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Article

Trap Culture Technique for Propagation of Arbuscular Mycorrhizal Fungi using Different Host Plants

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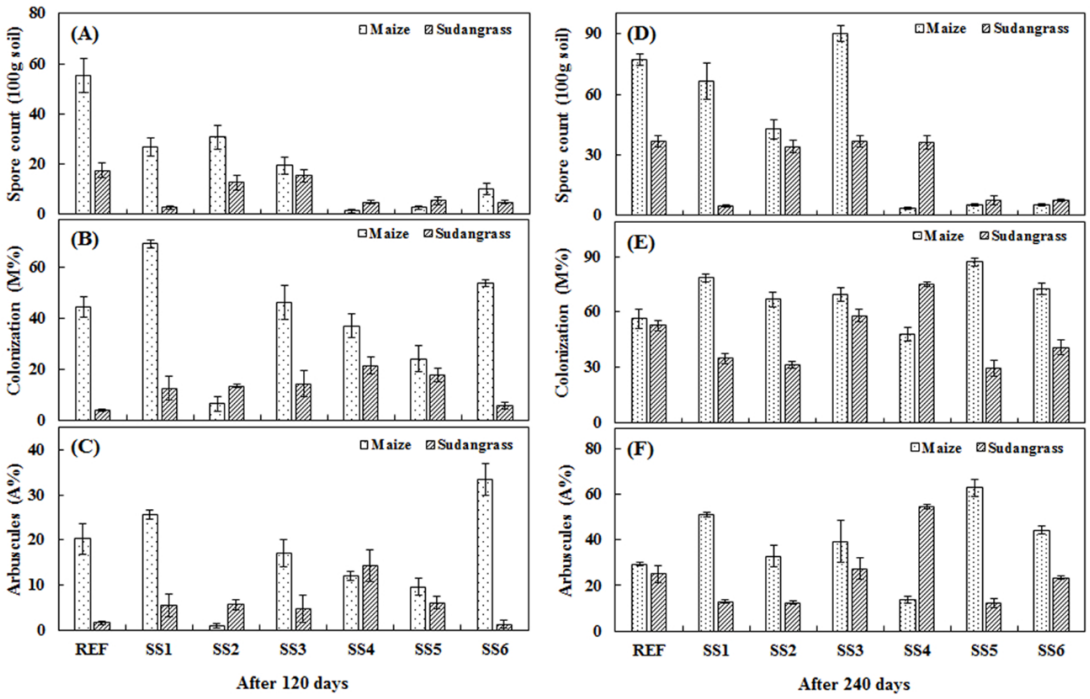
Arbuscular mycorrhizal fungi (AMF) spore propagation and long term maintenance is still a complicated technique for farmers. The use of AMF for their ability to promote plant growth and protect plants against pathogen attack and environmental stresses demands AMF propagation for large scale application. This study aimed to propagate AMF spores by trap culture technique and assess their ability to propagate with different host plants in a continuous plant cycle. Mycorrhizal inoculation by trap culture in maize resulted in longer shoots and roots than sudangrass plants. Increase in dry weight with higher percentage also was observed for maize plants. After first and second plant cycle, maize plants had the higher percentage of mycorrhizal response in terms of colonization and arbuscules than sudangrass. Maximum in spore count also achieved in the pots of maize plants. The results show that maize plant is more suitable host plant for AMF spore propagation and trap culture technique can be used effectively to maintain the AMF culture for long time.

Key words: Arbuscular mycorrhizal fungi, Trap culture, Maize, Sudangrass, Spore propagation



Mycorrhizal response to different host plants after two plant cycle. (A) and (D) AMF spore count; (B) and (E) mycorrhizal colonization; (C) and (F) arbuscules abundance.

