fermenters for all experiments were sterilized for 3 h at 121°C and 103 kPa pressure. After cooling, the carboys were aseptically seeded with 100 ml of the *F. oxysporum* M12-4A seed culture produced in PDB. Aeration and mixing of system contents was achieved by introducing forced sterile air (0.2 μm sterile filter) into the bottom of each carboy. Each treatment was replicated either two or three times. At harvest, all material (straw + fungus) was collected in a metal sieve lined with sterile cloth, air-dried, and weighed. Since the harvested material included a mixture of mycelia and spores in straw powder, evaluations were made using a modified serial dilution plate technique as described by Nash and Snyder (1962) and Stapleton and Devay (1982). Samples from each fermenter were ground in a coffee grinder and a 0.02 g subsample was series diluted (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>) in 0.1% water agar (WA) and plated on a *Fusarium*-selective medium containing PDB, pentachloronitrobenzene and chloramphenicol (Fauzi & Paulitz, 1994). Viable colony forming unit (CFU) counts were determined after 48–72 h of incubation at 26°C and corrected for sample moisture content.

CFU counts represent the number of infective propagules and can be comprised of chlamydo spores, mycelia, and/or microconidia that are loose or embedded within the straw material. Microscopic observations were used to determine the type of spores present in the powder. However, no effective technique was found to separate the fungal propagules from the fibrous materials. For this reason, data comparisons between treatments are discussed in terms of total colony forming units per litre of medium rather than by chlamydo spore counts. Experiments having three replicates were statistically analysed, while the mean and standard error are presented for experiments with two replicates.

*Viability of the powder.* Propagule survival within the harvested material was determined after 6–8 months for trials comparing the different types of substrates as well as the various straw concentrations. The dried harvested material from each fermenter was kept in sealed containers at ambient temperature ( $20 \pm 2^\circ\text{C}$ ). Three sub-samples were evaluated for each replicate. CFU counts were determined as described above.

### **Effect of Carbon, Nitrogen and Sorghum Root Exudates on Germination of M12-4A Chlamydo spores**

*Role of glucose and nitrogen.* Dialysis membrane pieces ( $1 \text{ cm}^2$ ) were first dipped into the different carbon/nitrogen (C/N) test solutions and then placed on glass slides in sterile moist chamber (Petri dishes). The solutions were: (1) Control (double distilled water, ddH<sub>2</sub>O); (2) 0.025 M-glucose; (3) 0.05 M-glucose; (4) 0.05 M-glucose + 0.025 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; (5) 0.05 M-glucose + 0.05 M-KNO<sub>3</sub>; (6) 0.05 M-glucose + 0.025 M-urea [(CO)<sub>2</sub>NH<sub>2</sub>]; (7) 0.05 M-glucose + 0.019 M-NH<sub>4</sub>Cl; (8) 0.025 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; (9) 0.05 M-KNO<sub>3</sub>; (10) 0.025 M-urea. The C/N ratio for treatments 4–6 and 7 were 5 and 14, respectively. Subsequently, 0.001 g ( $\sim 10^4$  CFU) of air-dried fermented material was placed on each membrane. The chambers were sealed with Parafilm and placed under fluorescent light ( $100 \mu\text{Em}^{-2} \text{ s}^{-1}$  PAR) at  $26^\circ\text{C}$  for 16 h. Observations and measurements were made using  $\times 440$  magnification on three replicates for each treatment (15 germ tube measurements per replicate). The experiment was repeated with two replicates per treatment. Data were expressed as mean germ tube lengths ( $\mu\text{m}$ ).

*Effect of the C/N ratio.* This experiment was established on the basis of results obtained from the previous experiment and was designed to compare the effect of various combinations of C and N at different C/N ratios on chlamydo spore germ tube length. Sterile moist chamber mounts were used as described above. The amount of glucose was constant (0.05 M) and the C/N ratios were obtained by varying the nitrogen level as follows: C/N of 2.5–0.1 M-KNO<sub>3</sub>, 0.051 M-NH<sub>4</sub>NO<sub>3</sub>, or 0.052 M-urea; C/N of 5–0.05 M-KNO<sub>3</sub>, 0.02 M-NH<sub>4</sub>NO<sub>3</sub>, or 0.025 M-urea; and for C/N of 10–0.026 M-KNO<sub>3</sub>, 0.013 M-NH<sub>4</sub>NO<sub>3</sub>, or 0.013 M-urea. Observations and measurements were made using  $\times 440$  magnification on three replicates per treatment (15 germ tube measurements per replicate) as described above. The experiment was repeated once.

*Effects of sorghum root exudates and arabic gum.* Sorghum root exudates (SRE) were collected from sorghum plants by growing seedlings in several mineral solutions. Sorghum seeds were surface-sterilized in 0.6% NaOCl for 15 min, rinsed in sterile water, and placed on moistened filter paper (Whatman) in a glass Petri dish. Three-day-old germlings (radicle just emerged) were transferred onto a nylon mesh placed over a 20% Long Ashton (LA) mineral solution for 3 days (Hewitt, 1966), rinsed in sterile water, and transferred to disposable 15 ml plastic test tubes containing either: (1) distilled water (SRE-1); (2) 40% LA solution (SRE-2); (3) 40% LA solution plus 68 ppm urea (SRE-3); (4) 68 ppm urea (SRE-4); or (5) 40% LA solution plus 68 ppm urea for 7 days and then the solution replaced with distilled water for 2 days (SRE-5). The various growth media served as controls. With

their roots immersed in the solutions, seedlings were incubated in controlled environment chambers at 28/26°C day/night temperatures with 16-h photoperiod at  $400 \mu\text{Em}^{-2} \text{s}^{-1}$  PAR. Test tubes were refilled with the appropriate solution every second day. After 9 days, sorghum seedlings were removed from the test tubes, the solutions in each tube thoroughly mixed, and dialysis membrane pieces ( $1 \text{ cm}^2$ ) wetted with the solutions. In addition to the SRE solutions, arabic gum (Type CS, Daminco Inc.) solutions of 10, 20 and 40% w/v were also prepared in distilled water and used to wet dialysis membranes. Moist chamber mounts, application of  $0.001 \text{ g}$  ( $\sim 10^4$  CFU) of fermented material, and spore germination tests were performed as described above. The experiment was repeated once.

