Journal of Biological Control, 32(3): 165-171, 2018, DOI: 10.18311/jbc/2018/21580

Research Article

Establishment of *Metarhizium anisopliae* (Metchnikoff) sorokin as endophyte in maize and sorghum

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ABSTRACT: Glasshouse experiments were conducted to establish an indigenous soil-derived strain of *Metarhizium anisopliae* (ICAR-NBAIR Ma-35) as endophyte in maize and sorghum by artificial inoculation through foliar spray of conidial suspension. Colonization of *M. anisopliae* were studied at 15, 30, 45, 60, 75 and 90 Days After Treatment (DAT) in stem and leaf tissues of maize and sorghum by plating technique and PCR method. *Metarhizium anisopliae* showed variation with regard to the extent of colonization in stem and leaf tissues of maize and sorghum. In maize, colonization was observed at 30DAT in stem and 30-45DAT in leaf. In sorghum, colonization of *M. anisopliae* was observed during 15-60DAT in stem and 15-75DAT in leaf indicating longer persistence compared to maize. Colonization of *M. anisopliae* was observed in young growing stem tips and leaves (unsprayed) indicating the internal spread of *M. anisopliae* in maize and sorghum plants. *M. anisopliae* was not detected in the untreated stem and leaf tissues of maize and sorghum. The positive results of colonization of *M. anisopliae* in maize and sorghum stem/leaf tissues observed in plating technique were confirmed by the PCR amplification.

KEY WORDS: Endophyte, entomopathogenic fungi, maize, *Metarhizium anisopliae*, sorghum

(Article chronicle: Received: 26-07-2018; Revised: 12-09-2018; Accepted: 25-09-2018)

INTRODUCTION

Metarhizium anisopliae (Ascomycota: Hypocreales) is one of the extensively researched entomopathogenic fungus for biological control of insects pests through augmentative application. Successful control of insect pests through augmentative applications of entomofungal pathogens is highly dependent on the prevalence of favourable environmental conditions like moderate temperatures and high humidity, lack of which result in failure of pest management. Recently *M. anisopliae* also found to occur naturally as endophyte in maize (Akello, 2012); soybean (Khan *et al.,* 2012) and has also been established as an endophyte by artificial inoculation through seed treatment, soil drench and foliar application in certain crops like, tomato (Elena *et al.,* 2011; Garcia *et al.,* 2011), maize (Akello, 2012; Kabaluk and Ericsson, 2007), soybean (Khan *et al.,* 2012), cabbage (Razinger *et al.,* 2014), sorghum (Mantzoukas *et al.,* 2015), common bean (Mutune *et al.,* 2016; Parsa *et al.,* 2016, 2018), broad bean (Akello and Sikora, 2012), rapeseed (Batta, 2013), cassava (Greenfield *et al.,* 2016) and tea (Kaushik and Dutta, 2016). *M. anisopliae* strains with the ability to establish as endophyte in crop plants are more advantageous than non-endophytic strains as they are inside the plant system, protected from abiotic stress factors like temperature and humidity leading to season long protection against insect pests and are cost effective because of limited application. Endophytic strains of *M. anisopliae* are reported to offer protection against *Plutella xylostella* on *Brassica napus* (Batta, 2013). In maize, an endophytic strain of *M. anisopliae* derived from maize plants was established as endophyte by seed treatment and found to reduce 30% of leaf damage caused by stem borer, *Chilo partellus* in Kenya (Akello, 2012). In sorghum, *M. robertsii* was established as endophyte by foliar application and caused 70-100% larval mortality of stalk borer, *Sesamia nonagrioides* in Greece (Mantzoukas *et al.,* 2015). Kabaluk and Ericsson, 2007 reported that, maize seeds treated with conidia of *M. anisopliae* resulted in significant increase in yield may be due to wireworm control in Canada.

Maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) are important cereal crops as a human food, animal feed as well as fodder. In India, maize occupying nearly 37 percent of area under cultivation with the annual production of 66,192 tonnes (Anon, 2011) and sorghum is about 61.61 lakh ha with an annual production of 54.45 lakh tonne. Stem borers like, *Chilo partellus* Swinhoe (Crambidae: Lepidoptera) and *Sesamia inferens* Walker (Noctuidae: Lepidoptera) are important pests of maize and sorghum in India. The crop losses caused by these pests ranges from 24

to 83 percent in maize (Sarup *et al.,* 1987, Sekhar *et al.,* 2008) and 18–53% yield losses in sorghum (Gethi *et al.,* 2001). The chemical insecticides were mainly used for the management of these borer pests. Control of these borer pests by chemical insecticides is extremely difficult because of its cryptic life cycle, expensive and has adverse effects on environment as well as human health. Hence there is a need for development of an alternative, safe protection technology using endophytic entomofungal pathogens.

In India, the entomopathogenic fungus, *M. anisopliae* has not been exploited till date as an endophyte in maize and sorghum for management of stem borers. At ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, Karnataka, India, an excellent collection of *M. anisopliae* strains (65) from insect/soil from different agro-climatic zones of India was made and characterized. A soil derived strain of *M. anisopliae* (ICAR-NBAIR Ma-35) from these collections was found to cause 90% mortality of maize and sorghum stem borer, *C. partellus* in the laboratory bioassay studies (Ramanujam *et al.,* 2015). The present study was undertaken to determine the ability of this isolate of *M. anisopliae* (ICAR-NBAIR Ma-35) to colonize the stem and leaf tissues of maize and sorghum and establish it as an endophyte by artificial inoculation.

MATERIALS AND METHODS

Glasshouse experiments were conducted to establish *Metarhizium anisopliae* as endophyte in stem and leaf tissues of maize and sorghum.

Maize and sorghum plants

Seeds of maize (Var. Nithyashree) and sorghum (Var. Maldandi M-35) extensively cultivated in Karnataka region were selected for the study. Seeds were surface sterilized initially with sodium hypochlorite (3%) for three minutes and later with ethanol (70%) for two minutes, rinsed in sterile distilled water for three times and sown in plastic pots containing sterile sandy loam soil (autoclaved at 121°C for 20min) (Tefera and Vidal 2009). The plants were maintained in the glasshouse at 25 to 28°C, 60 to 80% RH, with a 12-h photoperiod and irrigated regularly.

Fungal culture

Promising isolate of *Metarhizium anisopliae* (ICAR-NBAIR Ma-35) collected from ICAR-NBAIR culture repository was used in the study. Conidia of this isolate was produced on 100gms of sterilized rice grains taken in polypropylene bags (25x30cm) by inoculating 10ml of 4 days old shaker culture and incubated at 26 ± 1^0C for 15 days. Conidial suspension was prepared by suspending one gram of conidiated rice in sterile distilled water with 0.01% Tween 80 (0.01ml/100ml). The suspension was filtrated through three layers of muslin cloth to get hyphal-free conidial suspension. The concentration of the conidia in the suspension was adjusted to $1x10^8$ spores/ml using Neubauer's improved haemocytometer.

Foliar application

The conidial suspension of ICAR-NBAIR-Ma-35 isolate was sprayed on the maize and sorghum seedlings (1 X 10^8 conidia/ml; 5 ml/seedling) at 15 and 30 days after germination. The control plants were sprayed with sterile distilled water with 0.01 % Tween 80. Top of the each pot was covered with aluminum foil to avoid conidial contact with soil.

Studies on colonization of *Metarhizium anisopliae* in stem and leaf tissues of maize and sorghum

Colonization of *M. anisopliae* in stem and leaf tissues of maize and sorghum were studied at different sampling periods (15, 30, 45, 60, 75 and 90 Days After Treatment (DAT) after first spray) using plating technique (re-isolation) and by PCR method.

