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Tracing an inoculated arbuscular mycorrhizal fungus, *Funneliformis mosseae,* in a field experiment using molecular tools

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Abstract

The aim of this study was to assess the presence of inoculated AM fungus *Funneliformis mosseae (*earlier called *Glomus mosseae)*, applied as part of the microbial consortia *(*MC*)* in the root zone of chilly *(Capsicum annuum* L.*)* plants from the field experiment through nested PCR based approach. A field experiment was conducted with 100% recommended level of chemical fertilizers and 50% recommended level of chemical fertilizers plus selected microbial consortia i.e., *Funneliformis mosseae* + *Bacillus sonorensis* in order to find out the possibility of reducing the recommended level of chemical fertilizer for cultivation of chilly. The introduced inoculum *Funneliformis mosseae* was tracked from the field by amplifying a region of rRNA gene using specific primers followed by sequencing. Total DNA was extracted from the roots of chilly plants. The universal eukaryote primer pair NS5/ITS4 was initially used for the first amplification and further amplified by Glomeraceae specific primer GLOM1310, in conjugation with the universal primer ITS4i. The amplicon was obtained only from plant root inoculated with microbial consortia and its sequence validated the presence of *F. mosseae.*

Key words – AM fungus – Chilly – Nested PCR

Introduction

Arbuscular mycorrhizal fungi **(**AMF**)** are obligate biotrophs, known to play an important role in sustainable agriculture**.** AMF involve in plant nutrient acquisition by the spread of their hyphae into the root zone soil and colonize within plant roots**.** In recent years, much effort has been dedicated to finding suitable formulations for AM fungal propagules and appropriate means for their application to the field **(**Gianinazzi & Vosatka 2004**)**. Given the cost of producing, and the effort of applying, AM fungal inocula on a large scale, it is important to seek ways to verify whether inoculated AM fungi establish functional symbioses, and whether they contribute to yield improvements. Molecular identification of AMF in plant roots would clearly prove the presence, establishment, and persistence of AMF inoculants in the field**.** Polymerase chain reaction **(**PCR**)** method using AM fungi-specific and its taxon-specific primers has been well developed to detect AM fungal DNA in roots**.** AMF have traditionally been identified and classified by the morphological features of the asexually produced spores**.** Spores of very similar morphologies are now known to be produced by

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phylogenetically distant AMF (Walker et al. 2007). Several biases are implicit to this approach, namely the overestimation of the importance of prolific spore producers and on the other side the non-detectability of others that scarcely form spores. Another drawback of morphologically monitoring AMF by their resting spores (Oehl et al. 2005, Wang et al. 2008) is that the presence of spores may not reflect a symbiotically active community of the fungi**.** Molecular studies have also shown that spore population in the root zone soil do not always reflect the AM fungal communities present in roots (Clapp et al. 1995, Rosendahl & Stukenbrock 2004).

Molecular methods have been developed to avoid these potential problems, which allow the detection of AMF within colonized roots. During the past decade, PCR based molecular methods and DNA sequencing has been routinely used to identify AMF. Prior to availability of molecular tools presence of inoculated AMF could only be assessed via microscopic measurements of root and soil colonization and via biometric and nutritional analyses of the inoculated host plants (McGonigle 1988, Lekberg & Koide 2005). However, the molecular genetic tool box now enables highly specific and sensitive tracing of AMF in any type of environmental sample (Helgason et al. 1998, Redecker 2000, Krüger et al. 2009) by either sequencing (Opik et al. 2009, Jumpponnen et al. 2010, Tedersoo et al. 2010) or molecular fingerprinting (Vandenkoornhuyse et al. 2003, Gollotte et al. 2004) or a mixture of both (Mathimaran et al. 2007, Borstler et al. 2010, Kivlin & Hawkes 2011).