### **Efficacy of *F. oxysporum* M12-4A Inoculum to Suppress *S. hermonthica* Emergence Under Natural Conditions**

*Field trials in Mali, West Africa.* Inocula were produced on solid substrate and in a liquid fermentation system. Solid substrate of the *F. oxysporum* M12-4A isolate was prepared by placing 40 g of sorghum straw pieces ( $2 \text{ cm}^3$ ) in 2 l cylindrical jars containing 300 ml of water and soaking overnight (16 h). Excess water was drained off and jars were autoclaved at 121°C (103.5 kPa) for 20 min. Jars were cooled and inoculated with eight agar plugs (4-mm diameter) obtained from a 5-day old PDA *F. oxysporum* M12-4A culture. Jars were sealed with a filter screw cap ( $0.2 \mu\text{m}$ ) and shaken daily. Straw pieces were colonized within 5 days then the inoculum was air-dried for 3–5 h, and ground using a coffee mill. Liquid fermentation inoculum was produced in 20 l carboys as described earlier. Fermenter-harvested material was stored for 6 months at 20°C and consisted largely of chlamydo spores ( $1 \times 10^7$  CFU  $\text{g}^{-1}$  of fermenter harvested material).

Field trials were carried out during the rainy season (June–November, 1997) in Sikasso, Mali. Each experimental plot consisted of a 2.4 m row of sorghum, with seed pockets and rows spaced 0.4 and 0.8 m apart, respectively. At planting, all plots received a single fertilizer application of ammonium phosphate fertilizer (18-46-0) at  $100 \text{ kg ha}^{-1}$ . The factorial experiment was arranged as a randomized complete block design (RCBD) with five replicates. Two factors were examined. The first factor was inoculum with eight levels: (1) no material; (2) 10 g sterilized sorghum straw control; (3) 2.6 g sterilized powdered sorghum straw control; (4) 2.6 g *F. oxysporum* M12-4A solid substrate ground inoculum; (5) 0.5 g of chlamydo spore powder; (6) 0.5 g of chlamydo spore powder plus 10 g of straw; (7) 1 g of chlamydo spore powder; and (8) 1 g of chlamydo spore powder plus 10 g of straw. The second factor was supplemental fertilizer with two levels; no urea or  $65 \text{ kg ha}^{-1}$  urea side-dressed 15 days after sowing (DAS). The inoculum was placed in each seeding hole to a depth of 6 cm, covered with 2 cm of soil, sorghum seeds (var. Tiémaring) were sown just above the inoculum at a depth of 4 cm, and seeding holes filled with soil. Number of emerged *S. hermonthica* was recorded 105 DAS. Due to excessive bird damage, sorghum grain yield data was not recorded.

*Pot experiment in Mali, West Africa.* In 1998, fermenter-harvested inoculum, consisting mainly of chlamydo spores ( $10^7$  CFU  $\text{g}^{-1}$  material) was evaluated for *S. hermonthica* suppression in a pot experiment under field conditions in Mali. Black plastic bags (pots) (10-cm diameter) filled with soil were used to grow the sorghum. The pots were artificially infested with *S. hermonthica* ( $\sim 6000$  seeds/pot) and seeded with six seeds of CSM 388, a locally grown susceptible sorghum variety. The combined effect of *F. oxysporum* M12-4A inoculum and fertilizer was tested. Two factors were examined. The first factor was incorporated inoculum, with three levels: (1) none (control); (2) 0.5 g chlamydo spores powder; or (3) inoculum-coated sorghum seeds. The second factor was fertilization with two levels, none (control) or ammonium phosphate (20 pellets/pot). Additional pots were sown with sorghum in *Striga*-free soil receiving no fertilizer and served as checks. Inoculum-coated sorghum seeds were prepared as follows: sorghum seeds were covered with a

40% w/v solution of arabic gum in water, sprinkled with *F. oxysporum* M12-4A dry powder inoculum (20 g of inoculum/1000 sorghum seeds) and air-dried. Pots were filled to a depth of 8 cm with soil, the inoculum (or nothing) dispersed on the surface, 3 cm of soil, 1 cm of *S. hermonthica*-infested soil, 3 cm of soil, then the fertilizer, 3 cm of soil, sorghum seeds (six seeds inoculum-coated or not), and followed by a final 2 cm of soil. The factorial experiment was arranged as a RCBD with four replicates. *S. hermonthica* emergence, plant height, and sorghum growth were evaluated weekly. At harvest, sorghum and *S. hermonthica* plants were cut at the soil line, dried and weighed.

### Data Analyses

Statistical analyses (ANOVA or MANOVA) were performed on non-transformed data or on square-root transformed data when required, and means were separated using the Student–Neumann–Keuls Multiple Range Test (SNK) at an 0.05 significance level.