Plating technique

At each sampling period, three plants each from treated and untreated control were uprooted randomly and washed thoroughly in tap water. From each plant, two older leaves (sprayed leaves) and two young leaves (unsprayed leaves) and two pieces of older stem (sprayed stem) and two growing tips of the stem (emerged after spray) were used for plating. Stem and leaf samples were surface sterilized with sodium hypochlorite (1%) for 3 minutes, ethanol (70%) for 30 seconds and then rinsed in sterile distilled water for 3 times and dried on sterile blotting paper for three minutes in a laminar flow. The surface sterilized sample parts of maize and sorghum were cut into small sections of 5mm x 5mm (length x width) from treated and untreated control and were placed on Sabouraud's Dextrose Yeast extract Agar (SDYA) medium (Dextrose 40g, Mycological peptone 10g, yeast extract 5g, agar 20g in 1L of distilled water) containing 0.1g penicillin, 0.2g streptomycin sulphate, 0.25g chloramphenicol and 0.05g tetracycline to avoid bacterial contamination. The final rinsed water (0.1ml) was also plated on the SDYA plates to check the effectiveness of surface sterilization. The plates were then incubated at 26 ± 1ºC for seven days for the development of fungal growth. The fungal growth from the plated stem and leaf bits were examined under light microscope for confirmation of *M. anisopliae* growth.

Molecular detection using PCR

DNA was extracted from the surface sterilized old and young maize and sorghum stem/leaf samples (both treated as well as untreated) at 15, 30, 45, 60, 75 and 90 DAT by CTAB method (Cetyl Trimethyl Ammonium Bromide) (Hi-media instruction manual).

The DNA samples of maize and sorghum old and young stem and leaf tissues (both treated as well as untreated) at 15, 30, 45, 60, 75 and 90 DAT were assessed for PCR using *M. anisopliae* specific primer ITS Met: 5' TCTGAATTTTTTATAAGTAT 3' with ITS4 (5' TCCTCCGCTTATTGATATGC 3') as reverse primer (Destéfano *et al.,* 2004; Bechara *et al.,* 2011) to amplify the target DNA Sequence of *M. anisopliae.* The PCR mixture consisting of 50ng DNA, 10x Taq buffer with 2.5 mM $MgCl_2$ was prepared, 1.25mM of each dATP, dGTP, dTTP, dCTP, 10pmol of Forward and Reverse primer, 3 units of Taq DNA polymerase and made upto 50µl volume with sterile de-ionized water. The DNA amplification was carried out using a thermocycler (BioRad) with a program: initial denaturation at 95ºC for 5min, followed by 35 cycles of denaturation at 94ºC for 1min, annealing at 48ºC for 1min, extension at 72ºC for 1min, and final extension at 72ºC for 10min and stored at 4ºC. The PCR products were then visualized in 1.4% agarose gel stained with ethidium bromide. The molecular weight of the amplified fragment size was calculated (Ling *et al.,* 1987).

RESULTS AND DISCUSSION

Plating technique

The old and young stem and leaf bits having *Metarhizium anisopliae* colonization showed yellowish white hyphal growth from the margins of the bits and these fungal growths when examined under light microscope showed typical conidophores, phialides and conidia of *M. anisopliae*. No such fungal growth was observed from the old and young stem and leaf bits of untreated control.

Table 1. Colonization of *Metarhizium anisopliae* ICAR-NBAIR-Ma-35 in old and young stem/leaf tissues of Maize

Days after treatment	Maize			
	Old stem	Young stem	Old leaf	Young leaf
15DAT	-	-	-	-
30DAT	+	+	+	+
45DAT	-	-	+	+
60DAT	-	-	-	-
75DAT	-	-	-	-
90DAT	-	-	-	-

- indicates no colonization
+ indicates colonization

Maize

Colonization of *M. anisopliae* was observed in old and young stem tissues at 30DAT. In old and young leaf tissues, colonization was observed at 30 DAT and at 45DAT. No colonization of *M. anisopliae* was observed after 45 DAT (Table 1). In the untreated stem and leaf tissues, no colonization of *M. anisopliae* was observed (Figure 1).

