# DIFFERENTIAL ENDOPHYTIC COLONIZATION OF SORGHUM PLANT BY EIGHT ENTOMOPATHOGENIC FUNGAL ISOLATES AND *IN VITRO* EVALUATION OF CONIDIA VIRULENCE.

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## ABSTRACT

Studies were conducted to investigate the potentials of three strains of *B. bassiana*, two strains each of *I. farinosa* and *Metarhizium anisopliae* and a strain of *I. fumosorosea* to colonise sorghum endophytically after application of conidia to rhizosphere and the effect on the plant growth characteristics. Virulence of the conidia before and after endophytic growth phases were assessed using *Galleria mellonella* larvae mortality bioassay in-vitro. All the strains of the fungi colonised the sorghum plant. The strains of *I. farinosa* and *B. bassiana* were detected in the roots, the stem and the leaves while *M. anisopliae* was detected in the root only after 20 days. The shoot length of the sorghum plants inoculated with the strain of *Beauveria*, BB 315 were significantly higher than the control while none of the strains significantly affected the length of the root. One strain of *I. farinosa* (ARSEF 5081) and a strain of *B. bassiana* (432.99) significantly reduced the plant vigour and the length of the shoot when compared with the control. *M. anisopliae* strains had no significant impact on the plant growth characteristics. Conidia of 14 days old cultures of the fungal isolates (primary conidia) and that of the first growth on Sabouraud Dextrose Agar (SDA) after the endophytic growth phase in sorghum (Secondary conidia), were tested for virulence using *Galleria mellonella* bioassay system. The $LT_{50}$ of *G mellonella* larvae increased significantly ($P<0.05$) when they were inoculated with the secondary conidia of some of the strains, compared to virulence of the primary conidia. However, virulence of the primary and secondary conidia of two strains of *B. bassiana,* BB 315 and 776.05 were not significantly different. The desirable roles of entomopathogenic endophytes for use under marginal environmental conditions were discussed.

**Key words:** Virulence, Conidia, Sorghum, Shoot length, Root length

## INTRODUCTION

Fungal entomopathogens have considerable capabilities to regulate insect pest populations (Contreras *et al*., 2014; Imoulan and Elmeziane, 2014) and they have been isolated from plant tissues where they grow as natural endophytes (Brownbridge *et al.* 2012). Many studies have shown that colonization of plant tissues by entomopathogens through artificial inoculation of seeds or seedlings with their conidia offer crops some level of resistance against pathogens (Ownley *et al*., 2010) and promote plant growth (Khan *et al.,* 2012). The presence of an entomopathogenic fungus as an endophyte in a plant has been reported to deter foliage feeding pests (Hartley and Gange, 2009). They have been successfully introduced to many crops with positive outcomes, for example in cocoa seed dressing for the control of the pod borer, *Conopomorpha cramerella* (Amin *et al*., 2014). Entomopathogenic fungal endophytes are known to act against insect vectors of plant diseases (Gao *et al*., 2010) and induce antibiosis against transmitted pathogens, such that the vector and the pathogen might be suppressed (El-Deeb *et al*., 2012).

Little information is available on colonization of important staple crops by fungal entomopathogens under tropical environmental conditions. In the tropics, ambient temperature may be a limiting factor to their growth and establishment in the target crop. Borisade and Magan (2014) investigated the optimum and upper temperature boundaries for growth and sporulation of 19 strains of entomopathogenic fungi from the anamorphs of Hypocreales, *Beauveria bassiana, Isaria farinosa, Metarhizium anisopliae* and *I. fumosorosea* groups. The study showed that only a few isolates could grow at 35-37°C while the optimal temperature for growth and sporulation was 25-30°C and this was strain-dependent.

Failure of entomopathogenic fungi to achieve adequate control of pest population has been associated with interactions of abiotic factors; temperature and relative humidity (Sharififard *et*

*al*., 2012), which are often unfavourable in the field, particularly in the tropics. It is expected that future climate change scenarios will among other consequences lead to global temperature increase (a condition which may be more severe in the tropics) and the levels of success of biocontrol trials with fungi may be unpredictable (Borisade and Magan, 2014). Therefore, a method of delivery of conidia which would rely on optimal environmental factors for a short period of time to become established (strains with endophytic growth capabilities) may be more adaptable for use under marginal abiotic conditions. To become established as an endophyte, entomopathogenic fungal propagules may be applied to planting materials, plant foliage or directly to the rhizosphere to offer protection to crops against important entomological pests. Under such conditions, they grow internally in the plant to offer the required protection. The conidia, which are sensitive to abiotic interactions (temperature and water availability) are not involved in pathogenesis during endophytic growth after the fungi have established within the plant. For example, the mode of action of entomopathogenic fungi while growing as an endophyte has been suggested to be a toxin strategy rather than infection of insects by the fungal spores (Lewis *et al*., 1996).