## RESULTS

### Defining Parameters for Optimal Chlamydospore Production Using a Liquid Fermentation Process

*Growth parameters.* Fungal response (in CFU) to various light environments during the fermentation process revealed the stimulating effect of black light on fungal sporulation (Figure 1(a)). The increase in propagule yield under NUV light, although not significant,

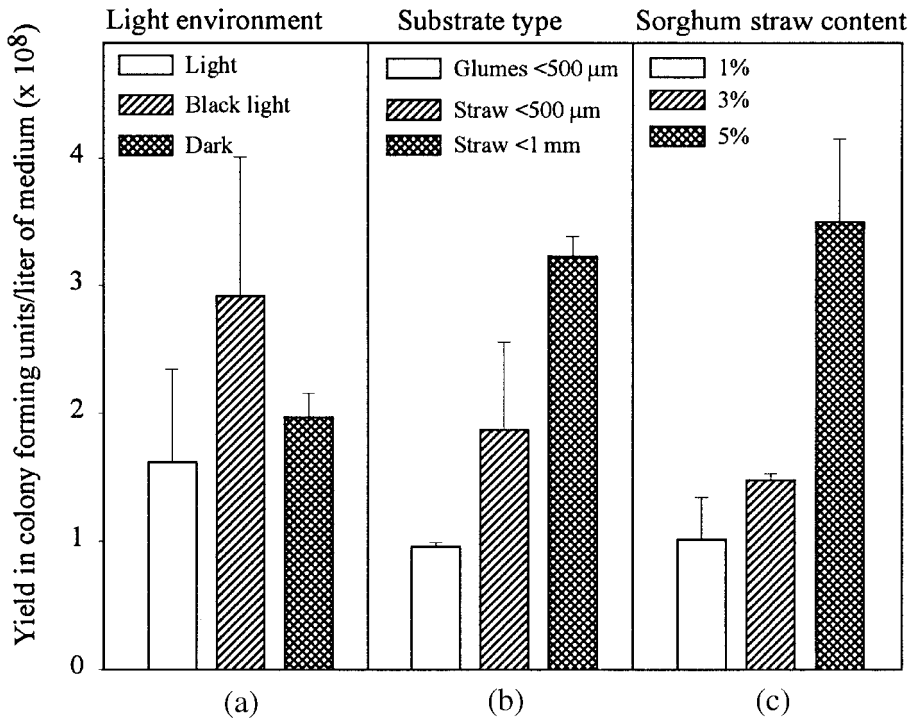


FIGURE 1. Yield, in colony forming units, of *F. oxysporum* M12-4A isolate grown in a one-stage agitated 15-l submerged fermentation system at 21°C for 21 days exposed to: (a) different light environments (fluorescent/incandescent light; black light (NUV); dark); (b) to various substrates (glumes < 500 μm; straw < 500 μm; straw < 1 mm); or (c) grown in a two-stage (submerged/solid state) fermentation system at 21°C for 14 days comparing substrate densities (1%, 3%, 5% w/v). Vertical lines on bars are standard errors.

was observed on several occasions. Local agricultural by-products such as sorghum straw (C/N of 192) and glumes proved to be adequate substrates for stimulating *F. oxysporum* isolate M12-4A growth and sporulation (Figure 1(b)). Medium containing larger-sized sorghum straw fragments (< 1 mm) yielded the highest CFU ( $3.23 \times 10^8$  CFU l<sup>-1</sup> of growth medium), while ground glumes supported only a third of this population (Figure 1(b)). Large size straw materials were colonized by M12-4A mycelia and they either remained as mycelial fragments or converted into chlamydo spores. Furthermore, fungal propagules embedded in these straw fragments were the first to germinate and grow rapidly in response to moisture availability (discussed below).

A significant ( $P < 0.05$ ) increase in production (CFU yield) was obtained by increasing the substrate concentration of straw from 3 to 5% straw (w/v) in a two-stage fermentation system maintained at 30°C. Beyond this substrate concentration, adequate aeration and straw mixing during the liquid phase could not be achieved using the 20 l fermenters and the aeration system employed. At the 5% substrate concentration, total CFU per fermenter was increased by nearly 3.5 times compared with yields obtained with a 1% substrate concentration; however, the CFU rate per g of substrate was greatest at the 1% concentration level (Figure 1(c)). During fermentation, mycelia and microconidia were present after 5 days and chlamydo spore development started at 10 days. After 14 days, the inoculum was composed mainly of chlamydo spores with some microconidia and few macroconidia present. Although overall optimal propagule production per g of substrate was obtained using the single stage fermentation with 1% w/v sorghum straw powder (< 1 mm) at 21°C for 21 days under black light ( $3.23 \times 10^8$  CFU l<sup>-1</sup> medium), the two-stage fermentation system using 5% w/v straw concentration operated for 14 days at 30°C under black light yielded the highest number of propagules ( $3.5 \times 10^8$  CFU l<sup>-1</sup> medium).

**Survival.** Viability of the fermented material decreased following 6 and 8 months' storage at room temperature (20°C) (Table 1). Variability in CFU values indicates the heterogeneous nature of the harvested inoculum. A reduction in the number of viable propagules occurred with the inoculum produced on glumes (50%) compared with inoculum produced on large sorghum straw fragments (25%) (Table 1(a)). Nevertheless, even with 25% reduction in viability, there was still more than  $3.5 \times 10^7$  viable propagules per g after 8 months' storage. Mean loss of viability obtained for samples grown in the two-stage fermentation system was less variable, with lowest survival rates (42%) from the 3% straw substrate (Table 1(b)).

TABLE 1. Shelf life of fermenter-produced *F. oxysporum* M12-4A inoculum stored at 20°C (a)

Substrates	Initial CFU <sup>a</sup>	CFU after 8 month shelf-life	Mean loss of viability (%) <sup>b</sup>
Straw < 1 mm	$4.65(0.79)^c \times 10^7$	$3.54(0.84) \times 10^7$	24.9
Straw < 500 μm	$3.04(1.45) \times 10^7$	$1.89(0.69) \times 10^7$	33.3
Glumes < 500 μm	$1.36(0.03) \times 10^7$	$6.82(0.32) \times 10^6$	50.3

(b)

Amount of substrate	Initial CFU <sup>a</sup>	CFU after 6 month shelf-life	Mean loss of viability (%) <sup>b</sup>
Straw 1%	$1.49(0.51)^c \times 10^7$	$9.38(1.12) \times 10^6$	30.7
Straw 3%	$6.87(0.13) \times 10^6$	$4.00(0.13) \times 10^6$	41.7
Straw 5%	$1.03(0.17) \times 10^7$	$6.88(1.75) \times 10^6$	34.6

<sup>a</sup>Colony forming units per gram of dry material collected from fermenters at harvest.