Sorghum

Colonization of *M. anisopliae* was observed in old and young stem tissues at 15, 45 and 60DAT. In old leaf tissues, colonization was observed during 15-75DAT and in young tissues colonization was observed in 15, 60 and 75DAT respectively. No colonization of *M. anisopliae* was observed after 75 DAT (Table 2). In the untreated stem and leaf tissues, no colonization of *M. anisopliae* was observed (Figure 2).

PCR amplification

The positive results of colonization of *M. anisopliae* in old and young stem and leaf tissues observed in plating technique were confirmed by the PCR amplification. The genomic DNA extracted from treated stem and leaf tissues of maize and

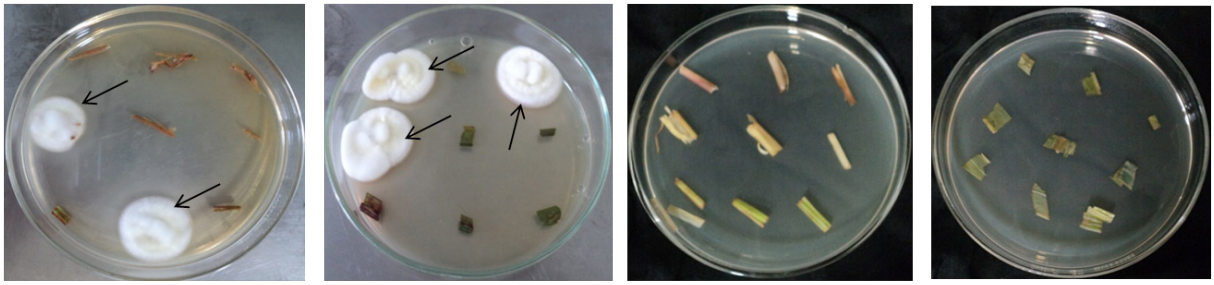

Fig. 1. A. Stem bits from maize treated with *Metarhizium anisopliae* (Ma-35) showing growth of the fungus; B: Treated maize leaf bits showing Ma-35 growth; C: No growth of Ma-35 in untreated maize stem bits; D: No growth of Ma-35 in untreated maize leaf bits.

Table 2. Colonization of *Metarhizium anisopliae* ICAR-NBAIR-Ma-35 in old and young stem/leaf tissues of sorghum

Days after treatment	Sorghum			
	Old stem	Young stem	Old leaf	Young leaf
15DAT	+	+	+	+
30DAT	-	-	+	-
45DAT	+	+	+	-
60DAT	+	+	+	+
75DAT	-	-	+	+
90DAT	-	-	-	-

- indicates no colonization
+ indicates colonization

sorghum showed amplification at 450bp with specific primer. The genomic DNA extracted from untreated tissues (control) failed to amplify any PCR product indicating that specific primer did not bind to any region of sorghum and maize plant genome. The amplification observed during different sampling periods (15, 30, 45, 60, 75 and 90 DAT) indicated the presence or absence of *M. anisopliae* in the old and young stem and leaf tissues of maize and sorghum (Figure 3 and 4).

DISCUSSION

The results of the present study indicated the ability of a soil-derived indigenous strain of *Metarhizium anisopliae* to colonize stem and leaf tissues of maize and sorghum and establish as endophyte when applied through foliar spray. Similar results of confirmation were observed in plating technique and PCR method. Stem and leaf samples from untreated control plants of maize and sorghum did not show the presence of the test strain in plating technique as well as in PCR study. No symptoms of physical damage were observed in the *M. anisopliae* treated plants of maize and sorghum. Akello, 2012 reported that, colonization of *M. anisopliae* (an endophytic strain derived from maize plants) in maize stem and leaf by artificial inoculation through seed treatment in Kenya.

