Applied Soil Ecology 87 (2015) 1–10

Contents lists available at ScienceDirect

Applied Soil Ecology

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

Characterization and crop production efficiency of diazotrophic isolates from the rhizosphere of semi-arid tropical grasses of India

C. Sarathambal [a,*], K. Ilamurugu [b], D. Balachandar [b], C. Chinnadurai [b], Yogita Gharde [a]

[a] Directorate of Weed Science Research, Jabalpur, Madhya Pradesh 482 004, India
[b] Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu 641 003, India

ARTICLE INFO

Article history:
Received 13 February 2014
Received in revised form 28 October 2014
Accepted 2 November 2014
Available online 29 November 2014

Keywords:
Grass species
Semi-arid tropics
Diazotrophs
16S rRNA sequencing
Plant growth-promoting rhizobacteria (PGPR)
Rice

ABSTRACT

The search for diverse plant growth-promoting diazotrophic bacteria is gaining momentum as efforts are made to exploit them as bioinoculants for various crops. In particular, the use of strains with multiple plant growth promoting properties would help to increase crop productivity on a sustainable basis. This study investigated the effects of plant growth promoting potential of diazotrophs isolated from rhizosphere of semi-arid tropical grasses and evaluated their inoculation effects on the growth of rice plants under *in vitro* and *in vivo* conditions. The diazotrophic isolates from grass species were characterized for nitrogenase activity by acetylene reduction assay (ARA) and 16S rRNA gene sequencing. The ARA activity of the isolates ranged from 50.83 to 172.25 nmol ethylene/mg protein/h and the putative diazotrophs from rhizosphere of grass species were identified by *nifH* gene amplification. The 16S rRNA gene sequence analysis identified the isolates as belonging to class of alpha Proteobacteria and Firmicutes. Plant growth promoting traits of all the selected diazotrophic isolates were analysed and results revealed that the diazotrophs were found to produce phytohormone, siderophores, HCN, solubilized minerals such as P, K and Zn. Diazotrophs also produced enzyme such as ACC deaminase that can modulate plant growth and development. Based on the presence of multiple plant growth promoting traits, the isolates were selected for inoculation studies. In gnotobiotic experiment, inoculation of diazotrophic isolates significantly improves the growth of rice. In the field experiment, *Serratia* sp. (CB2) and *K. pneumoniae* (CR3) treated plots, grain yields were recorded more by 31 and 28%, respectively, over yield obtained using full doses of fertilizers. This trait of improving growth parameters and yield of rice indicates that the diazotrophs isolated from grass species can be utilized as bioinoculant for rice.

1. Introduction

In the context of increasing global concern for food security and environmental quality, the use of bioinoculants like plant growth-promoting diazotrophs in agriculture is a potentially important issue for more grain yield and to less chemical inputs (Dey et al., 2004; Minorsky, 2008). The improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties, where both rely on soil biological processes and soil biodiversity.

Nitrogen fixation by plant-symbiotic bacteria, an eco-friendly biological process has been effectively exploited for important leguminous crop species. Although associations of diazotrophic bacteria with non-leguminous plants such as grasses have been known for decades (Dobereiner, 1977), they have been less studied in other crop plants except for a few cases; for example, associative bacteria of some tropical species of rice and maize (Reis et al., 2000). Diazotrophs may become selectively enriched to promote plant growth because of their competitive advantage in C-rich and N-poor environments (Cocking, 2005). A more complete understanding of the diversity and function of diazotrophic microorganisms, especially those that have clear relationships with commercially important non-leguminous plant species is of great value for research and application (Doty et al., 2009). The rhizosphere microbiology of native plants is important in view of the *in situ* conservation of the biodiversity associated with such niches to sustain delicate ecological processes in the oligotrophic ecosystem. These diazotrophs could be very useful in the formulation of new microbial inocula and could be applied most profitably to economically important non-legume crops (Cocking, 2005).

For instance, apart from its ability to convert atmospheric dinitrogen (N_2) into ammonia (NH_3) that can be used by plants, *Azospirillum* sp. also possesses an array of other plant growth

* Corresponding author. Tel.: +91 7612353129; fax: +91 7612353129.
E-mail address: saratha6@gmail.com (C. Sarathambal).

http://dx.doi.org/10.1016/j.apsoil.2014.11.004
0929-1393/

promoting traits, such as nutrient solubilization, uptake and enhanced stress resistance (Dobbelaere et al., 2003). In this regard, N_2-fixing bacterias belonging to the genera *Azospirillum*, *Herbaspirillum*, *Burkholderia*, and *Pseudomonas* appear to be frequent colonizers of important crop plants and have been extensively studied (Mirza et al., 2006). Thus, different plant growth promoting bacteria may promote growth of crop plants by several mechanism, *viz.* by synthesizing various phytohormones such as indole 3-acetic acid (IAA), producing siderophores that can provide iron to plants, solubilizing minerals such as P, and synthesizing enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase that can modulate plant growth and development (Lucy et al., 2004). A particular plant growth promoting bacteria may enhance plant growth and development using any one or more of these mechanisms. Inoculation of plants with plant growth promoting bacteria (PGPB) not only increases plant growth but also improves total NPK uptake (Shaharoona et al., 2007).

The association of diazotrophs with rice is one alternative that has been strategically thought to replace part of the N fertilizer required by the plant and in addition, indirectly helping the plant to assess other nutrients added or naturally present in the soil. Mahadevappa and Shenoy (2000) reported that free-living heterotrophic N_2-fixers are potentially important source of N_2-fixation in rice fields, and many researchers have addressed the beneficial effects of N_2-fixing systems on rice growth using different strains under greenhouse and field conditions (Ladha and Reddy, 2003; Muthukumarasamy et al., 2007; Choudhury and Kennedy, 2004).