Tefera and Vidal (2009) reported the potential of *B.bassiana* to establish as an endophyte in sorghum and the study showed that there was a significant reduction in stem borer attack without any negative consequences on the host. Similarly, Reddy *et al.* (2009) reported that *B. bassiana* colonized sorghum plants and incidents of stem borer tunnelling reduced significantly. In either of the studies, only a single fungal strain was examined. Thus, there is need for more information on other important members of this group of fungi.

The objectives of this study were to (a) screen 3 strains each of *B. bassiana* and *M anisopliae,* 2 stains of *I. farinosa* and a strain of *I. fumosorosea* for their potentials to colonise sorghum endophytically after application of conidia to the root of seedlings (b) evaluate the effect on growth characteristics of the host under ambient conditions in tropical agro-climate and (c) test virulence of conidia using *G.mellonella* bioassay system.

## MATERIALS AND METHODS

### Source of Fungi

The strains of *B. bassiana* and *M. anisopliae* were obtained from the International Institute of Tropical Agriculture (IITA), Republic du Benin, West Africa, and Dr. Dave Chandler (Warwick University, U.K.). The *I. farinosa* isolates and *I. fumosorosea* with previously reported potential virulence against insects were provided by the USDA Agricultural Research Service (ARSEF), USA. Table 1 shows the geographical origin and the host of the entomopathogens used in this study where known.

Table 1. Entomopathogenic Fungal Strains, Their Hosts (where known) and Geographical Origin.

| Entomopathogen | Strain | Host/ Substrate | Geographic origin |
|---|---|---|---|
| *B. bassiana* | BB 315 | Soil | Brazil |
| *B. bassiana* | BB 432.99 | Insect (Coleoptera) | United States |
| *B. bassiana* | BB776.05 | Insect (Coleoptera) | United Kingdom |
| | | | |
| *I. farinosa* | ARSEF 5081 | Insect (Aleyrodidae) | Pakistan |
| *I. farinosa* | ARSEF 2376 | Insect (Araneidae) | Philippines |
| | | | |
| *I. fumosoroseus* | IF 790.05 | Unknown | United Kingdom |
| | | | |
| *M.anisopliae* | V275 | Lepidoptera | Austria |
| *M.anisopliae* | Ma275.86DC (Ma 43) | * | * |

*Asterik: Fungal strain which is already registered as a bio-insecticide

**Fungal Culture and Preparation of Inoculum**

Agar plug of each strain was inoculated on Sabouraud Dextrose Agar (SDA) plate and incubated in the dark at 25 °C for 14 days. Thereafter, 10ml of sterile de-ionised water which contained 0.05 ml Tween-80 liter$^{-1}$ as a surfactant were poured on each plate and the conidia was gently dislodged with a drigalsky spatula. Conidia count was done using an improved Neubauer Haemocytometer under X400 objective of a compound microscope and the conidia suspension was standardized to 1.0 x $10^6$ spores ml$^{-1}$ by serial dilution.

**Planting of Sorghum and Application of Inoculum**

Nursery pots were filled with 900 g of homogenized loam soil and seeds of sorghum cv. KSV8 was planted into the nursery pots at the rate of four seeds per hole and maintained under natural daylight and darkness in screenhouse (Temperature = 28 ± 2 °C) . They were thinned to two seedlings per hole at 10 days after planting and water was applied to the plants as required. The rhizosphere of each of 10 day-old sorghum seedlings was inoculated with 5ml of 1.0 x $10^6$ conidia ml$^{-1}$ of each strain. The control experiment consisted of sorghum plants without inoculum. The design was a randomized block design with 6 replicates.

