ISSN 0367-6315 (Print) / ISSN 2288-2162 (Online)
Korean J. Soil Sci. Fert. 46(5), 373-378 (2013)
http://dx.doi.org/10.7745/KJSSF.2013.46.5.373

Article

Effect of Dispersed and Proximate Inoculation Methods of *Glomus etunicatum* on Root Colonization of Sorghum-Sudangrass Hybrid

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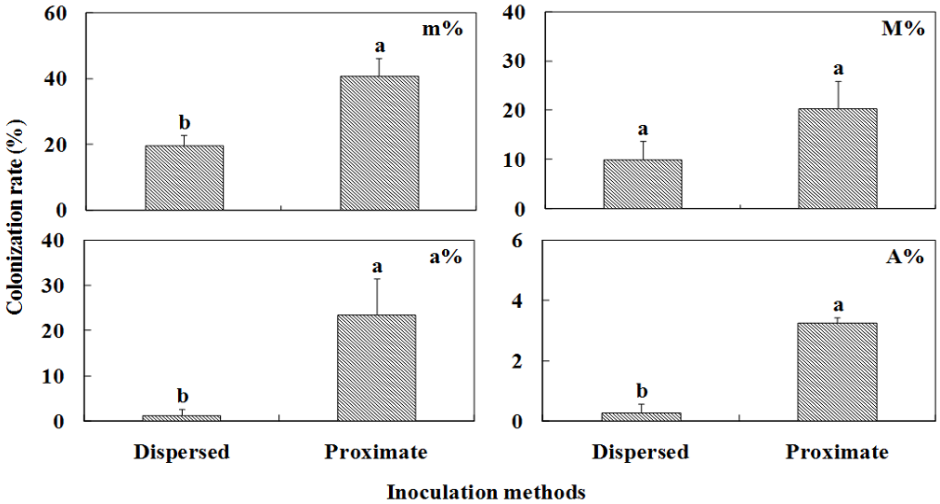
(Received: August 30 2013, Accepted: September 27 2013)

Information on the effective application method of arbuscular mycorrhizal fungi (AMF) inoculum is still inadequate. This work was performed to assess two AMF inoculation methods (dispersed and proximate) on root colonization of sorghum- sudangrass hybrid (*Sorghum bicolor* L.). In dispersed inoculation method, spores were inoculated in 2 kg pots of soil in which 5 day-old seedlings were transplanted and maintained for 50 days. In the proximate inoculation method, spores were first introduced in 500 mL pots where seeds were sown. After 10 days, the seedlings with the 500 mL soil were transferred to 2 kg pots without disturbing the contents. After 50 days of growth, root colonization and arbuscule abundance significantly increased (over 100%) in proximate method of inoculation. Moreover, sorghum-sudangrass hybrid had higher shoot growth (182.5 cm) and Glomalin related soil protein (GRSP) production in proximate method. Nutrient accumulation, particularly total nitrogen (82.61 mg plant^{-1}), was also found to be higher in proximate method of inoculation. Our results demonstrate that the proximate method of inoculation may improve the early stage mycorrhizal symbiosis and inoculum performance in Saemangeum reclaimed soil.

Key words: Arbuscular mycorrhizal fungi, Glomalin, Inoculation methods, Plant growth, Saemangum reclaimed land



Proxiamte method of inoculation improved root colonization and arbuscule abundance in sorghum-sudangrass hybrid

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§Acknowledgement: This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009287)" Rural Development Administration, Republic of Korea.

Introduction

Arbuscular mycorrhizal fungi (AMF) from the Phylum Glomeromycota are known to form symbiotic association with more than 80% of land plants (Helgason and Fitter, 2009). In a nutrient depleted zone, AMF extraradical hyphae can extend out into the soil to uptake nutrients more efficiently from non-rhizosphere soil. It is widely recognized that AMF play an important role in improving the uptake of low mobile ions, in phosphate (PO_4^{3-}) and ammonium (NH^{4+}) phases (Smith and Read, 1997; Marschner, 2007; Martin et al., 2007). The presence of AMF can reduce soil-borne fungal and nematode attacks on roots (Smith and Read, 1997, 2008) and also increase resistance to biotic and abiotic stresses (Smith and Read, 1997; Khan, 2006; Singh, 2006; Martin et al., 2007). In addition, the hyphae-produced glycoprotein called glomalin related soil proteins (GRSP) help in improving soil aggregation (Wright and Upadhyaya, 1998). Even though AMF is known to improve plant growth, its effective application in the field may not be fully realized (Solaiman and Hirata, 1997). This is due to the obligatory nature of growth of AMF and requires signaling from plant for spore germination and hyphal growth. Currently, large scale AMF inoculum application use carriers and is applied through broadcasting or placing directly near the planting hole (in case of transplanted crops). However, with these methods the contact of AMF with the plant roots is considerably low, which eventually leads to reduced efficiency of the applied AMF inoculums (Gadkar et al., 2001). The success of AMF in improving plant growth is highly dependent on the percentage of root colonization (Baird et al., 2010). In an agricultural field, inoculated AMF spores have to compete with the native organisms and require plant signaling for spore germination and colonization. To overcome these problems, proper inoculation methods should be implemented.