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Introduction

Fertilizers are instant nutrient suppliers used to improve plant growth in agriculture. Most of the countries utilize chemical fertilizers and only few countries practice organic farming. Among the organic fertilizers, bio-fertilizers received a great interest among the farmers as it is ecofriendly. Arbuscular mycorrhizal fungi (AMF) are wide spread soil-borne fungi and form symbiotic and mutualistic interaction with most of the plant species (Smith and Read 2008). AMF has been shown to improve plant growth even at adverse environmental conditions against biotic and abiotic stresses. The use of AMF is increasing in agriculture, forestry and environmental reclamation, to improve crop yield and soil health (Johansson et al., 2004). The obligate biotrophic nature of this fungi limits large scale production in a cost-efficient way. However, the conventional method like trap culturing can be a useful tool to propagate this fungi for plant growth and land reclamation purposes in small scale cost-efficient way (Guar and Adholeya, 2002).

AMF culture has been obtained through different methods of propagation technique using different host plants. The most widely used method is substrate-based, however, the spores produced through this method are contaminated with other microbes (Douds et al., 2006). The *in vitro* culture method provide pure cultures of AMF propagules, however, the production of AMF for large scale application is still in its infancy. Guar and Adholeya (2002) reported that the efficiency of mycorrhizal propagation varies depending on the host plant used. In addition, there is no proper procedure for long-term storage of this propagated AMF. Trejo-Aguilar et al. (2013) reported that long-term subculturing reduced the diversity of AMF spores in trap culture when a single host was used. During continuous propagation cycle, dilution of this trap culture inoculum may favor the diverse AMF species than the field (Wang et al., 2008). A trap culture contains spores, hyphae and colonized root bits as an inoculum and can promote diverse AMF species. In contrast, Schalamuk and Cabello (2010) reported that trap culture inoculum favored higher propagation of Glomeraceae family while lower number of spores was obtained from other families. Therefore, we replace it to hypothesize that suitable host plants with periodic rotation may reduce the loss of AMF diversity in trap culture.

The present study aimed to assess the efficiency of trap culture inoculum on plant growth and the effectiveness of AMF propagation by trap culture using different host plants.

Materials and Methods

Study area and soil sample collection Saemangeum is one of the world's largest reclamation sites adding about 400 km^2 to South Korea's total geographical area. A total of six rhizosphere soil samples (SS1 −SS6) were collected from different plant species of Saemangeum reclamation land to obtain different AMF isolates. Each rhizosphere soil sample was collected in sterilized polyvinyl chloride bags along with roots and was transported immediately to the laboratory in icebox and kept at 4°C until use. Initial spore count of the samples were assessed using wet sieving and decanting method as described in Daniels and Skipper (1982) followed by sucrose centrifugation method as described in Utobo et al. (2011).

Pot preparation and application of fertilizer and compost Soil samples were collected from rice field and were consecutively sterilized for five days at 121°C for 15 min. The sterilized soil was placed in 4 kg pots. Urea (nitrogen content 46%), fused superphosphate (phosphate content 20%) and potassium chloride (potassium content 60%) were used as N, P, K fertilizer. Fertilizer application rate was determined based on the recommended basal application rate for maize

Table 1. Inocula used in this study and initial spore count of the cultures.

Inoculum	Initial spore count	Maize	Sorghum-sudangrass
	---- (per 100 g) ----	---------- (Inoculum g) ----------	
CON	0	0	0
REF	50.67±2.03	250	250
SS1	32.33±2.73	250	250
SS2	19.67±2.19	250	250
SS3	17.33±0.88	250	250
SS4	12.33±0.88	250	250
SS5	66.67±5.78	250	250
SS6	32.00±2.08	250	250

CON − control, REF − reference spore (*Claroideoglomus etunicatum*), SS1 to SS6 − trap cultures collected from the rhizosphere of different plant species in Saemangeum reclaimed land.

(N : P : K = 17.4 : 3.0 : 6.9, kg 10 a^{-1}) and sorghum- sudangrass hybrid (N : P : K = 20.0 : 15.0 : 15.0, kg 10 a^{-1}). Compost (60% chicken dung, 20% bark and 20% saw dust) was added at 20.0 g kg^{-1} of soil. Fertilizer and compost were mixed with the soil before pot filling.

