European Journal of Soil Biology 74 (2016) 39–44

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Propagation technique of arbuscular mycorrhizal fungi isolated from coastal reclamation land

Gopal Selvakumar, Ramasamy Krishnamoorthy [1], Kiyoon Kim, Tongmin Sa*

Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

ARTICLE INFO

Article history:
Received 24 November 2015
Received in revised form
2 March 2016
Accepted 14 March 2016
Available online 25 March 2016

Handling Editor: Prof. C.C. Tebbe

Keywords:
Mass production
Arbuscular mycorrhizal fungi
Slide method
Gigaspora margarita
Claroideoglomus lamellosum

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are well known for their plant growth promoting potential and stress tolerance ability. AMF promotes plant growth even at adverse environmental conditions and improve plant nutrient uptake. This study aimed to propagate AMF using a single spore inoculation technique. Soil samples were collected from salt affected Saemangeum reclaimed land. Collected soil samples were subjected to soil analysis. In the single spore inoculation method, sorghum sudangrass (*Sorghum bicolor* L.) was inoculated with a single spore. Among the 150 inoculants, six spores were able to germinate *in vitro*. Geminated spores were transferred to 1 kg pots containing sterilized field soil. After 120 days, the contents were mixed and transferred to 2.5 kg pots and maintained for another 120 days. After 240 days, spore count and colonization were checked. The propagated spores were identified using nested PCR followed by sequencing. The 18S rDNA sequencing of spores revealed that the spores belonged to *Gigaspora margarita* and *Claroideoglomus lamellosum*. Among the 6 inoculants, *Claroideoglomus lamellosum* S-11 (391 spore per 100 g of soil) had the highest spore count followed by *Gigaspora margarita* S-23 (235 spore per 100 g of soil). Slide method allowed visual monitoring of spore germination *in vitro* as well as being able to mass produce pure cultures of AMF for bio-inoculation purposes.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are widespread soil fungi which form mutualistic symbiosis with more than 80% of land plants and are believed to be obligate symbiotic biotrophs. Only the intraradical hypha of this fungus takes up hexose as carbon source in the apoplast of the plant root cortex cells to complete their life cycle and sporulate [1]. Therefore, the ability of AMF to utilize externally supplied carbon source in asymbiotic medium is limited. Although AMF are indigenous to agricultural soils, inoculation of these fungi improves plant growth by enhancing the uptake of immobile soil mineral nutrients and water [2]. AMF have increased the growth of various field-grown crops, including cotton, tomato, orange, pepper and onions [3]. In addition to enhancement of nutrient uptake by plants, AMF also help plants withstand various biotic and abiotic stresses such as pathogen attack [4], drought stress [5] and salt stress [6].

Enhanced plant growth through AMF inoculation can be ensured by two principal ways (1) selection and inoculation of efficient mycorrhizal fungi and (2) promotion of the efficient native mycorrhizal fungi [7]. Although previous reports support that some AMF are beneficial to a wide variety of plant species [8], some reports argue that host specificity of AMF might reduce the mycorrhizal interaction between plant and fungi [9,10]. AMF inocula that are commercially available come in a variety of forms at low to high concentration and from single inoculum to multiple combined inoculum in a carrier material. However, their performance in non-indigenous soil tends to decrease due to their lack of host specificity and differences in agricultural management practices [11]. Estrada et al. [12] reported that native AMF species from Mediterranean saline soil are more effective in improving plant growth than the introduced AMF species. In addition, reintroduction of native AMF inocula may be necessary to overcome the harmful effects of previous agricultural management, e.g. excessive use of fungicide.

Purchasing large amounts of inoculum necessary for large-scale

* Corresponding author. Department of Environmental and Biological Chemistry, College of Agriculture, Life and Environment Sciences, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea.
E-mail address: tomsa@chungbuk.ac.kr (T. Sa).
[1] Present address: Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, India.

http://dx.doi.org/10.1016/j.ejsobi.2016.03.005
1164-5563/

agriculture is not cost-effective. Moreover, even though inocula of AMF are commercially available, production of pure cultures of host specific AMF is essential. Development of pure culture also enables the study of the morphological characteristics of new AMF species and their individual effect on plant growth. Previously, pure cultures of AMF have been obtained *in vitro* using Ri-TDNA transformed root organ culture [13,14], aeroponics [15,16] and bi-compartmental Petri plates [17]. However, these *in vitro* methods limit their application in the field due to their requirement of precolonized plants and different growth medium [18]. Although pot culture and monoxenic culture propagated spores share similar ontogeny, daughter spores from monoxenic cultures usually differentiate into smaller spores with paler pigmentation and thinner laminated spore walls [19,20]. The inocula produced by substrate-free cultivation method may require carrier material before their application in large scale to improve their survivability. In addition, inocula produced through soil-based method are least artificial, most adopted and can be applied directly in the field [11]. The present study aimed to propagate the AMF spores isolated from Saemangeum reclaimed land using single spores as starter inoculum.