Proximate or nursery method of inoculation was found to be highly effective in root colonization and plant growth of vegetable crops (Al-Karaki, 2006; Temperini et al., 2009). Nursery inoculation method realizes two main benefits from introducing mycorrhizal fungi to the plants: superior colonization in the nursery and improved performance after planting in field (Giananazzi et al., 2002). Inoculation with AM fungi at very early stages of plant growth (e.g., seedling stage) has been found to result in higher crop uniformity and reduced transplant mortality (Waterer and Coltman, 1988), and higher yields after transplanting in the field (Lovato et al., 1996). Since in most soils the indigenous populations of AM fungi are present, the pre-inoculation of seedlings in nurseries gives the introduced fungal strain a special advantage over the indigenous fungi after transplanting in field (Dubsky et al., 2002). Furthermore, root colonization by AMF is considered to be an important parameter to improve nutrient uptake and plant growth under stress conditions (Daei et al., 2009; Estrada et al., 2013). Salinity is one of the major problems which affect plant growth adversely. High salinity in soil causes physiological water deficit in plants and inhibits plant nutrient uptake (Jahromi et al., 2008).

Saemangeum is one of the world's largest reclamation sites and more than 30% of its land area has been allotted for agricultural purposes. But the unequal distribution of soil salinity inhibits good crop establishment. Thus, this study aimed to evaluate different methods of AMF inoculation for improved root colonization and growth of sorghum-sudangrass hybrid in Saemangeum reclaimed soil.

Materials and Methods

Greenhouse experiment and treatments Soil samples were collected from low salt affected sites in Saemangeum reclaimed land and analyzed. The soil chemical properties were as follows: Electrical conductivity (EC), 0.49 dS m^{-1}; Organic matter (OM), 0.13 g kg^{-1}; Av.P_2O_5, 20.1 mg kg^{-1}; Na 1.8 and Ca, 0.6 $cmol_c$ kg^{-1}. *Glomus etunicatum* was used as a mycorrhizal culture which was maintained in 5 kg sterilized arable soil with sorghum-sudangrass hybrid as a host plant. Spores were isolated from the soil using wet sieving and decanting method (Gerdemann and Nicholson, 1963). In the dispersed inoculation method, 50 spores were spread approximately 1 cm below the surface soil of 2 kg pot and 5 day-old sorghum-sudangrass hybrid seedlings were planted and maintained for another 50 days. In the proximate method of inoculation, 50 spores were placed approximately 1 cm below the surface soil of 500 g pot and 5 day-old seedlings were planted and maintained for 10 days. After ten days, the seedlings together with contents of the pot were transferred to 2 kg pot and maintained for another 40 days.

Fertilizer and compost application Urea (nitrogen content 46%), fused superphosphate (phosphate content 20%) and potassium chloride (potassium content 60%) were used. Fertilizer application rate was determined based on the recommended basal application rate for

sorghum-sudangrass hybrid (N : P : K = 20.0 : 15.0 : 15.0, kg $10a^{-1}$). 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.

Root staining and colonization measurement Fifty days after planting (DAP), the plants were harvested. The plant roots were washed and cut into 1 cm fragments. Five ml of 10% (w/v) KOH was added to the fragments and incubated at 90℃ for 15 min. After incubation, KOH was decanted; washed and 2% HCl was added and incubated at room temperature for 15 min. After decanting the HCl, 5 mL of staining solution (0.05% of trypan blue in lactoglycerol) was added and incubated at 90℃ for 15 min. Staining solution was decanted and the root fragments were destained using lactoglycerol (Phillips and Hayman, 1970).

Stained root fragments were arranged in glass slides and observed under the microscope for the presence of hypha, vesicles and arbuscules. Scoring was done based on the intensity of colonization (0 to 5) and based on the arbuscules intensity (A0 to A3) as described by Trouvelot et al. (1986). A total of 30 root fragments were observed for each treatment. Intensity of the mycorrhizal root colonization was estimated as the amount of cortex cell that 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 and nutrient accumulation Inoculation effect of AMF (*Glomus etunicatum*) on plant height, root length and dry biomass of sorghum-sudangrass hybrid was measured at 50 DAP. Total nitrogen (T-N) in plants was determined using Kjeldahl Autoanalyzer 1030. Root and shoot samples (200 mg) were dried, powdered and digested with sulfuric acid and potassium sulfate on a hot plate until mixture becomes clear liquid form. Total N in the digested samples was then analyzed using Kjeldahl Autoanalyzer. Total phosphorus (T-P) content was analyzed by UV/Vis spectrophotometer at 470 nm. The values were expressed as T-N and T-P uptake per unit dry weight of each plant.