Greenhouse experiment and treatments Soil samples collected from Saemangeum reclaimed land were used as inoculants. The reference spore, *Claroideoglomus etunicatum* was obtained from the Forest department, Chungbuk National University and used as a positive control for mycorrhizal culture. The culture was maintained in 5 kg sterilized arable soil with sorghum-sudangrass hybrid as a host plant. The inoculum and initial spore count were given in Table 1. Two hundred fifty grams of Saemangeum rhizosphere soil sample was used as inoculum in each 4 kg pot. Inoculants contain active propagules of AMF spores, hyphae and colonized root pits. Three to five day old seedlings of maize and sorghum-sudangrass were used as host plants. Maize seeds were surface sterilized by immersing in 70% ethanol for 1 min and 6% NaOCl for 5 min, followed by thorough rinsing with sterile distilled water for 7-10 times. Sorghum-sudangrass hybrid seeds were surface sterilized by immersing in 70% ethanol for 2 min and 1% NaOCl for 3 min and thoroughly rinsed with sterile distilled water for 7-10 times. Surface sterilized maize and sorghum-sudangrass hybrid seeds were sown in seedling trays and allowed to germinate for 5 days. The germinated seedlings were transplanted to pots containing trap culture inoculum. After 120 days, the plants were harvested and the pot contents were mixed thoroughly. Soil and root samples were collected and analyzed for spore count and mycorrhizal colonization. New seedlings were planted in the respective pots and maintained again for 120 days. Soil and root samples were collected again for spore count and mycorrhizal colonization analysis. During the experiment, each pot received 100 ml of modified Hoagland's nutrient solution (pH 6.2) every week.

Root staining and colonization measurement The roots of maize and sorghum-sudangrass were harvested after 120 days of planting. The plant roots were washed with tab water and cut into 1 cm fragments. Five ml of 10% (w/v) KOH was added to the fragments in test tubes and incubated at 90°C for 15 min. After incubation, KOH was decanted; washed with tap water and 2% HCl was added and again incubated at room temperature for 10 min to soften the root. HCl was decanted and 5 ml of staining solution (0.05% of trypan blue in lactoglycerol) was added and incubated at 90°C for 10 min. Staining solution was decanted and washed with tap water then the root fragments were destained by the addition of 5 ml of lactoglycerol (Phillips and Hayman, 1970).

Stained root fragments were arranged in glass slides and observed under the microscope for the presence of hyphae, vesicles and arbuscules. Scoring was done based on the intensity of colonization (0 to 5) and based on the arbuscule intensity (A0 to A3) as described by Trouvelot et al. (1986). A total of 30 root fragments were observed for each treatment. The intensity of the mycorrhizal root colonization was estimated as the amount of cortex cell that was colonized by mycorrhiza relative to the whole root system (M%) and to the root fragments (m%). Abundance of arbuscule was estimated as the arbuscule richness in the whole root system (A%) and in the mycorrhizal parts of the root fragments (a%). The *Mycocalc* software was used to determine the M%, m%, A% and a%.

Plant growth parameters After 120 days of plant growth, plant shoot length, root length and fresh weight were measured. After drying the samples in oven for 72 h at 70°C, the dry weight was measured for maize and sorghum-sudangrass plants.

Results and Discussion

Arbuscular mycorrhizal fungi is well studied for their ability to improve plant growth. However, the propagation and maintenance of AMF culture is critical due to their obligate biotrophic nature. Trap culture method of AMF spore development is widely used to obtain a mixed inoculum. Although the trap culture method is comparable to other method, the culture from this method is not pure. Freshly collected soil samples can be maintained in this method to minimize the loss or viability of the AMF spores (Brundrett et al., 1999). Depending on the dominant AMF species and host plant involved, the propagation of AMF varies in trap culture.