<sup>b</sup>Mean loss of viability percentages was calculated using values obtained from each replicate.

<sup>c</sup>Values in parentheses are standard errors.

## Germination of M12-4A Chlamydo spores as Influenced by Inorganic Carbon and Nitrogen

*Role of glucose and nitrogen.* Microconidia, few macroconidia, and chlamydo spores of *F. oxysporum* M12-4A were formed in liquid media or within the straw fragments during the fermentation process. The response of spore types to various nitrogen compounds differed, but only chlamydo spore germination will be discussed here. All results and analyses (Table 2) presented were obtained from pooled data of two trials (total of five replicates). The various nutrients had a significant effect ( $P < 0.001$ ) on chlamydo spore germ tube lengths (Table 2). A mixture of glucose and urea yielded the longest germ tubes. Glucose and potassium nitrate alone had no significant effect on chlamydo spore germ tube length, while urea and ammonium sulfate alone and glucose when combined with a nitrogen compound significantly increased chlamydo spore germination (Table 2).

Significant differences ( $P < 0.001$ ) in chlamydo spore behaviour were observed when exposed to various nitrogen solutions having different C/N ratios (Table 3). Potassium

TABLE 2. Effect of inorganic nitrogen and carbon on *F. oxysporum* M12-4A chlamydo spore germ tube lengths

Treatments	Chlamydo spore germ tube length ( $\mu\text{m}$ )		
Control (H <sub>2</sub> O)	54.2 <sup>a</sup>	(11.2) <sup>b</sup>	a <sup>c</sup>
Glucose (0.025 M)	62.2	(7.2)	a
Glucose (0.05 M)	85.7	(5.4)	a
Glucose (0.05 M) + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (0.025 M)	221.5	(16.5)	c
Glucose (0.05 M) + KNO <sub>3</sub>	179.0	(20.0)	bc
Glucose (0.05 M) + CO(NH <sub>2</sub> ) <sub>2</sub> (0.025 M)	291.4	(27.3)	d
Glucose (0.05 M) + NH <sub>4</sub> Cl (0.019 M)	230.0	(17.8)	c
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (0.025 M)	189.2	(21.7)	bc
KNO <sub>3</sub> (0.05 M)	98.1	(6.1)	a
CO(NH <sub>2</sub> ) <sub>2</sub> (0.025 M)	148.2	(14.5)	b

<sup>a</sup>Chlamydo spore germ-tube lengths ( $\mu\text{m}$ ) were obtained by placing *F. oxysporum* M12-4A chlamydo spores, from liquid fermentation, on dialysis membranes imbibed with nitrogen and carbon solutions, and held in sealed in moist chambers for 16 h at 26°C. Mean of 30 measurements.

<sup>b</sup>Values in parentheses are standard errors.

<sup>c</sup>Chlamydo spore germ tube length values having the same letter are not significantly different at the  $\alpha = 0.05$  level of significance according to the Student–Neumann–Keuls multiple range test.

TABLE 3. The influence of nitrogen source and solution C/N ratio on *F. oxysporum* M12-4A chlamydo spore germ tube length

C/N ratios <sup>a</sup>	Nitrogen amendment		
	KNO <sub>3</sub>	NH <sub>4</sub> NO <sub>3</sub>	CO(NH <sub>2</sub> ) <sub>2</sub>
2.5	141.8 <sup>b</sup> Aa <sup>b</sup>	267.9 Ab	247.2 Ab
5	156.8 Aa	278.3 Ab	332.5 Bb
10	180.8 Aa	297.1 Ab	387.3 Cc

<sup>a</sup>Glucose remained constant (0.05 M) and the C/N ratios obtained by varying the nitrogen content.

<sup>b</sup>Mean values for chlamydo spore germ-tube lengths ( $\mu\text{m}$ ) were obtained by placing *F. oxysporum* M12-4A chlamydo spores, from liquid fermentation, on dialysis membranes wetted with nitrogen compounds at selected C/N ratios and sealed in moist chambers for 16 h at 26°C.

<sup>c</sup>For each nitrogen source (uppercase) or C/N ratio (lowercase), chlamydo spore germ tube length values having the same letter are not significantly different at the  $\alpha = 0.05$  level of significance according to the Student–Neumann–Keuls multiple range test.

nitrate had the lowest stimulatory effect on germ tube elongation while urea at a C/N of 10 had the greatest stimulating effect on germ tube elongation. Chlamydospore germ tube lengths were affected in a similar manner when exposed to different C/N ratios for both ammonium and potassium nitrate treatments. In contrast, germ tube lengths of chlamydospores exposed to urea were significantly higher as the C/N ratios were increased (Table 3). However, at a C/N ratio of 2.5 germ tube lysis was observed for urea-treated samples. Of interest, potassium nitrate treatments produced new generations of microconidia within 16 h.