Metarhizium anisopliae showed variation with regard to the extent of colonization in stem and leaf tissues of maize and sorghum. In maize, colonization was observed at 30DAT in stem and 30-45DAT in leaf. Colonization of *M. anisopliae* for a period of one month was reported in maize stem and leaf with seed treatment by Akello, 2012. In sorghum, colonization of *M. anisopliae* was observed during 15-60DAT in stem and 15-75DAT in leaf indicating longer persistence compared

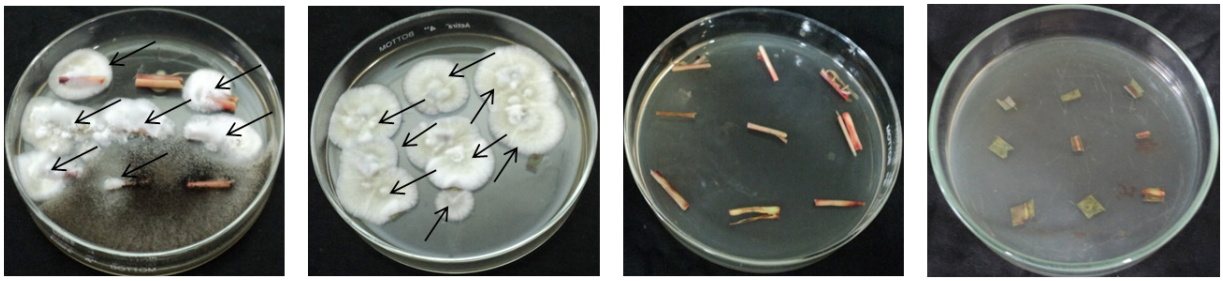

Fig. 2. A: Stem bits from sorghum treated with *Metarhizium anisopliae* (Ma-35) showing growth of the fungus; B: Treated sorghum leaf bits showing Ma-35 growth; C: No growth of Ma-35 in untreated sorghum stem bits; D: No growth of Ma-35 in untreated sorghum leaf bits.

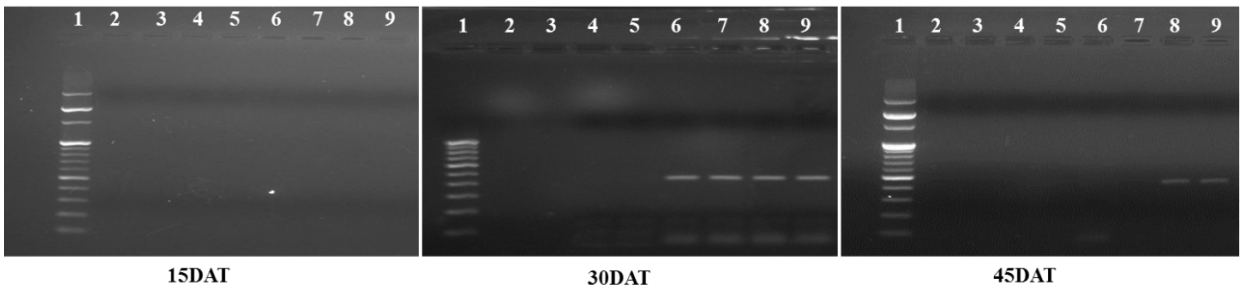

Fig. 3. PCR amplification of genomic DNA extracted from the *Metarhizium anisopliae* (Ma-35) treated and untreated control old/young stem and leaf tissues of maize. Lane 1-9: 1- 100bp ladder, 2- Control old stem, 3- Control young stem, 4- Control old leaf, 5- Control young leaf, 6- Ma-35 old stem, 7- Ma-35 young stem, 4- Ma-35 old leaf, 5- Ma-35 young leaf.