2. Materials and methods

2.1. 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 thirty five rhizosphere soil samples (10 cm radius and 15 cm depth; approximately, one kg for each sample) have been collected from Saemangeum reclamation land. The distance between each sample was at least 10 m, so that the chances of sharing the same AMF species between samples are reduced. Based on the initial spore count, ten soil samples which had over 10 healthy spores were used in this study. Soils from rhizospheres of dominant plant species such as *Phragmites australis*, *Cyperus polystachyos* and *Miscanthus sinensis* were chosen to obtain different AMF isolates. Each rhizosphere soil sample along with the plant roots was collected in a sterilized polyvinyl chloride bag and transported immediately to the laboratory in icebox and kept at 4 °C until use.

2.2. Soil analysis and initial spore enumeration

All the collected soil samples were subjected to initial spore count and determination of electrical conductivity (EC) value (1:5 dilution method). The initial spore count of the soil samples were assessed using wet sieving and decanting method as described by Daniels and Skipper [21] followed by sucrose centrifugation method. Soil properties such as pH, cation exchange capacity (CEC), organic matter, micro and macro nutrient contents were examined.

2.3. Single spore - slide method

Freshly isolated spores were examined under the microscope and only the healthy and damage-free spores were used for propagating the AMF *in vitro*. Slide method was conducted to visually monitor spore germination. Briefly, sterilized filter paper (Whatman No. 6) was excised to ¾ of the glass slide and tied with rubber band and kept in a 50 ml sterile falcon tube containing the mixture of plant root exudates and sterilized Hoagland's nutrient solution. The bottom of the filter paper was in contact with the solution so that the moisture can be maintained throughout the experiment. The plant root exudates were obtained as described in Panwar et al. [22]. Sorghum sudangrass seeds were surface sterilized by immersing them in 70% ethanol for 2 min followed by 1% sodium hypochlorite (NaOCl) for 3 min and thoroughly rinsed with sterile distilled water for seven to ten times. The surface sterilized seeds were placed in a petri dish containing sterile moist filter paper and kept in the growth chamber for 2–3 days. The growth chamber conditions were as follows: 12 h of light at 25 °C and 12 h of dark at 20 °C and maintenance of 70% humidity. The germinated seeds were transferred to slides and tied with rubber band without harming the radicles. A healthy single spore was placed near the growing root (Fig. 1). A total of 150 inoculants were made. The setup was kept in the growth chamber with the above mentioned conditions for two weeks. Every 3 days the spore was examined under the microscope for germination. After 2 weeks, six slides with successfully germinated spores were transferred to 1 kg pots containing sterilized soil without disturbing the setup and new pre-germinated sorghum seeds were sown. Sterilized field soil consecutively autoclaved for three days was added to fill up the remaining space in the pots. New pre-germinated sorghum seeds were sown and the plants were allowed to grow for 120 days. Hoagland's nutrient solution was poured on every pot every week. After 105 days, the plants were subjected to drought stress by withholding water or Hoagland's nutrient solution for 15 days to induce spore production.

After 120 days, the soil from each pot was mixed and transferred to 2.5 kg pot with sterilized soil. Pre-germinated sorghum seeds were sown and grown for another 120 days following the above mentioned procedure. After 240 days, root and soil samples were collected and examined for mycorrhizal colonization and spore numbers. Briefly, the roots were first washed with 10% KOH for 10 min in water bath at 90 °C. Then the roots were washed with tap water and immersed in 2% HCl for 10 min at room temperature. After discarding the HCl, 0.5% trypan blue in lactoglycerol was added and the roots were allowed to stain at 90 °C for 10 min. The staining solution was discarded and the stained roots were washed with tap water. Roots were immersed in destaining solution for overnight to remove excess staining and were checked for colonization [23].

The stained root fragments (1 cm) 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 (0–5) and based on the arbuscules intensity (A0–A3) as described by Trouvelot et al. [24]. 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%). Abundance of arbuscule was estimated as the arbuscule richness in the whole root system (A%). The Mycocalc software was used to determine the M%, and A%.

2.4. 18S rDNA sequencing

Five healthy spores from successfully propagated slide method pots were taken in microcentrifuge tube and surface sterilized with 2% chloramine-T and 100 μg/ml streptomycin for 30 min. Then the spores were transferred to a sterilized PCR tube containing 10 μl of 1:1 ratio of 10X PCR buffer and sterilized distilled water. Spores were aseptically crushed with a sterilized blunt end pasteur pipette. The 18S rDNA of arbuscular mycorrhizal fungal spores were amplified using the nested PCR [25]. In the first round of PCR, eukaryotic genes were targeted using universal eukaryotic primers GeoA2 (5′-CCAGTAGTCATATGCTTGTCTC-3′) and Geo11 (5′-ACCTTGTTACGACTTTTACTTCC-3′) which amplified the genes with the product length of 1800 bp. The final product was diluted 1:100 ratio with TE buffer [26] and used as template for next PCR. In the second round of PCR, AMF specific primer AM1 (5′-GTTTCCCGTAAGGCGCCGAA-3′) was used to amplify AMF specific