*Effect of sorghum root exudate and arabic gum.* Sorghum root exudates (SRE) strongly inhibited chlamydospore germ tube elongation (Table 4). Chlamydospores subjected to mineral solutions, or to SRE of sorghum plants grown in water (SRE-1), in urea (SRE-4), or in a mineral solution and transferred in water (SRE-5), produced significantly shorter germ tubes than in water (Table 4). In contrast, chlamydospores exposed to SRE from sorghum plants grown in LA solutions (118.8, 125.6  $\mu\text{m}$ ) or chlamydospores added to a urea solution (145.3  $\mu\text{m}$ ) produced much longer germ tubes. In our study, several chlamydospores exposed to sorghum root exudate in urea or water had still not germinated or exhibited suppressed germ tube growth following 72 h exposure. Nonetheless a significant stimulatory effect on *Fusarium* germ tube elongation was observed for all treatments involving arabic gum (Table 4). Profuse mycelial growth originating from propagules embedded in the sorghum straw fragments in the arabic gum treatment was observed after only 12 h. The presence of newly formed chlamydospores within the mycelia was observed for treatments with arabic gum and for chlamydospores immersed in urea. This new generation of thick layered spores was produced on short conidiophores and was characterized by the oval shape as opposed to the typical round shaped chlamydospore produced by M12-4A.

TABLE 4. Effect of sorghum root exudates, nutrient solution, and arabic gum on *F. oxysporum* M12-4A germ tube length

Treatments <sup>a</sup>	Chlamydospore germ tube length ( $\mu\text{m}$ )		
Control (H <sub>2</sub> O)	181.5 <sup>b</sup>	(30.2) <sup>c</sup>	d <sup>d</sup>
Mineral solution (Long Ashton)	47.3	(6.0)	ab
Urea (68 ppm)	145.3	(22.0)	cd
Mineral solution + urea	64.4	(13.8)	abc
SRE <sup>e</sup> -1 (water)	37.6	(9.2)	ab
SRE-2 (mineral solution)	125.6	(10.2)	bcd
SRE-3 (mineral solution + urea)	118.8	(43.9)	bcd
SRE-4 (urea)	3.3	(3.3)	a
SRE-5 (mineral solution + urea, water)	56.7	(12.9)	ab
Arabic gum 10% (w/v)	347.6	(24.6)	e
Arabic gum 20% (w/v)	435.1	(24.9)	f
Arabic gum 40% (w/v)	407.2	(10.6)	ef

<sup>a</sup>*F. oxysporum* M12-4A chlamydospores from liquid fermentation were placed on dialysis membranes wetted with various solutions, sealed in moist chambers for 16 h at 26°C, and germ tube lengths measured.

<sup>b</sup>Mean of 30 measurements.

<sup>c</sup>Values in parentheses are standard errors.

<sup>d</sup>Values having the same letter are not significantly different at the  $\alpha = 0.05$  level of significance according to the Student-Neumann-Keuls multiple range test.

<sup>e</sup>Sorghum root exudates (SRE) were collected from sorghum seedlings growing in several solutions for 9 days. In SRE-5 the original solution was replaced with distilled water for the last 2 days.

TABLE 5. Analysis of variance on the influence of *F. oxysporum* M12-4A inoculum type and urea on *S. hermonthica* emergence (a), and treatment effects on *S. hermonthica* emergence in the field (b)

(a)

Source of variation	Degrees of freedom	F-ratio	Significance level
A (Inoculum)	7	4.25	0.007
B (Fertilizer)	1	0.26	0.614
AB	7	0.62	0.736
Residual	64		
Total (corrected)	79		

The analysis was performed on square-root transformed data. There were five replicates.

(b)

Treatments: Inoculum	<i>Striga</i> plants per plot		
Control (no straw material incorporated)	32.1 <sup>a</sup>	(17.3) <sup>b</sup>	a <sup>c</sup>
Sterilized straw control (10 g)	16.8	(6.2)	ab
Sterilized ground straw control (2.6 g)	21.3	(12.5)	ab
Solid substrate ground inoculum (2.6 g)	7.9	(4.5)	b
Chlamyospore powder (0.5 g)	6.9	(4.9)	b
Chlamyospore powder (0.5 g) + sterilized straw (10 g)	3.6	(1.9)	b
Chlamyospore powder (1 g)	2.7	(1.7)	b
Chlamyospore powder (1 g) + sterilized straw (10 g)	2.5	(1.4)	b

<sup>a</sup>Mean number of *S. hermonthica* in plots inoculated with *F. oxysporum* M12-4A. The inoculum was incorporated with or without straw amendments at planting with the sorghum seeds. Supplemental fertilizer in the form of urea (65 kg ha<sup>-1</sup>) was applied on half of the experiment 15 days after sowing.

<sup>b</sup>Values in parentheses are standard errors.

<sup>c</sup>Emergence values having the same letter are not significantly different at  $\alpha=0.05$  according to the Student–Neumann–Keuls multiple range test.

### Efficacy of *F. oxysporum* M12-4A Inoculum to Suppress *S. hermonthica* Emergence Under Natural Conditions

*Field experiments.* Contrary to results obtained in previous trials (Ciotola *et al.*, 1996b), the addition of urea 15 days after sowing did not have a significant effect on *S. hermonthica* emergence (Table 5(a)) while the incorporation of inoculum had a significant negative impact on *S. hermonthica* emergence (Table 5(a)). Chlamyospore powder treatments were very effective in reducing *S. hermonthica* emergence, ranging from 78 to 92%. The highest *Striga* suppression (92%) was observed with 1 g chlamyospore powder + 10 g straw amendment per seed pocket (Table 5(b)).