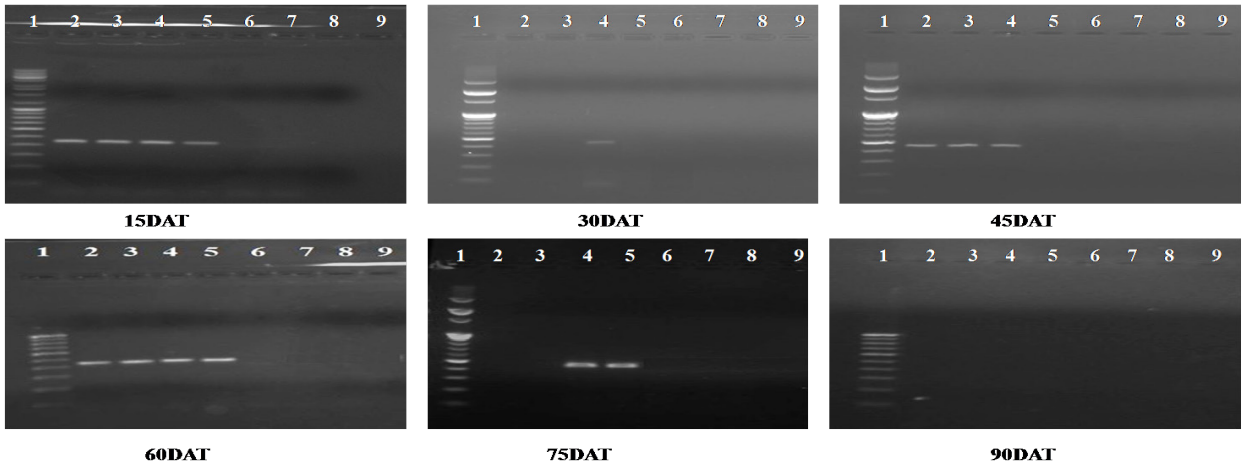

Fig. 4. PCR amplification of genomic DNA extracted from the *Metarhizium anisopliae* (Ma-35) treated and untreated control old/ young stem and leaf tissues of sorghum. Lane 1-9: 1- 100bp ladder, 2- Ma-35 old stem, 3- Ma-35 young stem, 4- Ma-35 old leaf, 5- Ma-35 young leaf, 6- Control old stem, 7- Control young stem, 8- Control old leaf, 9- Control young leaf.

to maize. The variations in colonization of *M. anisopliae* in maize and sorghum tissues may be due to the differential growth rates and ability to adjust to the nitch areas of the host plant species (Akutse *et al.,* 2013; Biswas *et al.,* 2013) and already associated endophytic fungi and bacteria of the host plant (Brownbridge *et al.,* 2012). Landa *et al.,* 2013 also reported that fungal colonization in the plant tissues was not uniform. Colonization of *Metarhizium robertsii* was reported in stems and leaf tissues of sweet sorghum upto 30 days after foliar application in Greece (Mantzoukas *et al.,* 2015).

In the present study, we have observed colonization of *M. anisopliae* in young growing stem tips and leaves (unsprayed) in addition to the old stem/leaves (Sprayed) indicating the internal spread of *M. anisopliae* in maize and sorghum plants. The colonization observed in the growing stem and leaf tissues may be due to translocation ability of the strain by passive transport within the xylem or through internal growth spread (Bing and Lewis, 1991). Light microscopy and Scanning Electron Microscopy (SEM) studies by Kaushik and Datta (2016) indicated the spread of *M. anisopliae* through intercellular spaces and vascular bundles in tea plant tissues. *M. anisopliae* also established as endophytes in oilseed rape (Batta, 2013), tomato (Dutta *et al.,* 2015) and tea (Kaushik and Datta, 2016) by foliar application.

CONCLUSION

Metarhizium anisopliae was established as endophyte in the maize and sorghum stem and leaf tissues. This study provides the basis for further investigations to exploit the endophytism of *M. anisopliae* for management of cryptic pests like stem borers (*C. partellus* and *Sesamia inferens*) of maize and sorghum.

ACKNOWLEDGMENTS

The authors are thankful to ICAR for financial support throughout the research and to the Director, ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bangalore, Karnataka, India for providing facilities for undertaking the work.

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