Arbuscular mycorrhizal fungal inoculation has been shown to increase the growth of most crop plant species. In addition to plant growth, AMF also help plants to withstand various environmental stress including salinity (Abdel Latef and Chaoxing 2011; Navarro et al., 2012), drought (Asrar and Elhindi 2011) and protects plant from pathogen attack (Nair et al., 2014). Hajiboland et al. (2010) reported that the mycorrhizal inoculation increased tomato plant growth at 5 dS m^{-1} salinity. Wu et al. (2010) reported that orange plants had low accumulation of reactive oxygen species content than non-mycorrhizal plants under salinity. Mycorrhizal inoculation reduced salinity effect and increased the plant growth of pepper (Kaya et al., 2009) and citrus (Wu and Zou 2009). However, propagation and storage of this obligate biotroph is still unachievable by all farmers.

Effect of mycorrhizal inoculation on the growth parameters The mycorrhizal inoculation collected from Saemangeum reclaimed land had more than one AMF species

as inoculum. Depending upon the plant rhizosphere, the amount of individual species varied. The trap culture mycorrhizal inoculum had an overall positive effect on plant growth in both maize and sudangrass. The percentage increment in height was higher in maize plants with an average of 23% (Fig. 1A and D) than in sorghum sudangrass with 4% height increment. Mycorrhizal maize plants had longer roots by an average of 22%, whereas, mycorrhizal sudangrass root length increment was by an average of 26% for all inoculants (Fig. 1B and E). This result is similar with the previous report by Lee et al. (2015) that mycorrhizal inoculation increased maize shoot and root length compared to non-mycorrhizal plants.

Mycorrhizal inoculum increased the shoot dry weight of maize and sudangrass. The average increase in dry weight for maize plants was 36%, whereas in sudangrass was 40% (Fig. 1C and F). The highest dry weight of maize plants was observed for the plants inoculated with SS3 inoculum, whereas sudangrass inoculated with SS4 had the highest dry weight. The increase in dry weight with mycorrhizal inoculation also reported by Wu and Zou (2009). Abdel-Fattah and Asrar (2012) reported that the mycorrhizal inoculation increased dry weight of wheat under salinity.

Mycorrhizal colonization and spore production in different hosts Mass production of mycorrhizal inoculum is vital for the application on a large scale. Several different methods were employed to propagate AMF such as single or monosporic (Fracchia et al., 2001; Selvakumar et al., 2016), hairy root (de Souza and Declerck 2003), solid substrate (Millner and Kitt 1992; Douds et al., 2010), aeroponic (Mohammad et al., 2000), and hydroponic (Tajini et al., 2009). However, storing the propagated AMF spore requires technical skills and preliminary knowledge, thus making it difficult for farmers. One of the common practices to maintain the propagated spores is trap culture method. The use of single host for several plant cycles reduce the diversity of AMF spores (Trejo-Aguilar et al., 2013), however, plant rotation from C_3 to C_4 or vice versa may promote diverse AMF species (INVAM – International Culture Collection of (Vesicular) Arbuscular Mycorrhizal fungi). In the present study, we used two different host plants which were commonly used for AMF propagation.

Mycorrhizal inoculation increased colonization in maize and sudangrass plants. Spore production increased after the 2nd cycle of plants in maize and sudangrass (Fig. 2A and D). After the first plant cycle, none of the trap culture showed higher spore count than reference spores. After the second