**Measurement of Length of Root and Stem**

The inoculated sorghum seedlings were irrigated daily to keep the soil moist and left to grow for additional 30 days during which the entomopathogens were expected to have established in the sorghum plants. Thereafter, 40ml sterile water was applied to the root area to soften the soil in order to allow uprooting of the plant without damage to the roots. The plants were rinsed in a stream of water and allowed to dry for one hour under ventilation at 30 °C. The shoot length was measured with a ruler from the base of the stem to the tip of the leaf while the shoot length was measured from the base of the stem to the tip of the longest main root.

**Assessment of Endophytic Colonization of Plant Parts**

The surface of all the plants to be assessed for endophytic colonization was sterilised by soaking in 3% hypochlorite (Hypo, No. CT-3282) for 3 minutes, followed by rinsing 2 times with sterile de-ionized water (Barnstead Thermo- Scientific, D11901). The plants were left to dry for one hour inside a Laminar flow cabinet (Airstream® LHC-3A1) and sprayed with 90% isopropanol solution (Sigma-Aldrich 197064) to achieve total surface sterilization. Randomly picked 20 plants were tested for sterility by appressing their leaves, stems and roots on prepared sterile Sabouraud Dextrose Agar (SDA, Oxoid Ltd. CM0041). The SDA plates were sealed with Parafilm and incubated for 8 days at 25 °C in alternating light and darkness (12 hr: 12 hr). There was no growth observed on the SDA plates after this period; an indication that the plant surfaces were sterile. Transverse sections were cut through roots, stems (5 cm up from the base of the stem) and leaves of all the uprooted sorghum using sterilized surgical blade (Swann-Morton B.S. 2982 I.S.O. 7740). The size of the root, stem and leave portions were 2-3 cm long. The blade was dipped in 70% isopropanol and flamed after each cut of the plant parts. The cut surface of each plant part was placed in separate Petridishes containing SDA media which was modified with 0.5mg liter$^{-1}$ chloramphenicol. Chloramphenicol was added to the SDA media to prevent bacteria growth. The control plant was similarly prepared and the concept was that, any growth in the control would render the result of the experiment invalid. The plates were sealed with Parafilm and incubated at 25 °C for 7-10 days during which fungal outgrowths from the plant tissues were observed. Presence or absence of endophytes were either scored as 'yes' (++) or 'no' (-) and frequencies of detection were recorded. The plates were left for additional 8 days to allow for sporulation and subsequent morphological examination of the growing fungi. A wet mount of the fungi which had grown out of the plant tissues was observed under a microscope and identification was done as described by Samson *et al.* (1988) to ascertain that the morphology was consistent with that of the entomopathogen which was used for inoculation. The experiment on the ability of the entomopathogen to colonize the sorghum plants and growth responses was repeated twice in space and time and the results of the two trials were combined (pooled).

**Evaluation of Virulence of Conidia**

Initial conidia (primary conidia) which were harvested from 14 days old SDA culture were assessed for virulence using *Galleria* model bioassay system. Thereafter, conidia of the isolates which grew out of sorghum (secondary conidia) were washed from the surface of SDA plates with sterile de-ionized water containing 0.05% Tween-80 and standardized to 1.0 $x10^6$ conidia $ml^{-1}$. These were used to assess virulence of the secondary conidia to confirm that the endophytes were indeed entomopathogenic. The post-endophytic fungal outgrowths were not sub-cultured before they were assessed for virulence. The *Galleria* bioassay was performed using 9 cm Petridish with 1 $cm^2$ ventilation hole on the lid. A Whatman filter paper was placed in each dish and ten *G. mellonella* larvae per Petridish was used. The ventilation hole was covered with muslin cloth which was held in place with a paper tape to prevent egress of the larvae. Each of the larva was dipped in the conidia suspension for 1-2 seconds and placed on the filter paper in the Petridish after which the lid was replaced. The function of the filter paper was to absorb excess water on the larvae to prevent death which may result from asphyxiation due to spiracle blockage. The control larvae were equally dipped in sterile de-ionized water containing 0.05% Tween-80. The set-up was replicated 3 times and Petridishes containing the inoculated larvae and the control were arranged separately inside plastic boxes (30 x 30 x 25 $cm^3$ volume). The lid of each plastic box had a ventilation hole (5 x 5 $cm^2$) which was lined with muslin cloth to allow for aeration of its contents. Two beakers each containing 500 ml sterile de-ionized water was placed inside each box for equilibration (Equilibrium relative humidity, ERH>98%) which may be necessary for fungal infection. The set-up was kept at 25°C in the dark and cumulative mortality of the larvae was recorded every 24 hours for 6 days. The bio-assay was a single dose experiment and virulence of the conidia was measured by the Median Lethal Time ($LT_{50}$) at 25ºC.

