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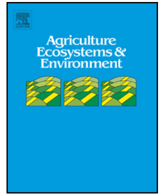

Short communication

Arbuscular mycorrhizal fungi and associated bacteria isolated from salt-affected soil enhances the tolerance of maize to salinity in coastal reclamation soil

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) have close association with bacteria in establishing a tripartite interaction with plants. The objective of this study was to assess the tripartite interactions among AMF, associated bacteria and maize on enhancing salt stress tolerance. AMF spores isolated from coastal reclamation land were identified as *Rhizophagus intraradices* and multiplied using monosporic mass culturing. From surface-decontaminated spores of *R. intraradices*, isolation of associated bacteria was carried out and the obtained isolate was identified as *Massilia* sp. RK4. The isolated bacterial strain was found to possess several plant growth promoting characteristics and for further studies, its effect on maize plant growth in coastal reclamation soil was evaluated under three different salt concentrations. Salt stress substantially reduced plant growth, root colonization and spore-producing ability of the *R. intraradices*. However, treatment with the AMF and a combination of AMF and associated bacteria alleviated the salt-induced reduction of plant growth, root colonization, nutrient accumulation and lowered leaf proline levels compared to control treatment. The co-inoculation of *R. intraradices* and *Massilia* sp. RK4 exhibited significant impact on AMF root colonization and nutrient accumulation in plants compared to inoculation with *R. intraradices* only. Inoculation of *R. intraradices* and *Massilia* sp. RK4 improved the salinity tolerance of maize through the dual effect exerted by AMF and its associated bacteria.

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1. Introduction

Maize is the third most important food crop in the world and has been classified as a salt-sensitive plant (Estrada et al., 2013a), with its production is severely affected by salt stress. Plant growth in salt-affected soil is inhibited by the multifaceted interactions involving ion toxicity, oxidative stress, osmotic stress and nutrient imbalance which act solitarily or in-combination to affect plant health. Plants can overcome salt stress by interacting with various soil microorganisms, such as arbuscular mycorrhizal fungi (AMF) and plant growth-promoting bacteria (PGPB). AMF belonging to the phylum *Glomeromycota*, has been found to be associated with more than 80% of terrestrial plants (Smith and Read, 2008). Generally, AMF ameliorates salt stress by improving nutrient uptake, water uptake, photosynthesis, accumulation of organic solutes and increase the PGPB population in rhizosphere region (Abdel Latef and Chaoxing, 2011). Several studies have shown that the inoculation of AMF improve plant growth and productivity under salt stress conditions (Estrada et al., 2013a; Talaat and Shawky, 2014).

PGPB may improve salinity tolerance and plant growth by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, fixing nitrogen, phytohormone production, siderophore production and solubilizing insoluble phosphates. In addition, many PGPB show significant positive impact on AMF hyphal growth, root colonization and spore production (Hildebrandt et al., 2006). Enhancement of salt stress tolerance

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through a single/co-inoculation of AMF and PGPB have been reported in several crops including maize, peanut, and rice (Estrada et al., 2013a; El-Akhal et al., 2013; Bhusan Bal et al., 2013). In majority of such studies, non-native AMF/PGPB species were used to evaluate mitigation of salt stress. To obtain significant impact on plant growth with sustainable prospects, co-inoculation studies must strive to employ native microbiota and associative micro-organisms which are compatible and comparable with the original conditions. We hypothesize that co-inoculation of AMF and associated bacteria may enhance mycorrhizal activity as well as plant growth. To prove our hypothesis, we isolated *Rhizophagus intraradices* as well as its associated bacteria from the salt affected coastal reclamation soil and used them to enhance the salt tolerance of maize.

2. Materials and methods

2.1. AMF isolation, identification and spore surface sterilization

AMF spores isolated from the rhizosphere of the *Phragmites* sp. (Common Reed) grown in Saemangeum reclamation land, South Korea were used for the monosporic mass culturing of AMF. Sorghum-sudangrass hybrid plants were used as a host plant for AMF multiplications. After six months, the spores developed in monosporic mass culturing was used for the DNA isolation according to Lumini et al. (2007), and the DNA was used for nested PCR amplification. The first PCR was performed with the GeoA2/Geo11 primer set, which amplifies the fungal DNA, and the second PCR targeted the AMF 18 S rDNA using the NS31/AM1 primer set. The first PCR product was diluted 100-fold using TE buffer, and the diluted PCR product was used for NS31/AM1 amplification. The PCR reactions were performed in a final volume of 20 μl containing 2 μl of 10X PCR buffer (with Mg^{2+}), 400 μM dNTP mix, 0.2 μM primers, 1 unit of Taq DNA polymerase and 2 μl of template DNA. The thermal cycling conditions for the first PCR were: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 7 min. The second PCR was performed with one cycle of 94 °C for 1 min, 66 °C for 1 min, and 72 °C for 1.30 min, 30 cycles of 94 °C for 30 s, 66 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR product was sequenced using an ABI 3100 sequencer, and the sequence was compared with those in GenBank using the BlastN program. The isolated AMF was identified as *Rhizophagus intraradices* through 18S rDNA sequencing (GenBank accession number KJ792100).