Glomalin related soil protein extraction Glomalin related soil protein (GRSP) was extracted as described by Wright and Upadhyaya (1998). Easily extractable glomalin related soil protein (EE-GRSP) was extracted using 20 mM sodium citrate (pH 7.0). Briefly one gram of soil was mixed with 8 mL of 20 mM sodium citrate and autoclaved for 30 min at 121℃. The autoclaved content was centrifuged at 5,000g for 15 min and then supernatant was used to measure the protein content. The pellet was dissolved in 8 mL of 50 mM sodium citrate (pH 8.0) to extract total glomalin related soil protein (T-GRSP). The suspension was autoclaved at 121℃ for 1 h and centrifuged to collect the supernatant to measure T-GRSP. For total glomalin extraction, autoclaving and centrifugation process was repeated three times to effectively extract glomalin protein from soil. Protein content of the supernatant was measured using Bradford method.

Statistical analysis There were a total of 2 treatments with 4 replications arranged in a completely randomized design. Analysis of variance and LSD were calculated using SAS Version 9.1 (SAS 2009). Critical difference (CD) values were calculated at *P* level of 0.05%.

Results and Discussion

In Saemangeum reclaimed soil, sorghum-sudangrass hybrid was colonized by AMF in both the methods. With the proximate inoculation method mycorrhizal root colonization was greater than with the dispersed method. Root colonization in the whole root system (M%) of sorghum-sudangrass hybrid was 102.2% higher in the proximate method of inoculation. Mycorrhizal root colonization in the root fragments (m%), arbuscule abundance in the root fragments (a%) and arbuscule abundance in the root system (A%) were also significantly higher in the proximate method (Fig. 1).

Nursery method of AMF inoculation in dry and wet nursery showed 55% and 22% higher root colonization compared to control plants (Solaiman and Hirata, 1997). The nursery method has the similar principle with the proximate method applied in this study. Similar results were obtained by Gamalero et al. (2008) where AMF inoculation increased M% and A% in cucumber plant. Zubek et al. (2011) found higher M% and A% in medicinal plants when inoculated with AMF. Root colonization percentage may vary between plant species and also within the different cultivars of the same species. Red pepper cultivars showed different levels of colonization when AMF (*Glomus intraradices*) species were inoculated in the nursery (Temperini et al., 2009). In our work, proximate method of inoculation significantly increased root colonization in sorghum-sudangrass hybrid compared to dispersed method of inoculation.

Proximate method of inoculation improved root length

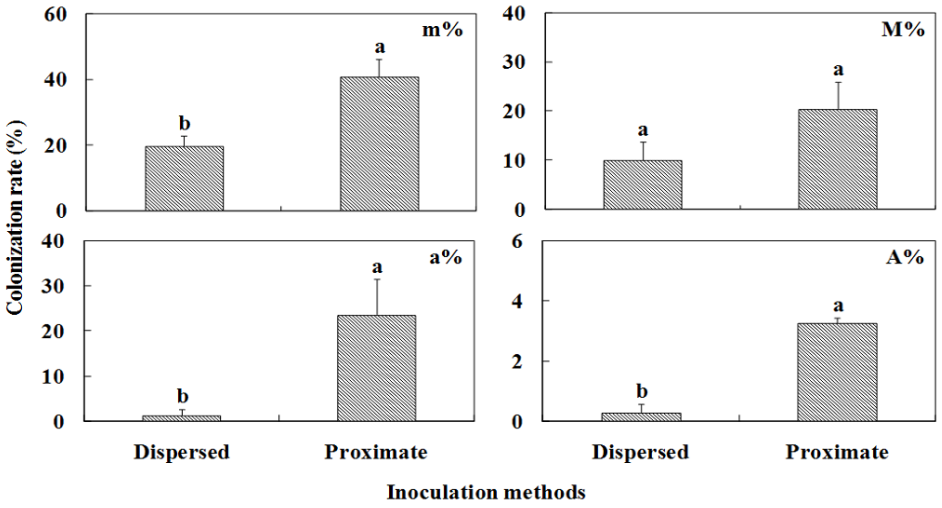

Fig. 1. Effect of dispersed and proximate methods on mycorrhizal root colonization of sorghum-sudangrass hybrid. Intensity of mycorrhizal root colonization in the root fragments (m%), Intensity of mycorrhizal root colonization in the root system (M%), Arbuscule abundance in mycorrhizal parts of root fragments (a%), Arbuscule abundance in the root system (A%). Each value represents the mean ± S.E. (n=4). Same letters are not significantly different from each other as determined by *t* test ($P \leq 0.05$).