**STATISTICAL ANALYSIS**

The data on plant growth characteristics were subjected to Analysis of Variance (ANOVA) procedure. Where significant differences were found, means were separated using Tukey's Honestly Significant Difference at P=0.05. Data on virulence of fungal conidia to *G. mellonella* was corrected for natural mortality (Abbott, 1925).Thereafter, a graph of the probit values of the percentage cumulative mortality against time was plotted and the $LT_{50}$ was determined from the regression equation of the curve. Data analysis was done using the IBM-SPSS 21 statistical software and graphs were plotted with MS Excel 2010.

**RESULTS**

**Parts of Sorghum with Endophytic Growth and Frequency of Detection**

All the tested isolates and strains colonized various parts (Figure 1) of the sorghum plants 20 days post-inoculation at varying degrees. *Beauveria bassiana* and *Isaria* strains were detected in the roots, stems and leaves while *M. anisopliae* was confined to the root (Figure 2). The variability in the frequency of detection in different parts of the plant was strain-dependent. BB 776.05 was detected in the roots of all the plants inoculated, while 80 and 75% of stems and leaves examined were colonized. BB 315 had the highest percentage of colonization of the root, stem and leaf among the *B. bassiana* isolates. *Metarhizium anisopliae* strains, V275 and Ma 275.86DC respectively colonized 83 and 75% of the roots of the inoculated plants.