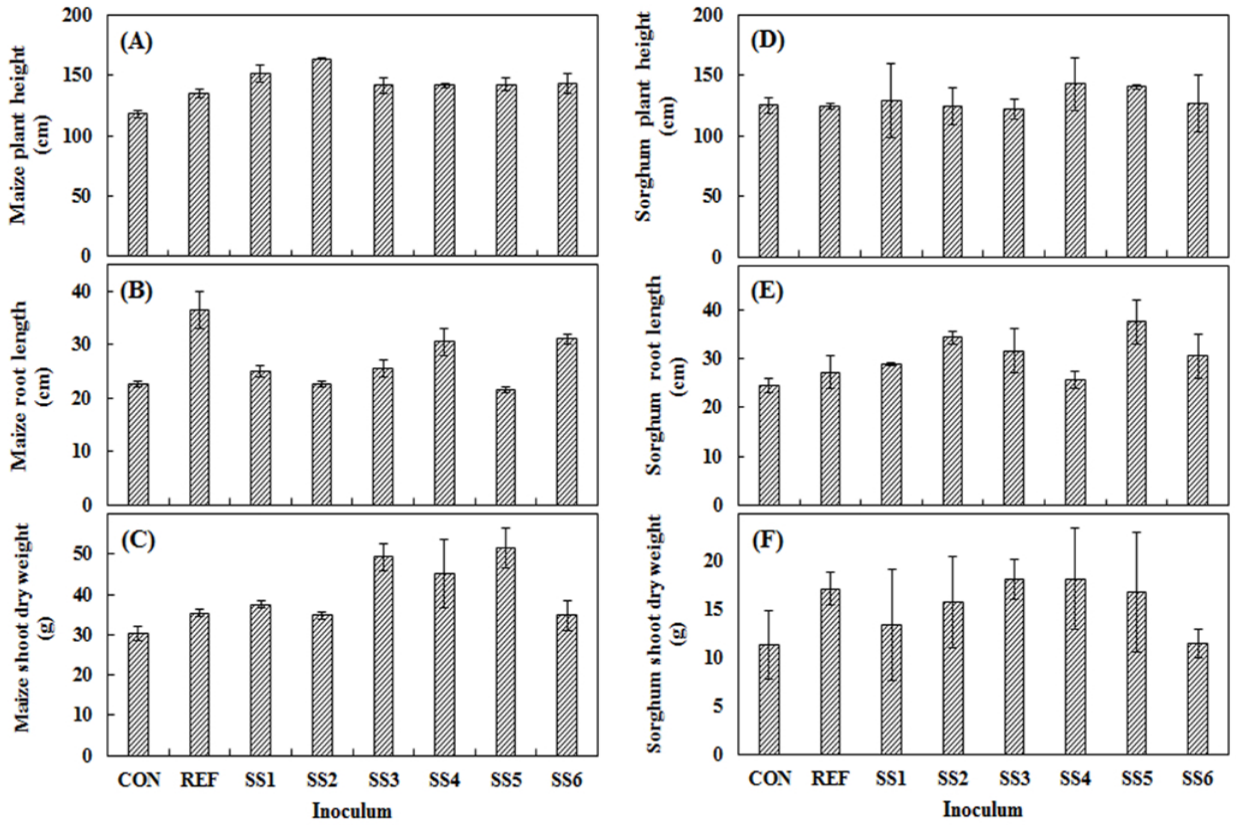

Fig. 1. AMF inoculation effect on maize and sorghum-sudangrass plant growth. (A) maize plant height; (B) maize root length; (C) maize shoot dry weight; (D) sorghum-sudangrass plant height; (E) sorghum-sudangrass root length; (f) sorghum-sudangrass shoot dry weight.