*Pot trial.* Both dry powder formulations of M12-4A completely inhibited *S. hermonthica* emergence. Since *S. hermonthica* emergence was prevented by the presence of *F. oxysporum* dry inoculum whether applied alone or coated on sorghum seeds, the effect of ammonium phosphate could only be evaluated in non-inoculated treatments (Figure 2). *S. hermonthica* height for plants subjected to the fertilizer treatment was significantly reduced ( $P < 0.001$ ) compared with plant height for the control treatment. During this experiment, sorghum growth within control pots receiving no amendments was relatively poor with most plants turning yellow early in the season. This reduced host vitality could explain the low levels of *S. hermonthica* emergence observed (Figure 2). Sorghum height was significantly increased in inoculated ( $P < 0.001$ ) and fertilized pots ( $P < 0.001$ ) (Table 6). Sorghum height increase

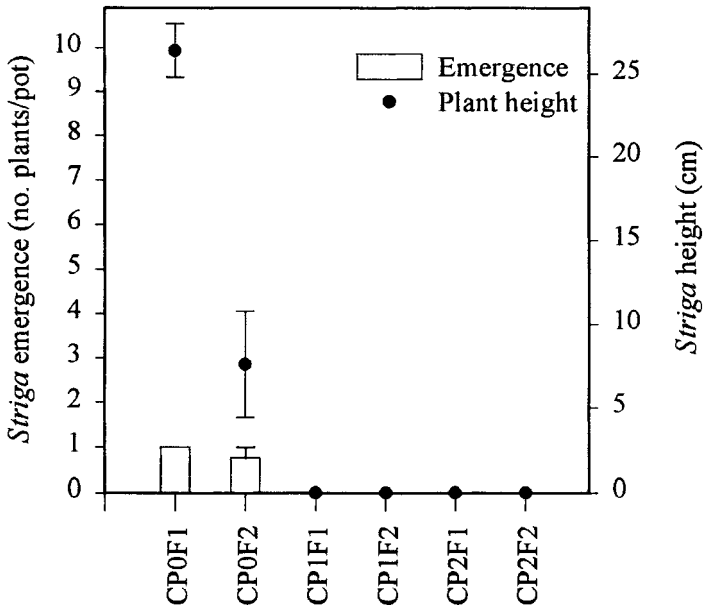


FIGURE 2. Growth and mean number of *S. hermonthica* grown in a pot experiment under Malian field conditions as affected by the incorporation of *F. oxysporum* M12-4A chlamyospore powder (CP0: control/no *F. oxysporum*; CP1: *F. oxysporum* chlamyospore powder 0.5 g/pot; CP2: *F. oxysporum* chlamyospore powder-coated sorghum seeds) in combination with/without fertilizer (F1: no added fertilizer, F2: 20 pellets of ammonium phosphate). Lines on each bar indicate standard errors.

TABLE 6. The influence of ammonium phosphate and *F. oxysporum* M12-4A inoculum on sorghum height at harvest for the pot experiment

Treatment: Inoculum	Sorghum height (cm)		
Control (no inoculum)	26.6 <sup>a</sup>	(2.5) <sup>b</sup>	a <sup>c</sup>
Inoculum powder	39.4	(5.3)	b
Inoculum-coated seeds	50.3	(5.6)	c

Treatment: Fertilizer	Sorghum height (cm)		
Control (no fertilizer)	31.0	(3.9)	a
Ammonium phosphate	46.5	(5.1)	b

<sup>a</sup>Mean sorghum height values at harvest of plants inoculated at planting with *F. oxysporum* M12-4A inoculum and fertilized with ammonium phosphate.

<sup>b</sup>Values in parentheses are standard errors.

<sup>c</sup>Height values in either main effect having the same letter are not significantly different at  $\alpha = 0.05$  according to the Student–Neumann–Keuls multiple range test.

was the greatest in the inoculum-coated sorghum seed treatment in both fertilized and unfertilized pots (Figure 3). Growth of sorghum from inoculum-coated seeds in presence of *S. hermonthica* was even greater than growth of sorghum in the absence of *S. hermonthica* (i.e. weed-free check) (Figure 3, Table 6).

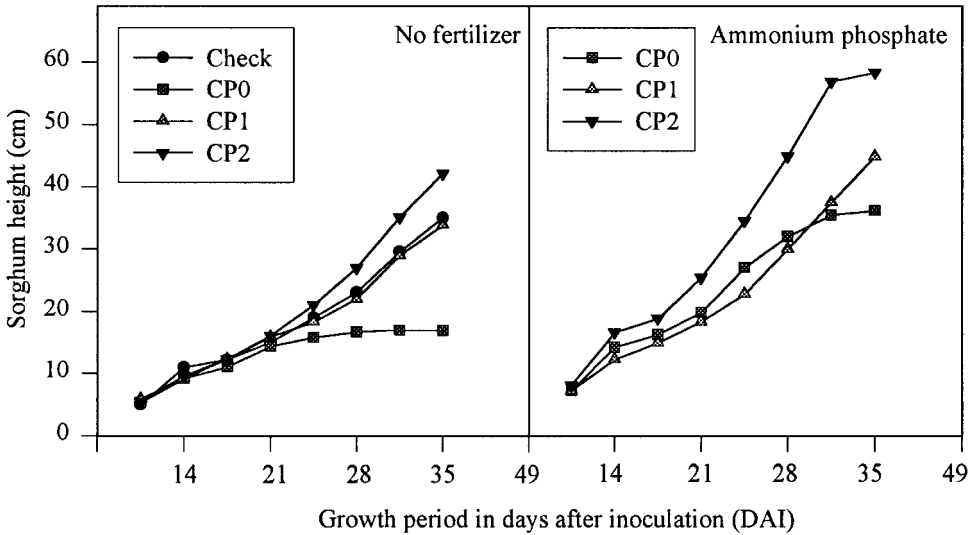


FIGURE 3. Sorghum growth in a pot experiment under field conditions in Mali, exposed to the incorporation of *F. oxysporum* M12-4A chlamyospore powder (CP0: control/no *F. oxysporum*; CP1: *F. oxysporum* chlamyospore powder 0.5 g/pot; CP2: *F. oxysporum* chlamyospore powder-coated sorghum seeds) in combination with/without fertilizer (F1: no added fertilizer, F2: 20 pellets of ammonium phosphate). The check treatment refers to sorghum grown in unfertilized *S. hermonthica*-free soil.