Simon et al. (1993) designed primers to identify AMF in colonized roots by PCR amplification of the 18S rDNA combined with the single-stranded conformation polymorphism analysis. Subsequently, Simon and Lalonde (Simon & Lalonde 1995) designed the primer VANS1, which amplified part of the 18S rDNA from AMF (*Glomus intraradices* and *Gigaspora margarita*) directly from colonized roots. Helgason et al. (1998) designed the general fungal primer AM1 which could be used to detect AMF inside colonized plant roots. Redecker (2000) designed some specific PCR primers to identify divergent clades of AMF within colonized roots. These primers targeted at five major phylogenetic subgroups of AMF (*Glomus, Acaulospora, Entrophospora, Scutellospora* and *Sclerocystis*). The primers also could facilitate specific amplification of ITS and 18S rDNA fragments from colonized roots in the absence of spores. Krüger et al. (2009), Lee et al. (2008) had developed universal AMF primer set which allowed us to detect AMF from both soil and inside plant roots. Recently, RNA Polymerase II has been introduced as new marker gene to study the AMF communities (*Stockinger* et al. 2014). Though SSU rRNA gene is the most frequently used as molecular marker (Lee et al. 2008, Opik et al. 2008), three rDNA region *viz.,* the partial small subunit (SSU) rRNA gene, the internal transcribed spacers (ITS1, 5.8S and ITS2) and the partial large subunit (LSU) rRNA gene in combination can be more distinguishable in closely related AMF species (Stockinger et al. 2010, Schoch et al. 2012).

In order to develop a microbial consortia for sustainable cultivation of chilly several AMF were screened for symbiotic response and *Funneliformis mosseae* proved to be best (Thilagar & Bagyaraj 2015). Similarly several PGPR were screened and *Bacillus sonorensis* proved to be best in improving growth and yield of chilly (Thilagar et al. 2016b). Interaction between *F. mosseae* and *B. sonorensis* was then studied and results brought out that interaction is synergistic and dual inoculation with these as a microbial consortia resulted in better growth and yield of chilly compared to inoculation with either one of them (Thilagar et al. 2014). All these studies were carried out at pot culture conditions. This was followed by a microplot experiment with the microbial consortia (*F. mosseae* + *B. sonorensis*) selected from the pot culture studies along with varying levels of fertilizers. The results suggested that inoculation with microbial consortia can result in saving of 50% of chemical fertilizer application with no loss in fruit yield. A large scale field trial was conducted at farmer's field to validate the results obtained in the microplot experiment with only two treatments *viz.,* 100 % NPK (T_1) and 50% NPK + microbial consortia (*F. mosseae* + *B. sonorensis*) (T_2). The results conclusively brought out that the application of chemical fertilizer to chilly crop can be reduced by 50 % through inoculation with *F. mosseae* + *B. sonorensis* (Thilagar et al. 2016a). The present study was carried out to track down the presence of inoculated AM fungus *F. mosseae* in the roots of inoculated treatment from the above mentioned farmer's field trial using molecular techniques.

Materials & Methods

Chilly field experiment

Chilly field experiment with two treatments i.e., 100% recommended NPK (T_1) and 50% recommended NPK + microbial consortia (*F. mosseae* and *B. sonorensis*) (T_2) was conducted in a farmer**'**s field at Gadenahalli village, outskirts of Bengaluru, Karnataka, India during *kharif* 2013-2014**.** The site was situated at 13°11' N latitude and 77°36' E longitude with an altitude of 920 meters above the Mean Sea Level**.** Bengaluru receives 970 mm rainfall per year**.** The soil sample from a depth of 0-20 cm was collected from the experimental site before imposing treatments**.** Further, the soil was analyzed for various physico-chemical properties by adopting appropriate methods**.** The texture of the field soil was red sandy loam having pH 6.9, organic carbon 0.4%, available nitrogen 249.6 kg/hectare, and available phosphorus and potassium of 22.4 kg/hectare and 217.3 kg/hectare, respectively**.** The experimental area was brought to a fine tilth and each plot size was 30m x 30m and half the dose of N in the form of urea, along with full dose of P in the form of single super phosphate and K in the form of muriate of potash was applied to T_1 at the time of planting while the remaining half of N was added 6 weeks later. Similarly 50% recommended NPK was added to T_2. The liquid inoculum of *B. sonorensis* was mass produced (12 litres)in LB broth and the liquid inoculum containing cfu 7 x 10^8/ml was mixed with ***F.*** *mosseae* mycorrhizal inoculum (12 kg) containing the IP 2.8 x 10^8/g. Ten grams of microbial consortia was applied to each planting hole of T_2. The most commonly used cultivar Namdhari chilly seeds (NS 1701) were used in the study. Chilly seedlings were raised in seedling trays and thirty days old uniform size seedlings were transplanted to the main field with spacing of 75 cm between rows and 45 cm between plants. Plants were irrigated whenever necessary. The chilly plants were cultivated following the usual practice and at harvest the roots from the two treatments were collected. The following methods were used to track the inoculated AM fungus in the chilly plant roots.