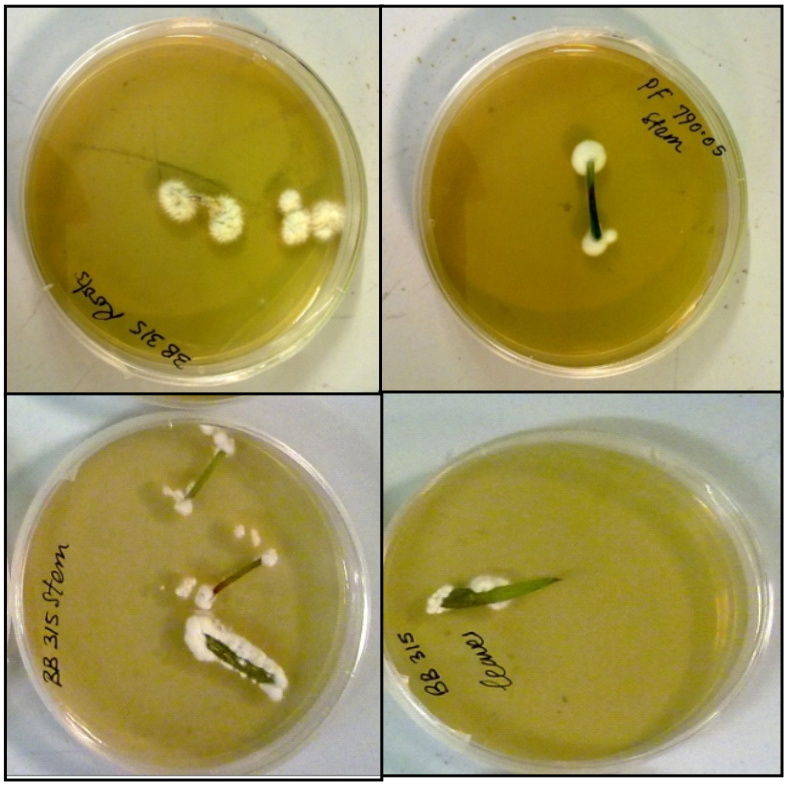


**Figure 1.** Entomopathogenic Fungi Growing out of Sorghum Plant Tissue, 20 days Post Inoculation

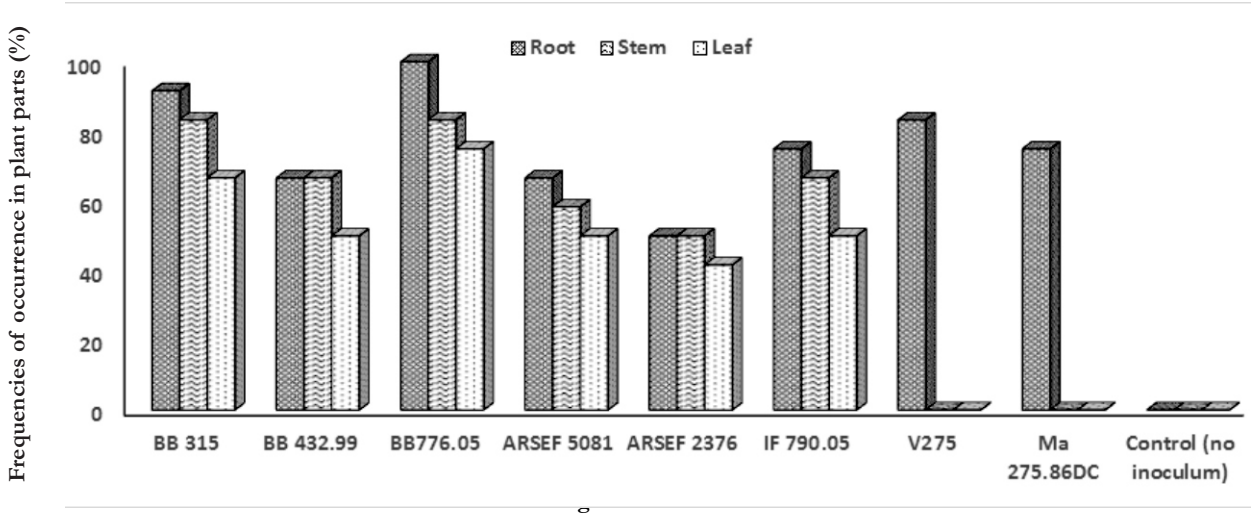


**Figure 2.** Frequencies of Detection of Entomopathogenic Endophytes in Plant Parts, 20 days Post Inoculation.

**Effect of Endophytes on Development of Root**

Endophytic colonization of the plant had no significant effect on the length of the roots compared with the control ($F_{8, 96}$=1.59, P=0.138; Figure 3). However, visual examinations showed that development of adventitious roots in the plants inoculated with the strain of *B. bassiana* (432.99), *Isaria* (5081), *I. fumosorosea* (790.05) and the two strains of *M. anisopliae* (V275 and 275.86) was poor.