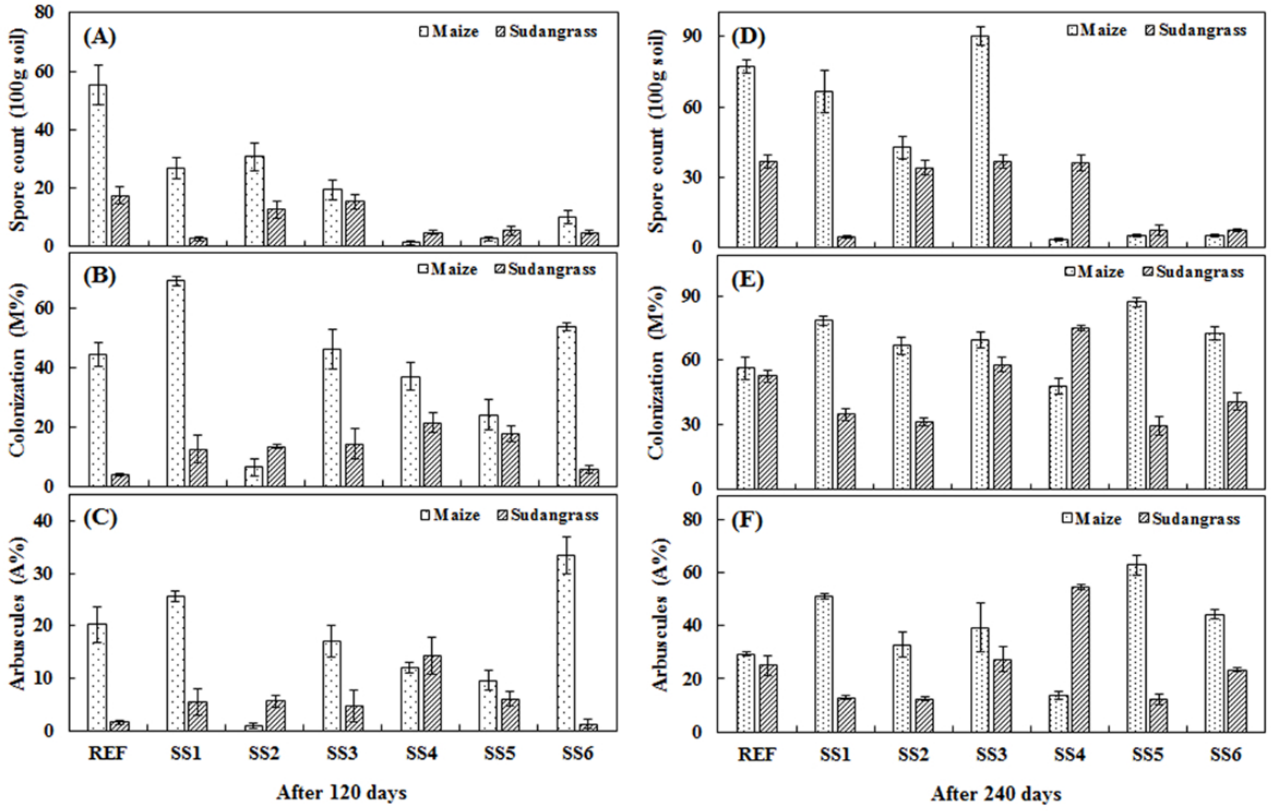

Fig. 2. Mycorrhizal response to different host plants after two plant cycles. (A) and (D) AMF spore count; (B) and (E) mycorrhizal colonization; (C) and (F) arbuscules abundance after first and second plant cycle, respectively.

plant cycle, only trap culture SS3 had higher spore count then reference spores. Initial spore counts were same for all treatments, however, the 1st cycle of propagation had lower count than reference AMF, which resulted in the lower spore inoculation of the 2nd plant cycle. However, higher propagated spores were observed in maize hosts than those in sudangrass after the 2nd plant cycle. In our previous study (Lee et al., 2015), we found that maize plant favored spore propagation when used with the same reference spores. After the first and second plant cycles, maize plants showed higher colonization than sudangrass (Fig. 2B and E). The same trend was observed for arbuscules abundance. After 120 days or the 1st plant cycle, only two trap cultures had higher arbuscules abundance than that of reference spores, however, after the 2nd plant cycle, five of the trap cultures had higher arbuscules abundance than reference spores (Fig. 2C and F).

Conclusions

In summary, mycorrhizal trap cultures had more positive effect on maize plants than sudangrass. Maize plants showed higher percentage of growth parameters than sudangrass in response to mycorrhizal inoculation. After multiple plant cycles, AMF propagation became active in trap culture method in terms of colonization. The efficiency of mycorrhizal propagation and mycorrhizal interaction with host should be checked after several plant cycles to understand the biology of AMF and to store for a long time. Further molecular identification of propagated AMF spores may help to understand compatibility of spores for trap culture technique.

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