Isolation of total genomic DNA

Chilly plant roots collected from the uninoculated and inoculated treatments were thoroughly cleaned with water to remove soil and other debris. Cleaned roots were stored at -20ºC until further use. Total DNA from root sample was extracted essentially by following the method of Zeze et al. (1998). Briefly, plant roots were powdered finely with liquid nitrogen in pre chilled and sterile mortar. To approximately 100 mg of the powder, 1 volume of extraction buffer (CTAB 2%, Tris-HCl 100 mM pH 8, EDTA 20 mM, NaCl 1.4 M and PVP 1%) preheated at 65ºC was added, properly mixed and incubated at 65ºC for 2-3 hours with regular mixing. 1 volume of Chloroform: Isoamyl alcohol mixture (24:1:: volume:volume) was then added and kept on ice for 20 minutes after mixing by vortexing briefly. Centrifugation was then carried out for 10 minutes at 12,000 rpm in a Microfuge. The aqueous supernatant was transferred to a sterile microfuge tube and 0.1 volume of 10% CTAB (CTAB 10% and NaCl 0.7 M) preheated to 65ºC was added along with 1 volume of Chloroform: Isoamyl alcohol mixture and incubated on ice for 20 minutes. The supernatant from the aqueous layer obtained after Centrifugation at 12,000 rpm for 15 minutes was transferred to a fresh microfuge tube and equal volume of CTAB precipitation buffer (CTAB 1%, Tris-HCl 50 mM pH 8 and EDTA 10 mM) was added and incubated at 4ºC. At the end of 1 hr the tube was centrifuged at 12,000 rpm for 15 minutes and supernatant was discarded. The pellet was suspended in 300 µl of TEN solution (Tris-HCl 10 mM pH 8, EDTA 10 mM and NaCl 1 M) and incubated at 65ºC for 10 minutes. To precipitate DNA, 2 volumes of cold ethanol was added and incubated overnight at 0ºC. After centrifugation the pellet was washed twice with 70% ethanol, air-dried and resuspended in 20 µl of MilliQ water and stored at 4ºC.

Polymerase Chain Reaction

The inoculated AM fungus (*Funneliformis mosseae*) applied as part of microbial consortia was tracked down in the roots of chilly plants from the field by following a part of the rRNA gene by a

nested PCR approach. The primers used for the nested PCR is listed in Table 1. and their positions on the rRNA are schematically shown in Fig. 1.

Table 1 List of primers used for nested PCR

Name	Primer Sequence	Reference
NS5	5´-AAC TTAAAG GAATTGACGG AAG-3´	White et al. 1990
ITS4	5´-TCCTCCGCTTATTGATATGC-3´	White et al. 1990
Glom1310	5´-AGCTAGGCTTAA CATTGTTA-3’	Redecker 2000
ITS4i	5´-TTGATATGCTTAAGTTCAGCG-3’	Redecker et al 2003