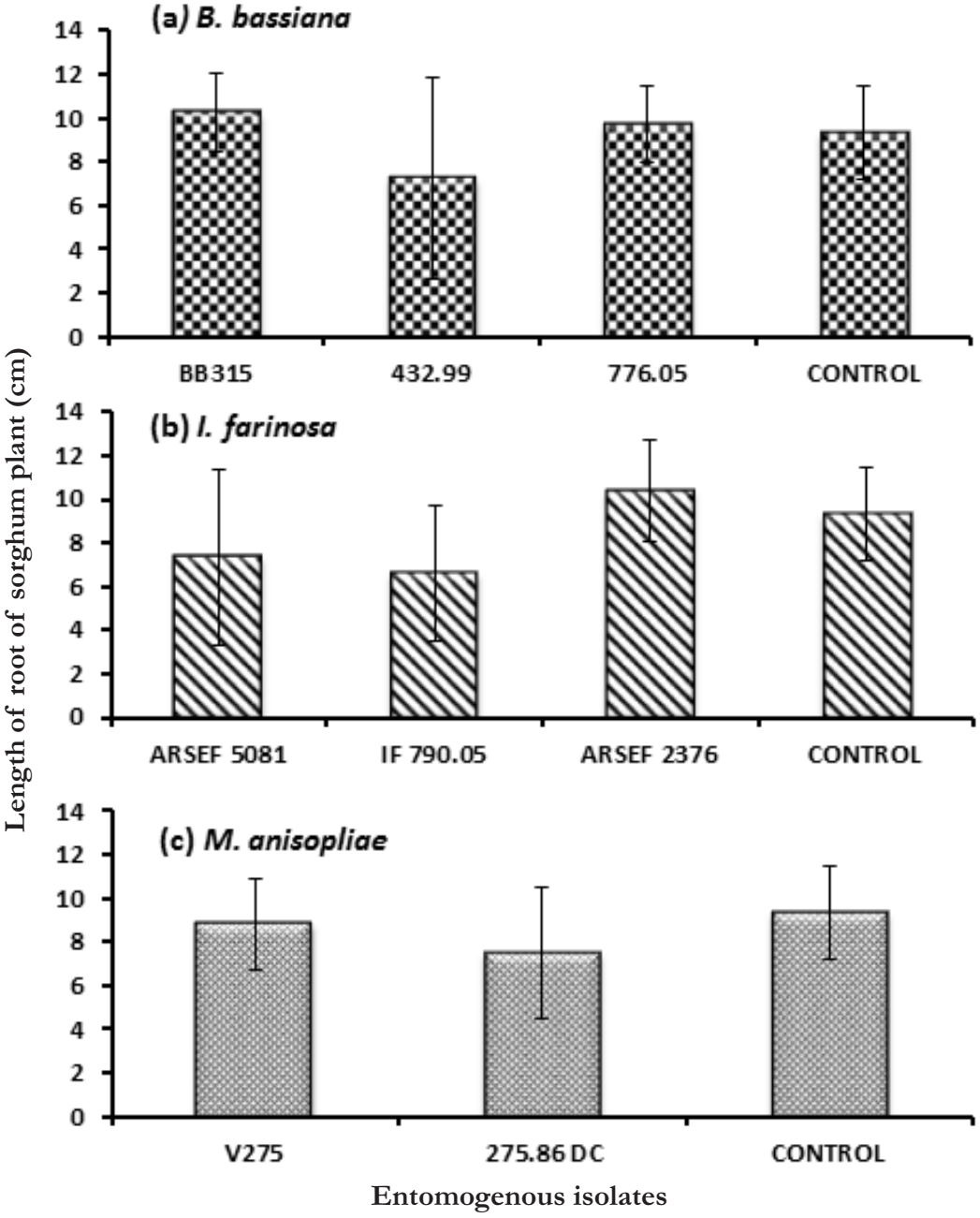


**Figure 3**. Effect of Endophytic Colonization on Length of the Root of Sorghum Plants. The bars are standard error of the means.

**Effect of Endophytes on Development of Stem**

There were variabilities in shoot development (Figure 4) among the plants inoculated with conidia of the different strains. The length of the stem in some of the treatments were significantly different from the control (F 8, 96=2.649, P=0.011). *Beauveria bassiana,* BB 315 increased

sorghum growth as the stems of the treated plants were significantly longer than those of the untreated control. In contrast, *B. bassiana,* BB 432.99 significantly reduced the plant growth. A similar significant reduction in growth was observed in the plants treated with *I. farinosa* (ARSEF 5081). The plants which were inoculated with the conidia of this strain of *Isaria* were the shortest of all the treated plants. The two strains of *M. anisopliae* had no significant effect on the shoots compared with the control.

**Evaluation of Virulence of Conidia**

Figure 5 compares pathogenicity of conidia of the fungi which grew out of the sorghum plant (secondary conidia) and the primary conidia to *G. mellonella* larvae. The secondary conidia of all the isolates were less virulent after the endophytic phase of their growth in sorghum except for the 2 strains of *B. bassiana* (BB 315 and 776.07) where virulence was not significantly affected ($p>0.05$). The $LT_{50}$ of the primary conidia of *Metarrhizium* strains, 275.86DC and V275 were 3.2 and 2.2 days respectively. However, the $LT_{50}$ of their secondary conidia respectively increased to 4.1 and 3 days. The $LT_{50}$ of the primary conidia of the strain of *I. farinosa,* ARSEF 2376 was 11 days and this also increased to 14 days after the endophytic growth phase.

