Annals of Agricultural Sciences xxx (xxxx) xxx–xxx

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Contents lists available at ScienceDirect

Annals of Agricultural Sciences

journal homepage: www.elsevier.com/locate/aoas

Genetic diversity of plant growth promoting rhizobacteria and their effects on the growth of maize plants under greenhouse conditions

Sameh H. Youseif

National Gene Bank and Genetic Resources, Agricultural Research Center, 12619 Giza, Egypt

ARTICLE INFO

Keywords:
Isolation
PGPR
Zea mays
Diversity
IAA
N_2- fixation
P- solubilization
Plant inoculation
Plant biomass production

ABSTRACT

Engineering of plant rhizosphere with beneficial plant growth promoting (PGP) bacteria offers a great promise for sustainable crop productivity. In this context, 49 rhizospheric/endophytic bacterial isolates were purified using N-free medium, screened *in vitro* for PGP characteristics and evaluated for their beneficial effects on the early growth of maize (*Zea mays* L.). The biodiversity of isolated bacteria was analyzed by amplified ribosomal DNA restriction analysis (ARDRA) using four restriction enzymes. Out of the 49 isolates, 7 produced high levels (32.1–82.8 μg mL^{-1}) of indole-3-acetic acid (IAA); 11 had potential phosphate solubilizing abilities (101–163 μg mL^{-1}), while significant acetylene reduction activities (100–1800 nmole C_2H_4 mg^{-1} protein h^{-1}) were observed in 12 isolates. Five ribogroups (A-E) were identified using ARDRA. The 16S rRNA-sequence analysis of bacterial representatives from different ribogroups revealed that, 89% of isolates belonged to phylum *Proteobacteria*, while 11% of them were assigned into phylum *Bacteroidetes*. Phylum *Proteobacteria* included *Achromobacter*, *Agrobacterium*, *Bordetella*, *Cupriavidus, Ochrobactrum*, *Pseudoxanthomonas* and *Stenotrophomonas* genera. While, phylum *Bacteroidetes* was represented by *Chryseobacterium* and *Flavobacterium* genera. Under greenhouse conditions, all the selected isolates significantly increased shoot and root fresh and dry biomass of maize plants, compared to the non-inoculated control. Inoculation with *Chryseobacterium* sp. NGB-29 and *Flavobacterium* sp. NGB-31 showed the highest beneficial effects on plant growth parameters that were tentatively associated with the high efficiency of these isolates to fix nitrogen and produce high amounts of IAA. The study indicates the potential use of these isolates for production of maize biofertilizers under field conditions.

1. Introduction

Maize is a major food source for the world and is a high-yield commodity crop. It is a vital source of food security in many developing countries in Africa (FAO, 1997). Furthermore, it serves as a main forage for the production of biogas. In world production, maize is ranked as the third major cereal crop after wheat and rice. In 2016, the total cultivated area of maize in Egypt was valued about 0.75 million hectares and produced 6 million metric tons (USDA, 2017). However, the local production of maize is not sufficient to satisfy the domestic consumption, in the same year, Egypt imported about 9 million tons of maize grains to fulfill the national demand (Wally and Beillard, 2017).

Tough a variety of biotic and abiotic stresses affect maize, soil fertility remains one of the main constraints facing the crop productivity, especially in arid and semi-arid countries. Because, nitrogen is considered to be the most critical factor in crop production, the rate of nitrogen application in Egypt is one of the highest rates in the world (FAO, 2005). Maize is a high fertilizer-demanding crop; under Egyptian soil conditions it requires 330–360 kg N ha^{-1} to get an optimum yield (FAO, 2005; Hafez and Abdelaal, 2015). Nevertheless, based on worldwide survey, the N fertilizer recovery efficiency has been found to be around 33% for maize (Krupnik et al., 2004). This means that, large portion of N fertilizer is lost from soil to the environment which not only increases the cost of production but also causes severe environmental problems (Tilman et al., 2002). Introduction of plant growth-promoting rhizobacteria (PGPR) as biofertilizers is a more ecological-friendly and economical approach to manage this problem and can be used as a sustainable option for improving nutrient availability, plant growth, and crop yields (Vessey, 2003).