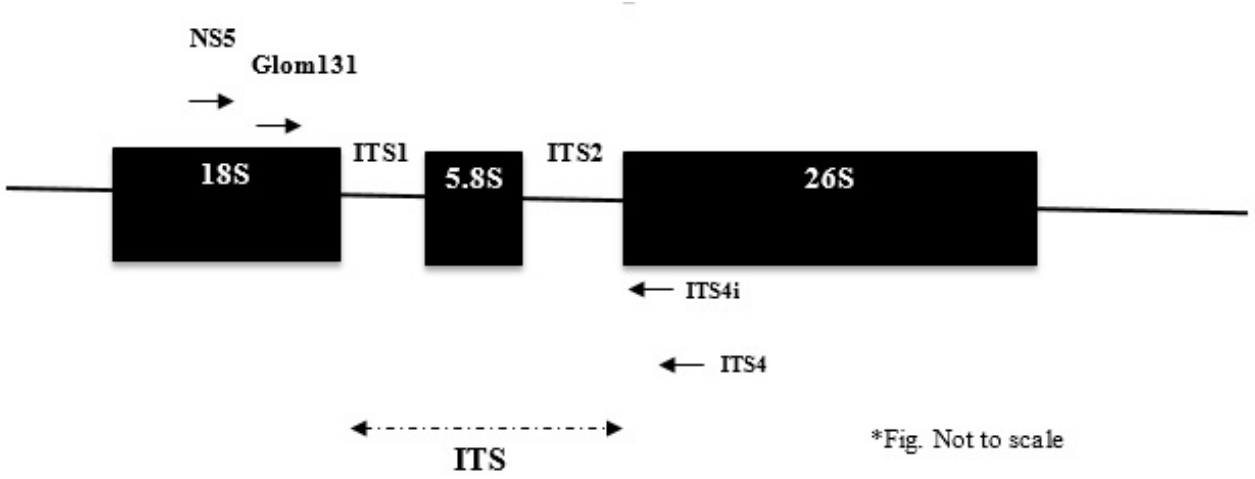

Fig. 1 – Schematic representation of 18S-5.8S-26S rRNA gene unit: coding regions (18S, 5.8S, 26S), spacer regions (ITS1, ITS2,) and binding sites of primers NS5, Glom1310 ITS4 and ITS4i used for analysis are indicated.

The first amplification was carried out using the universal primers NS5 and ITS4 (Table 1). The PCR was performed in a 20 µl volume having the following components: 1×*Taq* buffer (GeNei, India), 0.20 mM dNTPs (GeNei, India), 0.5 µM of forward and reverse primers, 1 mM $MgCl_2$ (GeNei, India), 10 – 20 ng of root DNA and 0.5 U of *Taq* DNA polymerase (GeNei, India). The reaction was carried out in a thermal cycler (MJ Mini, Bio-Rad Laboratories, USA) by incubating reactants at 94ºC for 5 minutes followed by 39 cycles of 94ºC for 2 minutes, 60ºC for 30 seconds, and 72ºC for 1.20 minutes with a final extension of 10 minutes at 72ºC. Amplicons from the above PCR using universal primers were diluted in 1:10 ratio and was used as template for second nested amplification using Glomeraceae specific primer GLOM1310, and a universal primer ITS4i (Table 1). The PCR was performed in a thermal cycler as described earlier.

PCR products were analyzed by electrophoresing on 1.5% agarose gel in a Tris buffer and stained with Ethidium Bromide (Sambrook et al. 1989). The DNA fragment was visualized in an UV trans-illuminator (DNR, Biotron Healthcare Pvt. Ltd.) and photographed. The size of separated DNA fragments was obtained by comparison with a DNA ladder. Both strands of the DNA fragments were sequenced at Merck Specialities Pvt. Ltd. Bengaluru, India. The qualities of the sequences were ascertained using a sequence analysis software (Geneious version 8.1.5). The similarity search of the sequence was done by nBLAST against NCBI sequence database (Altschul et al. 1990) as well as the mycorrhiza database MaarjAM (Opik et al. 2010). The target sequences were aligned with the other AMF sequences obtained from GeneBank sequence database.

Results

The results of the field experiment revealed that plant growth, dry biomass, fruit yield and nutrient uptake obtained from the treatment 50% recommended NPK + MC did not differ significantly from the plants grown with 100% recommended NPK (Thilagar et al. 2010a). In the present field investigation, the establishment and presence of the inoculated *F. mosseae* in the roots of chilly plants (treatment group) was traced through combination of PCR and sequence analysis.

Genomic DNA isolation

The total DNA (including fungal as well as plant DNA) from uninoculated and inoculated chilly roots with microbial consortia treatments was found to be of good quality (Fig. 2) indicating that the method is suitable for DNA isolation from chilly roots infected with fungus.

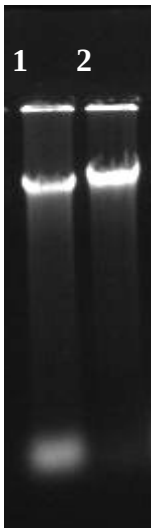

Fig. 2 – Agarose gel photograph of the DNA extracted from the chilly root samples. Lane 1 control plants un-inoculated. Lane 2 Experimental plants inoculated with the microbial consortium.

Nested PCR amplification

***Amplification with the universal primer pair NS5-ITS4*:** The DNA samples were appropriately diluted (generally in the range of 1:50 to 1:100) and used for PCR. A major amplicon of approximately 1185 bp (Fig. 3) was obtained in all plant root DNA samples (inoculated as well as uninoculated) amplifying the partial 18S and ITS region in all the samples. The size of the amplicon obtained is comparable to the expected product from PCR from a fungal DNA using the universal primers.