The rhizosphere of plant species growing profusely under stress-conditions harbors novel diazotrophs to meet their nitrogen requirement as observed in salt marsh grasses such as *Spartina alterniflora*, *Juncus roemerianus* (Bagwell and Lovell, 2000) and *Salicornia virginica* (Bagwell et al., 1998), oligotrophic habitant *Drosera villosa* (Albino et al., 2006) and desert growing *Lasiurus* grass (Chowdhury et al., 2009). Hence, it is widely accepted that the rhizosphere of any plant species is a unique niche harboring diversified bacterial and fungal communities, which serve as potential resource for bioprospecting. Accordingly, present investigation was undertaken to study diverse group of multiple plant growth promoting diazotrophs associated with semi arid tropical grasses and evaluated their inoculation effects on rice under gnotobiotic and field conditions.

2. Materials and methods

2.1. Rhizosphere soil sampling and isolation of diazotrophs

Based on their predominance in each physiographic region, a total of 10 different grass species were sampled and presented in Table 1 (Dixit et al., 2008). Plants were uprooted carefully and the soil adhering to the root was separated in a sterile petri dish and mixed thoroughly so as to make a composite sample for microbiological analysis. Soil samples collected were transported to laboratory in ice box for further analysis (Pramer and Schmidt, 1966). Diazotrophic microorganisms were isolated using serial dilution technique on four selective N-free media *viz.*, N-free malate medium (NFMM) (Piao et al., 2005), LGI-P (Reis et al., 2000), total diazotroph medium (TDM) (Dobereiner, 1989) and junior N-free bromothymol blue medium (JNFb) (Kirchhof et al., 1997). Aliquots (0.1 ml) from the serially diluted samples were added to four different media in petri plates and kept in an incubator at 30 °C. Five days after incubation, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies were picked from the petri dishes and sub-cultured to obtain pure cultures. Stock cultures were made in nutrient broth containing 50% (w/v) glycerol and stored at −80 °C.

2.2. Authentication of diazotrophy

The bacterial isolates grown in N-free media broth for 4 days at 28 ± 2 °C were assayed for nitrogenase activity by ARA using gas chromatograph (Chemito 7610) equipped with flame ionization detector and Poropak-N column by following standard procedure as described by Park et al. (2005).

The presence of *nifH* gene was determined by amplifying the 450 bp fragment using a pair of specific degenerated primers as described by Burgmann et al. (2004). For this, total DNA of the diazotrophs was isolated using the standard protocol of hexadecyltrimethyl ammonium bromide (CTAB) method (Melody, 1997) and dissolved in distilled water for final concentration of 20 ng/μl and stored at 4 °C. The *nifH* amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) with a 25 μl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 μM of each primer (Burgmann et al., 2004), 2.5 mM of $MgCl_2$, and 2.5 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme. PCR amplification was performed in a thermocycler (Eppendorf Master Cycler, Germany) using conditions: initial denaturation at 95 °C for 5 min, 35 cycles consisting of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (primer extension) and final extension at 72 °C for 5 min. The amplified products were analysed by electrophoresis in 1.5 percent agarose gels. After separation of the PCR products in agarose gel, it was viewed and photographed using InGenius gel documentation (Syngene, UK) and analysis system.

2.3. Identification of diazotrophs by 16S rRNA gene sequencing

Nearly full-length of 16S rRNA gene was amplified from elite isolates as described earlier using universal eubacterial primers, FD1 and RP2 (Weisburg et al., 1991) and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using pTZ57R/T vector supplied with TA cloning kit (Fermentas, USA)

Table 1
Details of grass species from different physiographical regions of India used for the present investigation.

Grass species	Sampling site	Latitude	Longitude	Physiographic region
Brachiaria reptans (water grass)	Barrackpur, Kolkata, West Bengal	88° 34′ 5.1″ E	22° 19′ 49.6″ N	Indo gangetic alluvial plain
Cenchrus glaucus (buffel grass)	Chadrapur, Ganjam, Orissa	88° 24′ 22.8″ E	19° 24′ 21.09″ N	Eastern ghats
Saccharum spontaneum (wild sugarcane)	Madan Mahal, Jabalpur, Madhya Pradesh	79° 40′ 50.33″ E	22° 51′ 17.03″ N	Central highlands
Panicum repens (torpedo Grass)	Maruteru, West Godaveri, Andrapradesh	80° 59′ 38.86″ E	16° 30′ 39.7″ N	Deccan plateau
Cyperus rotundus (nut grass)	Chickarasinikere, Mandya, Karnataka	77° 3′ 35.9″ E	12° 17′ 34.78″ N	Reverain land form
Dactyloctenium aegyptium (crowfoot grass)	Kasargod, Kerala	75° 7′ 59.81″ E	12° 24′ 31.4″ N	Kerala plains
Chloris barbata (finger grass)	Thavalakuppam, Pudhucherry	76° 46′ 54.7″ E	11° 23′ 12.6″ N	Coastal plains
Oryza rufipogon (wild rice)	Gudalur, Ooty, Tamil Nadu	79° 51′ 33.1″ E	11° 54′ 32.52″ N	Western ghats
Cyanodon dactylon (bermuda grass)	Navalur kutapattu, Trichy, TamilNadu	79° 46′ 34.9″ E	10° 33′ 21.32″ N	Reverain land form
Setaria verticillata (bristly foxtail)	Thirupoondi, Nagapattinam, Tamil Nadu	79° 53′ 37.6″ E	10° 46′ 25.67″ N	Coastal plains