## DISCUSSION

Liquid fermentation systems have made use of agricultural by-products (i.e. barley straw, corncobs, and soybean hull) as substrates for producing large quantities of chlamydo-spores of *Fusarium* species (Hildebrand & McCain, 1978; Hebbar *et al.*, 1996). Source, size and density of substrate affected the production and survival of *F. oxysporum* M12-4A propagules in agitated submerged cultures. Ground sorghum straw (C/N ratio of 192:1) of a relatively small size (< 1 mm) supported the production of large numbers of CFU, composed mainly of chlamydo-spores. It has been previously shown that a high C/N ratio (120:1) favours chlamydo-spore production over macroconidia production (Oritsejafor, 1986), and the carbon content of the growth media is the most important factor determining chlamydo-spore production (Qureshi & Page, 1969; Oritsejafor, 1986). In contrast, Hebbar *et al.* (1996) found that substrates having low utilizable carbon levels produced the highest numbers of chlamydo-spores regardless of the substrate C/N ratio.

NUV light has been shown to be the most effective wavelength region to induce sporulation in many fungi (Leach, 1962) and is known to influence chlamydo-spore development in several *F. oxysporum* species (Huang *et al.*, 1983). *F. oxysporum* M12-4A chlamydo-spore production was also generally enhanced by NUV light, but not significantly. Further studies are required to determine if NUV light exposure is necessary for optimum inoculum production. A system that does not have a light requirement would be preferable for a local low-technology cottage industry fermentation.

Our two-stage fermentation system produced high propagule density in 14 days at 30°C. The two-stage fermentation proved to be time and cost effective system, reducing the fermentation period from 21 to 14 days, whilst eliminating the possible temperature constraint since 30°C is more attainable than 21°C under local conditions in Mali where inoculum production is planned. Chlamydo-spore production was initiated 96 h prior to harvest, coinciding with the shift from liquid to solid state fermentation and removal of

aeration. The trigger for the induction of chlamydospore production may have been the sudden reduction in air supply to the system during the second stage of the fermentation process. Our findings are consistent with other studies reporting chlamydospore development increasing in liquid media having low dissolved oxygen levels (French & Nielsen, 1996; Hebbar *et al.*, 1997).

The shelf life of *F. oxysporum* M12-4A inoculum produced in submerged liquid culture is very favourable. M12-4A inoculum produced in submerged liquid culture remained viable (up to 75%) and effective (field experiment) after periods of 8 and 6 months, respectively. The differences in propagule survival observed for the different treatments might be linked to the physical nature of the substrate materials as well as their nutrient content. *F. oxysporum* f. sp. *cannabis* dry inoculum, produced on a barley straw medium, retained its virulence after 6 months, but a reduction in disease potential was observed after 9 months (Hildebrand & McCain, 1978).

*Fusarium* germination and soil colonization could be critical factors for effective *Striga* suppression. The addition of stimulatory compounds could hasten and augment the germination process, thus, enhancing mycelial growth and improving the efficacy of the fungus. Ammonium sulfate in combination with glucose (C/N of 5) was found to stimulate the germination of *F. solani* chlamydospores more than the nitrate form (Cook & Schroth, 1965). Glucose alone increased chlamydospore germination of *F. solani* f. sp. *phaseoli* and nitrogen was not required (Adams *et al.*, 1968). However, when *F. solani* chlamydospores originated from a high-density macroconidial source, both ammonium chloride and glucose were necessary for germination (Griffin, 1970). In a comprehensive review, Toussoun (1970) concluded that moderate levels of carbon and nitrogen were required for chlamydospore germination. A urea–glucose combination increased *F. oxysporum* M12-4A germ tube length more than potassium nitrate–glucose, but glucose alone had minimal effect on M12-4A. The C/N balance of urea had a substantial effect on germ tube growth of *F. oxysporum* M12-4A chlamydospores compared with potassium and ammonium nitrate. Moreover, at a C/N ratio of 10, germ tube elongation was doubled with urea when compared with potassium nitrate. Although ammonium nitrate and urea significantly hasten fungal growth, high levels of urea can severely reduce inoculum potential by causing germ tube lysis as was observed with M12-4A. Cook and Snyder (1965) also reported hyphal destruction in media having high nutrient concentrations.

This requirement for specific ammonium-based nitrogen amendments to increase fungal germination supports earlier findings in field trials. When *F. oxysporum* M12-4A was used in combination with ammonium nitrate, a synergy occurred significantly reducing *S. hermonthica* emergence and increasing crop yield (Ciotola *et al.*, 1996b). Ammonium-based fertilizers are reported to induce *Fusaria* diseases in several cropping systems (Maurer & Baker, 1965; Woltz & Engelhard, 1972; Woltz & Jones, 1973). Urea and ammonium-based fertilizers have been commonly reported to reduce *Striga* growth and infestations and to have a toxic effect on *Striga* germination (Pesch & Pieterse, 1982; Igbinnosa *et al.*, 1996; Mumera & Below, 1993; Kim *et al.*, 1997).

A variety of germination patterns were obtained for chlamydospores exposed to extracts collected from different sorghum growing media. Compounds exuded from sorghum roots in water inhibited germ tube growth, demonstrating a potential negative effect of this non-host crop on *F. oxysporum* M12-4A development. The presence of the mineral solution in the growing media was either masking the inhibitory effect of the exudates or influencing the composition of exuded materials. Differences in the type and quantity of compounds exuded from plants have been found to be correlated with ambient environmental conditions and affected by the form of nitrogen resulting in significant effects on disease development (Schroth *et al.*, 1963). Germ tube elongation of chlamydospores immersed directly in urea was similar to the water control and secondary chlamydospores were formed. However, germ tube growth of chlamydospores exposed to root exuded materials from sorghum grown in a urea solution was severely reduced. The suppressive effect on chlamydospore

germination, could be the result of exposure to specific plant exudates induced by urea or related to a modification of the solution pH from the conversion of urea into ammonia during sorghum growth, thus having a toxic effect on *Fusarium* (T. C. Paulitz, personal communication). Fungicidal activity of ammonia against *Fusarium* has also been documented (Smiley *et al.*, 1970, 1972; Gilpatrick, 1969).