Amplification with Glomeraceae specific primer: The PCR product obtained using universal primers were appropriately diluted and used for PCR using Glomeraceae specific primer Glom1310 and ITS4i (Table 1) The results showed that an amplicon of approximately 1000 bp was obtained with the PCR template only from plants inoculated with microbial consortia while PCR template from uninoculated plants did not yield amplification product (Fig. 4). Interestingly, the absence of amplification in the uninoculated plants indicated an absence of *Glomus* sp. in uninoculated treatment. However the amplification from inoculated plants with *F. mosseae* validated the fact that the roots of the treated plants are colonized by the MC containing *F. mosseae*.

Sequencing and BLAST analysis

The DNA fragments (Fig. 4 Lanes 2-5) obtained from the nested PCR using Glomeraceae specific primer from the inoculated plants were sequenced as described in Materials and Methods. The sequences obtained were manually checked for the quality using the Geneious® software v 8.1.7.

and three sequences were used for analysis. The multiple sequence alignment was performed using CLUSTAL algorithm built in the Geneious® software and is shown in Fig. 5.

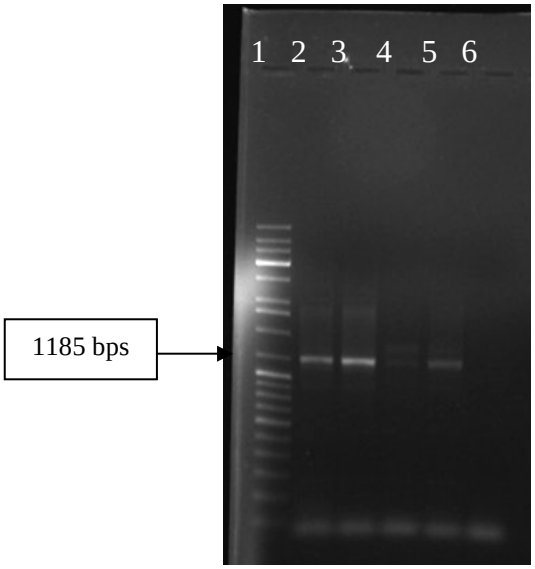

Fig. 3 – DNA fragments obtained from PCR using universal primers NS5-ITS4. Lane 1 DNA 100bp ladder**)**. Lanes 2-3 Microbial consortia inoculated plants. Lanes 4-5 Un-inoculated plants. Lane 6 Negative control.

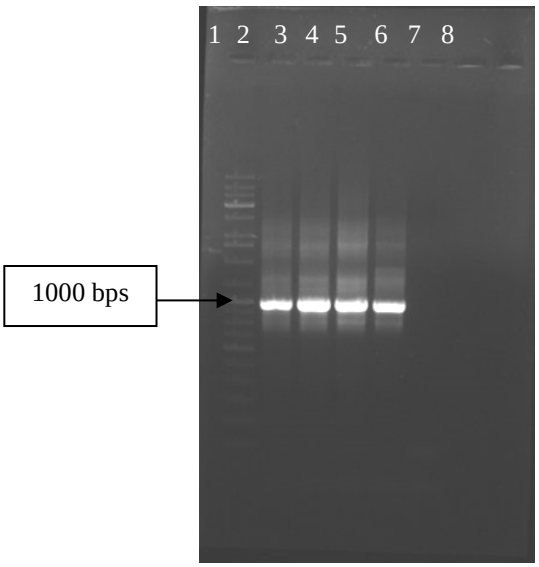

Fig. 4 – DNA fragments obtained from Nested PCR using Glomeraceae specific primer GLOM1310 and a universal primer ITS4i. Lane 1 100bp DNA ladder. Lanes 2-5 plants inoculated with Microbial consortia. Lanes 6-8 Un-inoculated plants.

The BLAST search of the three sequences indicated high degrees of similarity with *Funneliformis mosseae.* Representative rDNA sequences across AMF that are closely related to the *F. mosseae* were obtained from the NCBI database. A multiple sequence alignment was performed using the three sequences from this study and set of representative AM fungal sequences from NCBI and is shown in Fig. 6.

A consensus neighbor-joining phylogenetic tree using 1000 bootstrap values was constructed using the three sequences and the set of AM fungal sequences from NCBI (Fig. 7).