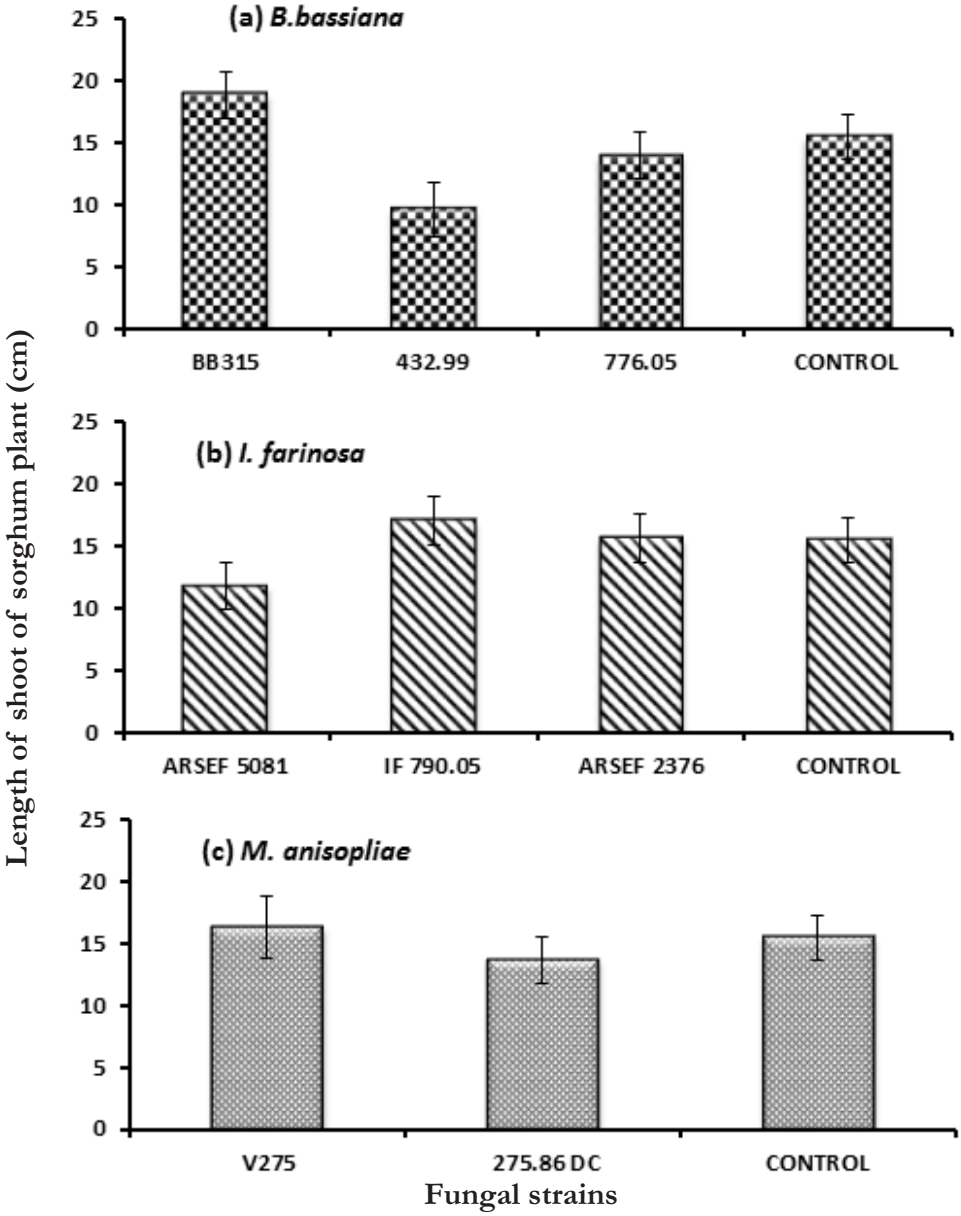


**Figure 4.** Effect of Endophytic Colonization on Length of Shoot of Sorghum Plants. Bars are standard errors of the means.