PGPR are plant-associated bacteria that aggressively colonize the rhizosphere/plant roots, imparting beneficial effects to plants (Panwar et al., 2014). PGPR enhance plant growth both directly and indirectly (Glick, 2012). Generally, PGPR promote plant growth directly by either facilitating nutrient availability and acquisition via N_2 fixation (Ahemad and Kibret, 2014) and P- solubilization (Zaidi et al., 2009) or modulating plant growth by providing or regulating various plant

Peer review under responsibility of Faculty of Agriculture, Ain-Shams University.
E-mail address: Samehheikal@hotmail.com.

https://doi.org/10.1016/j.aoas.2018.04.002
Received 25 February 2018; Received in revised form 18 April 2018; Accepted 25 April 2018

hormones including IAA, abscisic acid, gibberellic acid and cytokinin (Panwar et al., 2014). While, PGPR induce plant growth indirectly by decreasing the inhibitory effects of various pathogens on plant growth via production of antibiotics (Labuschagne et al., 2010), siderophores (Glick, 2012), and induced systemic resistance (Ramamoorthy et al., 2001). PGPR also improve plant defense mechanisms under stressed conditions by producing the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, facilitating plant growth by decreasing ethylene levels, inducing salt tolerance and reducing drought stress in plants (Zahir et al., 2009).

Previous reviews have described the diversity of PGPR in multiple plant species, especially those with agronomical significance (Barriuso et al., 2008; Ahemad and Kibret, 2014). *Azotobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Paenibacillus, Pseudomonas* and *Serratia* (Ahemad and Kibret, 2014) are genera that comprise common PGPR with known benefits on different crop plants. Research on PGPR has been increasing and many experimental studies have been carried out on different crops including maize (Zahid et al., 2015), wheat (Majeed et al., 2015) and rice (Sen and Chandrasekhar, 2014). These studies have shown the potential of PGPR to increase the growth and yield of such crops with minor inputs of agrochemicals.

With the increasing awareness about the economic and environmental concerns of the use of chemical fertilizers, it is important to identify and characterize plant associated rhizobacteria which can be used as potential plant growth promoters to enhance plants fitness and crops productivity. Therefore, the present study aimed at isolation of PGP bacteria from rhizosphere and root- endosphere of some agriculturally important crops (maize, peanut, rice and wheat) grown in Egypt. These bacteria were characterized and screened *in vitro* for their PGP activities and representative isolates were identified using 16S rRNA sequence analysis. The PGP potential effect of high efficient rhizobacteria on the growth promotion of maize plants at early growth stage was investigated under greenhouse conditions.

2. Materials and methods

2.1. Samples collection and bacterial isolation

Plant roots and rhizospheric soil samples from different crops including, maize (*Z. mays*), peanut (*Arachis hypogaea*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) grown in different governorates in Egypt were collected for this study. Soil samples were collected from 15 to 20 cm depth along with plant roots. Rhizospheric bacteria were isolated from 10 g soil tightly adhering to plant roots by serial dilution plating on Burk's N-free agar plates (Wilson and Knight, 1952) as described by Somasegaran and Hoben (1994). For endophytic bacteria, fresh roots were surface-sterilized with 70% ethanol for 5 min, followed by 1% of sodium hypochlorite for 2 min and then washed five times with sterilized distilled water (Kuan et al., 2016). The roots were streaked on Luria-Bertani (LB) agar and nutrient agar plates to check the sterilization efficiency and aseptically smashed with mortar and pestle to isolate the endophytic bacteria on Burk's N-free medium.