Discussion

The aim of this study was to verify whether the introduced *F. mosseae* could be found inside the plant root after plants were harvested from the field. AMF have a great influence, directly and indirectly, on the life on land, thus, their obvious importance makes it necessary to understand their fundamental biology, genetics, and genomics. Correct identification of AMF isolates is becoming more crucial for basic and applied studies. Almost all molecular identification systems for AMF are based on the rRNA genes. DNA amplification by PCR has been in use for a quite a while as a standard technique for AM fungus identification. In this study, the presence of the AM fungus *F. mosseae* in plant roots was determined by PCR and followed by sequencing. In the first step, the total DNA isolated from plant roots were used for nested PCR. DNA extracted from colonized roots consisted of plant and potentially many endophytes, including the AMF. The majority of DNA extracted from colonized roots is of plant origin and, therefore, nested PCR primers were used in order to increase specificity. Plant roots may contain various compounds that inhibit PCR amplification. To improve target specificity, nested PCRs were employed successfully (van Tuinen et al. 1998). Different primer systems have been developed and compared to characterize AMF communities (Kohout et al. 2014). Initial amplification by universal eukaryote primer pair NS5 / ITS4 revealed the amplification of eukaryotic partial 18S ITS region in both inoculated and uninoculated treatments. The ITS region has previously been used as a molecular genetic marker in environmental studies as it is highly variable among different species and isolates and is also polymorphic within single individuals of AMF (Redecker 2000, Renker et al. 2003).

Recent work using the SSU rRNA gene, the ITS region, and the LSU rRNA gene in combination clearly showed that species- or even isolate-level resolution can be reached (Krüger et al. 2009, Stockinger et al. 2010, Schoch et al. 2010). The sensitivity of detection was improved when an initial PCR amplification using the primers NS5 / ITS4 was followed by the amplification of *Glomus* specific primer pair GLOM1310 / ITS4i. The amplification by primer GLOM1310 / ITS4i, revealed the presence of *Glomus* only in the treated sample. Furthermore the sequence results validated the presence of *Funneliformis mosseae* (*Glomus mosseae)* in the inoculated plant roots while no amplification was obtained from uninoculated treatment indicating the absence of AM fungus, *F. mosseae* in the field. The phylogenetic analysis of three sequences obtained from inoculated treatment (CNBRCD_DJB_12, CNBRCD_DJB_22 and CNBRCD_DJB_23) group them within the clade of *Funneliformis mosseae* which shows us a proof-of-concept for the observed effects of the inoculated *F. mosseae* in chilly crop.

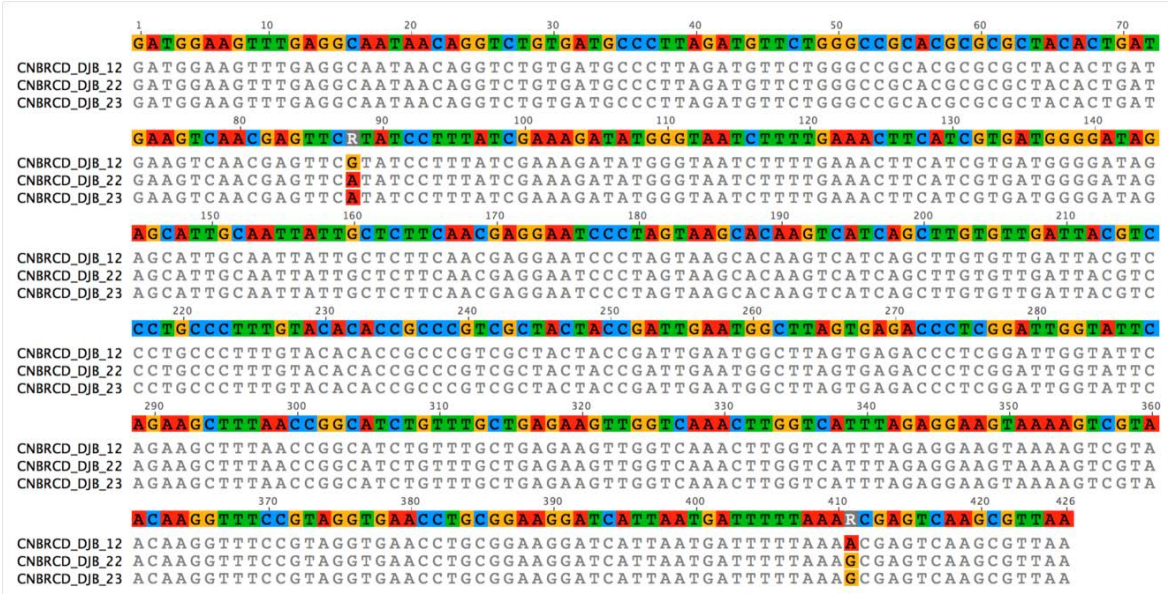

Fig. 5 – Alignment of sequences obtained from plants inoculated with microbial consortium (CNBRCD_DJB_12, CNBRCD_DJB_22 and CNBRCD_DJB_23).