Table 1. Effect of *G. etunicatum* inoculation methods on plant height and macro- nutrient accumulation of sorghum-sudangrass hybrid.

Methods	Shoot length	Root length	Total-N	Total-P
	(cm)		(mg plant^{-1})	
Dispersed	173.3 ± 9.96a	33.70 ± 1.12a	79.15 ± 2.6a	55.69 ± 2.7a
Proximate	182.5 ± 12.99a	34.13 ± 1.60a	82.61 ± 2.8a	55.1 ± 5.09a

Each value represents the average of four replicates per treatment ± S.E. (Standard Error). Same letters are not significantly different among different method of inoculation at $P \leq 0.05$ according to *t* test.

Table 2. Effect of *G. etunicatum* inoculation methods on dry matter accumulation of sorghum-sudangrass hybrid and glomalin related soil protein content.

Methods	Dry weight			EE-GRSP	T-GRSP
	Shoot	Root	Total		
	(g plant^{-1})			(mg g^{-1} of soil)	
Dispersed	16.10 ± 1.42a	3.85 ± 0.93a	19.95 ± 1.17a	0.22 ± 0.01a	0.46 ± 0.03a
Proximate	17.13 ± 2.04a	3.68 ± 0.24a	20.81 ± 1.04a	0.26 ± 0.02a	0.60 ± 0.08a

Each value represents the average of four replicates per treatment ± S.E. (Standard Error). Same letters are not significantly different among different method of inoculation at $P \leq 0.05$ according to *t* test.

by 1.3% in sorghum- sudangrass hybrid compared to the dispersed method (Table 1). Plant height was higher in proximate method of inoculation compared to the dispersed method of inoculation. Caravaca et al. (2005) reported that AMF inoculation improved shoot and root length of shrub plants. Similarly, shoot length increased when maize inoculated with *Glomus etunicatum* over non mycorrhizal plants (Zhu et al., 2010). Proximate method of inoculation increased dry weight of tomato under greenhouse condition (Al-Karaki, 2006). Adriano-Anaya et al. (2006) found that *Glomus intraradices* inoculation improved shoot and root dry weight of maize and sorghum plants. Similarly, in this study, proximate method of inoculation increased shoot dry weight and total dry weight of sorghum plants (Table 2). This suggests that with proximate method of inoculation, the possibility for effective early stage symbiosis between plant and AMF would be higher since the inoculants were applied very near the root zone.

In proximate method of inoculation, the abundance of arbuscules was higher than in the dispersed method. These arbuscules act as a nutrient exchange site. This

suggests that the greater abundance of arbuscules in the proximate method of inoculation might have improved nutrient exchange. For nutrient accumulation in plants, higher T-N content was determined in the proximate method of inoculation (Table 1). Wang et al. (2011) also observed that N and P accumulation in plants were increased when inoculated with *Glomus mosseae*. In contrast, total phosphorus accumulation in sorghum-sudangrass hybrid was found to be slightly lower or similar in both methods of inoculation. This suggests that though AMF inoculation improved phosphorous uptake in both the methods, proximate method of inoculation was more effective in terms of effective root colonization.

Glomalin related soil protein (GRSP) produced by mycorrhizal hypha improves soil structure by increasing soil aggregation. Aggregated soil structure possess improved water holding capacity and act as a carbon sink. Proximate inoculation increased the production of EEGRSP and T-GRSP with 16.6% and 30.3% increase over the dispersed method, respectively (Table 2). Similar result was obtained by Caravaca et al. (2005), where AMF application significantly improved GRSP production. Hammer and Rillig (2011) demonstrated that higher sodium in soil improved the glomalin related soil protein production by AMF hypha. There may be a relationship between the percentage of root colonization in sorghum-sudangrass hybrid and GRSP production. It was observed in this study that where there is high percentage of root colonization, GRSP production also is high. This suggests that higher percentage of root colonization may positively influence the soil structure through GRSP production.

Conclusion

Our results demonstrate that proximate method of *Glomus etunicatum* inoculation may improve effective root colonization of sorghum-sudangrass hybrid using Saemangeum reclaimed soil. Moreover, AMF inoculation closer to roots at the early stage of growth may increase the possibility of early mycorrhizal symbiosis which may lead to better plant performance. Long term study on these inoculation methods is needed to understand better the effectiveness of early root colonization on nutrient uptake and plant growth before large scale field application.

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