2.2. In-vitro screening of PGP activities

2.2.1. Nitrogen fixation by acetylene reduction assay (ARA)

Nitrogen-fixing ability of isolated diazotrophic bacteria was tested using ARA as previously described by Hardy et al. (1968). Pure bacterial colonies were inoculated in 7 mL airtight tubes containing 3 mL semi-solid (0.15% agar w/v) Jensen nitrogen-free medium (Jensen, 1951) and were grown at 28 °C for 72 h. Then after, the tubes were injected by 10% (v/v) acetylene and incubated at 28 °C for 24 h. Ethylene production was measured using Gas Chromatography with flame ionization (GC, DANI 1000, Italy). After completion of the ARA, the cells were predigested by adding 10% SDS and protein concentration was determined as described by Lowry et al. (1951). The rate of N_2 fixation was expressed as the quantity of accumulated ethylene (nmoles C_2H_4 mg^{-1} protein h^{-1}).

2.2.2. Phosphate solubilization

All isolates were screened for solubilization of tricalcium phosphate quantitatively in a liquid medium as described by King (1932). Briefly, bacterial isolates were inoculated in a 25 mL Pikovskaya's broth medium and incubated for 96 h at 28 °C. Bacterial cultures were centrifuged at 15,000 rpm for 30 min. The supernatant (1 mL) was mixed with 10 mL of chloromolibidic acid and the volume was made up to 45 mL with distilled water. Cholorostannous acid (0.25 mL) was added and the volume was made up to 50 mL with distilled water. The absorbance of the developing blue color was read at 600 nm. The amount of solubilized phosphate was detected from the standard curve of a pure substance of KH_2PO_4 (Sigma-Aldrich®, USA).

2.2.3. IAA production

For detection and quantification of IAA production, bacterial colonies were inoculated into LB medium containing 0.5 mg L-tryptophan/mL. The culture was incubated at 28 °C with continuous shaking at 125 rpm for 48 h (Rahman et al., 2010). Approximately 2 mL of culture was centrifuged at 15,000 rpm for 1 min, and a 1 mL aliquot of the supernatant was mixed with 2 mL of Salkowski's reagent (150 mL concentrated H_2SO_4, 250 mL distilled water, 7.5 mL 0.5 M $FeCl_3{\cdot}6H_2O$) and was incubated for 20 min in darkness at room temperature as described by Gordon and Weber (1951). IAA production was observed as the development of a pink-red color, and the absorbance was measured at 530 nm using a spectrophotometer (Thermo Scientific, USA). The concentration of IAA was determined using a standard curve prepared from pure IAA (Sigma-Aldrich®, USA) solutions.

2.2.4. Siderophore production

Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (Acros Organics®, Belgium) as described by Schwyn and Neilands (1987). Briefly, inoculum (10 µl of 10^8 CFU/mL) of bacterial isolates were spotted onto the CAS agar plates and incubated at 28 ± 2 °C for 72 h. Siderophore production was assessed on the basis of change in color of the medium from blue to orange. Quantitative estimation of siderophores was performed by CAS-liquid assay in which 0.5 mL of culture supernatant was mixed with 0.5 mL of CAS reagent (Payne, 1994). Absorbance was measured at 630 nm against a reference consisting of 0.5 mL of uninoculated broth and 0.5 mL of CAS reagent. Siderophore content in the aliquot was calculated by using the following formula:

$$\%\text{siderophore units} = Ar{-}As/Ar \times 100$$

where Ar = absorbance of the reference at 630 nm (CAS reagent) and As = absorbance of sample at 630 nm.

2.2.5. NH_3 production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 48–72 h at 28 °C. Five hundred microliters of Nessler's reagent (Fisher®, USA) were added in each tube. Development of brown to yellow color was a positive test for ammonia production (Cappuccino and Sherman, 1992).

2.3. Bacterial identification

2.3.1. PCR amplification of 16S rRNA

Genomic DNA of bacterial cells was isolated and purified using GeneJet™ Genomic DNA purification Kit (Thermo Scientific®, USA). The procedures were carried out according to manufacture instructions. Primers fD1 and rP2 (Weisburg et al., 1991) were used to amplify a near-full length, approximately 1500 bp fragment of 16S rDNA from those isolates (37) that showed high PGP activities *in vitro*. PCR was