Fig. 6 – Multiple sequence alignment from three sequences obtained from inoculated treatment (CNBRCD_DJB_12, CNBRCD_DJB_22 and CNBRCD_DJB_23) and set of representative AM fungal sequences from NCBI GenBank nucleotide database.

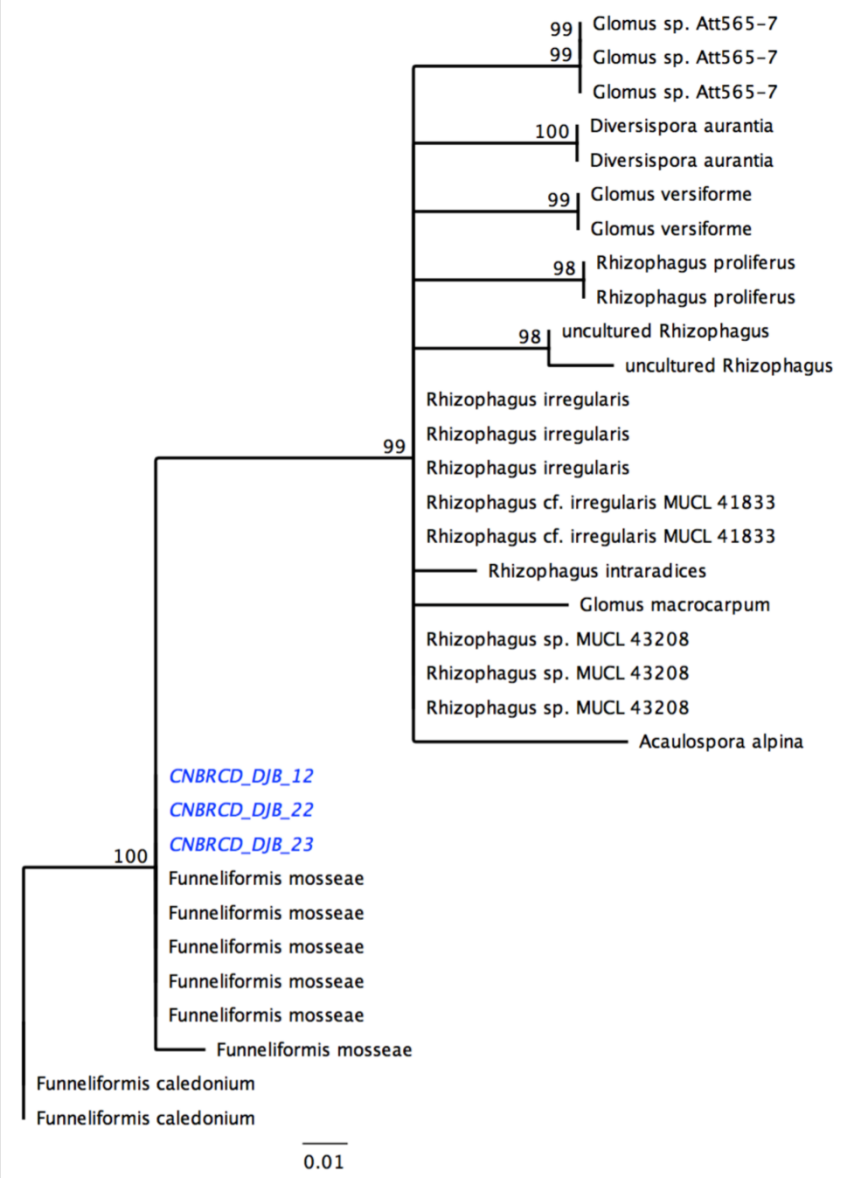

Fig. 7 – A Neighbor-Joining consensus tree constructed using sequences CNBRCD_DJB_12, CNBRCD_DJB_22 and CNBRCD_DJB_23 shows a common clade with *Funneliformis mosseae* sequences obtained from the NCBI GenBank nucleotide database.

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