There are many reports of chlamydo-spores from pathogenic *Fusaria* being stimulated by the presence of host-root exudates (Schroth & Hendrix, 1962; Schroth *et al.*, 1963; Cook & Schroth, 1965). Odunfa (1978) observed a stimulatory effect on *F. oxysporum* conidia germination when exposed to sorghum root exudates but also noted an inhibitory effect on mycelial growth. Few studies, however, have investigated the effect of non-host root environment on pathogenic *Fusaria* germination (Rice, 1984). Sorghum, a non-host crop of *F. oxysporum* M12-4A, is well known for its allelopathic properties on weeds and crops (Lehle & Putnam, 1983; Ben-Hammouda *et al.*, 1995) causing plant growth suppression, crop autotoxicity and herbicidal activity (Alsaadawi *et al.*, 1986; Hussain & Gadoon, 1981). Characterization of sorghum root extracts has revealed a vast array of chemical compounds including phenol acids such as p-coumaric and protocatechic (Burgos-Leon *et al.*, 1980), apigeninidin derivatives, luteolinidin and sorgoleones (Netzly *et al.*, 1988). Schutt and Netzly (1991) found that specific compounds (e.g. apigeninidin) from sorghum plant extracts exhibited inhibitory effects on *Fusarium* growth. *In vitro* results suggest that sorghum root exudates were involved in the inhibition of *F. oxysporum* M12-4A chlamydo-spore germination, but it is not known if the suppressive activity of the exudates was due to the presence of sorgoleones or other compounds released by sorghum plants. Further studies should be conducted in natural soil environments to elucidate mechanisms involved.

The inoculum production proposed for *F. oxysporum* M12-4A is to mass produce inoculum having high survival ability using locally-available, inexpensive unrefined agricultural by-products such as sorghum straw. The proposed delivery strategy is to use cereal crop seeds and arabic gum as inoculum carriers to facilitate the inoculation process as well as to place the inoculum in close proximity to the target, *S. hermonthica* seeds and germlings in the soil. The gum serves to temporarily glue the inoculum to sorghum seeds and subsequently releases the chlamydo-spores slowly into the rhizosphere environment. The arabic gum will also serve as a carbon source stimulating germ tube elongation and the production of secondary chlamydo-spores. The production of secondary chlamydo-spores increased the inoculum potential of *F. oxysporum* M12-4A in only 16 h. Secondary chlamydo-spore development results from exposure to a temporary nutrient source that triggers germination, vegetative growth and formation of new chlamydo-spores (Schroth & Hendrix, 1962). The production of replacement chlamydo-spores are known to increase *Fusaria* soil populations if, following germination, the pathogen fails to infect its host (Schroth & Hendrix, 1962; Papavizas *et al.*, 1968; Nash Smith, 1970). So in all this system, *F. oxysporum* M12-4A inoculum actually will multiply in the soil while lying in wait for its host, *S. hermonthica*.

Arabic gum is a viscous material exuded from wounds of leguminous *Acacia* tree species (mainly *Acacia senegal*) growing in desert regions of the Republic of Sudan, Nigeria, Senegal, and Mali. This natural gum is readily available at local markets in these countries and arabic gum has the highest water solubility index of all gums. The structural and chemical composition of this gum is known and is comprised of simple sugars including D-galactose, L-arabinose and L-rhamnose and (Whistler & BeMiller, 1995).

Arabic gum has been used as an adhesive to coat *Rhizobium* to legume seeds (Skerman *et al.*, 1988), while xantham gum was reported to increase both the germination and disease severity of *Colletotrichum truncatum* on *Desmodium tortuosum* (Florida beggarweed) when used as part of the growth medium (Cardina, 1986). In our study, arabic gum not only served as an adhesive for the inoculum and a stimulant for *Fusarium* germination and growth, but could also engender new generations of chlamydo-spores in the absence of the host. Further investigations in both *in vitro* and *in vivo* natural soil environments are

required to more fully elucidate the role of sorghum root exudates and their interactions with arabic gum in influencing *Fusarium* germination and growth. Nonetheless, the findings reported in this study suggest that arabic gum seed coated inoculum will impact future biocontrol strategies using soil-applied fungal inocula.

The strong within-treatment variability observed in field experiments performed in *Striga*-infested natural populations limits the power to statistically discriminate between treatment effects, and is most probably a consequence of the heterogeneous emergence and patchy spatial distribution of *Striga* (Webb & Smith, 1995). Notwithstanding, fermenter-harvested M12-4A inoculum, composed mainly of chlamydospores, applied at seeding significantly reduced *S. hermonthica* emergence under field conditions and controlled *S. hermonthica* emergence in a pot experiment.

*F. oxysporum* M12-4A inoculum produced in a 20 l carboy, can be stored or applied readily on arabic gum-coated sorghum seeds. We believe that the use of a soil-applied dry inoculum is best suited for the biocontrol of *S. hermonthica* in the semi-arid tropics where high temperatures and low-relative humidity prevail. The prolonged activity of the underground growing inoculum will ensure protection throughout the season and reduce *S. hermonthica* growth and seed production. The delivery of the cereal seed-coated inoculum (arabic gum and *F. oxysporum* M12-4A) is a time-efficient and uniform incorporation technique adapted to local needs and resources. Furthermore, arabic gum and urea were found to stimulate the development of secondary chlamydospores possibly resulting in substantial increases in inoculum potential, pathogenesis and survival of *Fusarium* soil populations in natural systems. A management system, integrating the biocontrol agent *F. oxysporum* M12-4A, arabic gum, and urea may provide an effective means of suppressing *Striga*.

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