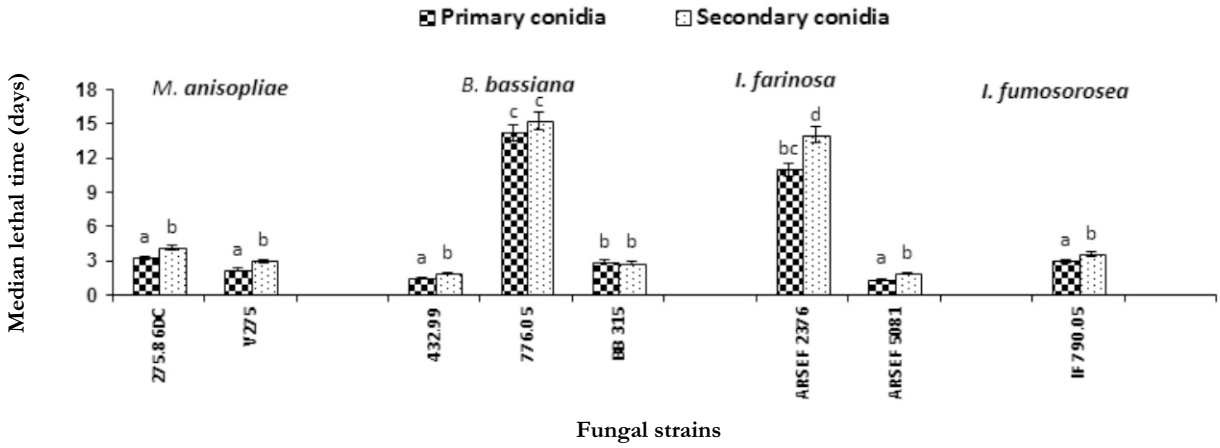


**Figure 5.** Comparison of Virulence of Conidia to *Galleria mellonella* Before and After Endophytic Growth Phases in Sorghum. Bars are standard error of the means where shown.

## DISCUSSION

The nine isolates of the entomopathogenic fungi tested in this study grew as endophytes in sorghum when the root region was inoculated with the conidia and there were variabilities in their endophytic colonization rates. At 20 days post inoculation of the roots, the growth of the two strains of *M. anisopliae* was confined within the roots. In contrast, both *Beauveria* and *Isaria* strains ramified throughout the plant tissues as they were detected in the roots, the stems and the leaves.

The extent of colonization of plants by entomopathogenic fungi has been reported to vary between different plant species and different species of fungi (Vega *et al.,* 2009) Entomopathogenic fungi with a potential to colonize all plant parts when applied to the roots may be especially useful in seed dressing for the management of sap sucking and disease transmitting aleyrodid pests such as *Bemisia tabaci*- the vector of mosaic virus in cassava and other important tropical crops. Endophytic growth may induce antibiosis against plant pathogens and initiate host preference behaviours to the vector (El-deeb *et al*., 2012). However, strains with such capabilities may require a significant risk assessment before their use in the protection of edible crops such as fruits and vegetables.

In terms of the effect of colonization on growth, none of the isolates significantly affected the development of the roots of the plant but *B. bassiana*, isolate 315 significantly influenced growth. It is interesting that the two isolates of *B. bassiana* (432.99) and *I. farinosa* (5081) which were associated with poor root development caused significant reduction in stem growth. Several agronomic studies have shown positive correlations between root development and growth rates in several crops (Nand, 2012). However, the influence of different entomopathogenic endophytes on ability of crops to uptake and utilize specific micro- or macro-nutrients have not been well reported. For example, zinc is a micronutrient which is especially necessary for root development and the effect which an endophyte may exert on its uptake and utilization would affect overall plant performance *in vitro*. Chemical and organic fertilizers can enhance plant development much more and the residual effect of endophytic colonization by entomopathogen may disappear in a short while in the field.

Khan *et al.* (2012) reported that a strain of *M anisopliae* reprogrammed soya bean to higher growth and improved tolerance to salt stress. Similarly, Elena *et al.* (2011) tested a strain of *M. anisopliae* in tomato plants and found a significant improvement in the length of the root and the shoot as well as higher dry weight of the plant biomass. *Metarhizium anisopliae* has also been

reported to significantly increase the yield of corn when applied for the control of wireworm (Kabaluk and Ericsson, 2007).

The effect which passage of the strains through endophytic phase of growth had on virulence of the secondary conidia was strain-dependent. Two strains of *B. bassiana* retained their virulence while *M. anisopli*ae, *I. farinosa* and *I. fumosorosea* were attenuated. Growth substrate has been shown to affect virulence of entomopathogenic fungal conidia (Safavi *et al.,* 2007) but the effect of endophytic growth phase on virulence of secondary conidia has not been well reported.

There are contrasting results on the effect of endophytic colonization of plants by entomopathogenic fungi in relation to plant growth characteristics. Screening of fungi for endophytic colonization potentials and the effect that such interactions may have on a particular crop are important. For example *M. anisopliae* strains which are more adaptable to the rhizosphere and confined within the plant roots may be especially useful for the management of root pests, while isolates with extensive endophytic colonization capabilities, such as the *Beauveria* and the *Isaria* strains being reported in this study may be more adaptable to the management of stem borers which are very important pests in sorghum.

## CONCLUSIONS

This study has demonstrated variabilities in the rate of colonization of sorghum endophytically by different isolates and strains of entomopathogens under tropical ambient conditions. This suggests that strains can be carefully selected and tested for biocontrol efficacy against specific pests in further studies. The results of this study showed that entomopathogens have great potentials in managing sorghum pests.

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