R. intraradices spores were surface-decontaminated following the method reported by Jargeat et al. (2004) with few modifications. Briefly, the spores were surface-decontaminated using 4% chloramine T for 35 min and washed five times with sterile distilled water to remove the chemical residues from the spore surface (Fig. S1). The spores were then incubated in an antibiotic solution containing 200 mg of streptomycin/lit and 100 mg of gentamicin/lit for 4 h and washed five times with sterile distilled water. One hundred microliter aliquots of the solution after the fifth wash and the surface-decontaminated spores were placed on nutrient agar medium to confirm the effectiveness of the surface decontamination process, and the plates were incubated at 25 °C for 72 h. If bacterial colonies grew (or were present) from the last wash or on the surface of the decontaminated spores, the whole batch was discarded. Spore DNA and associated bacteria isolation was performed only if no bacterial growth was observed after the last wash and on the surrounding spores. PCR confirmation of the surface decontamination process was performed using the solutions after the first wash (wash obtained before surface decontamination) and last wash (wash obtained after surface decontamination) with the universal bacterial primer set (27F/1492R) to completely ensure the absence of any bacteria on the spore surface (Mondo et al., 2012).

2.2. Isolation and characterization of associated bacteria

Surface-decontaminated *R. intraradices* spores were used for associated bacteria isolation. Briefly, the spores were crushed in a microcentrifuge tube (triplicate) containing 15 μl of sterile distilled water. The spore homogenate was centrifuged, and the supernatant (10 μl) was inoculated in 15 ml of 0.25X nutrient broth and incubated in a shaker for 72 h at 30 °C. After incubation, 1 ml was serially diluted up to 10^{-2}, and 100 μl were plated on 0.25X nutrient agar (NA) medium followed by incubation for 72 h at 30 °C. After isolation of the associated bacterium, the genomic DNA of the associated bacterial isolate was extracted and amplified using 27F and 1492R. The amplified product was sequenced using the fluorescent dye terminator method (ABI prism instrument and a BigdyeTM Terminator Cycle Sequencing-Ready reaction kit V.3.1). The sequences were aligned, and the closest neighbor was identified using the EzTaxon server. A phylogenetic tree was constructed using MEGA 6 after multiple alignments of the results using the ClustalW program. A neighbor-joining tree was constructed with bootstrap values of 1000. From phylogenetic analyses, the associated bacterial isolate was closely (97.92%) matched to *Massilia niastensis* (Fig. 1) and the sequence was submitted in GenBank with accession number KF438009 (*Massilia* sp. RK4). The *Massilia* sp. RK4 biochemical characterization was performed by following standard laboratory protocols. Qualitative and quantitative analysis of indole acetic acid (IAA) production, phosphate solubilization (tricalcium phosphate), siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, exopolysaccharides (EPS) was carried out. Salt tolerance of the *Massilia* sp. RK4 was tested by supplementing nutrient broth with different percentages of NaCl.

2.3. Pot culture experiment

2.3.1. Microbial inoculant preparation

Mycorrhizal inoculum was developed through monosporic mass culturing of *R. intraradices*. Ten gram of mycorrhizal inoculum contained approximately 35 spores and 10 root fragments with 70% colonization. Isolated associated bacteria (*Massilia* sp. RK4) was grown in nutrient broth for 24 h and then centrifuged at 8000 rpm for 10 min. The pellet was washed twice with 0.1 M phosphate buffer (pH 6.8) and resuspended in the same buffer. The optical density of the suspension was adjusted to approximately 1.0 (O.D. at 600 nm) by diluting with 0.1 M phosphate buffer. The population count of *Massilia* sp. RK4 was maintained at $\sim 1 \times 10^8$ cfu/ml at an O.D. of approximately 1.0.

2.3.2. Soil analysis and seedling preparation

One hundred grams of Saemangeum reclamation land (N 35°46′13.28 and E126°37′21.80) soil was used for the analysis of physical and chemical properties of the soil using standard laboratory protocols. Maize seeds (shrunken-2) were surface sterilized using 70% ethanol for 1 min, treated for 5 min with 6% NaOCl, and washed seven times with sterile distilled water. For bacterial treatment, the surface-sterilized seeds were imbibed in 10 ml of 0.1 M phosphate buffer (pH 6.8) containing $\sim 1 \times 10^8$ cfu/ml of *Massilia* sp. RK4 for 4 h before the seeds were sown in a seedling tray. For the control and *R. intraradices* single inoculation treatments, the seeds were treated with 0.1 M phosphate buffer (pH 6.8) without bacteria.

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