Microbiological Research 171 (2015) 78–89

Contents lists available at ScienceDirect

Microbiological Research

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

Influence of cyanobacterial inoculation on the culturable microbiome and growth of rice

Himani Priya, Radha Prasanna*, Balasubramanian Ramakrishnan, Ngangom Bidyarani, Santosh Babu, Shobit Thapa, Nirmal Renuka

Division of Microbiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi 110012, India

ARTICLE INFO

Article history:
Received 3 October 2014
Received in revised form
17 December 2014
Accepted 26 December 2014
Available online 3 January 2015

Keywords:
Bacteria
Cyanobacteria
FAME
Microbiome
Rice

ABSTRACT

Rice plants are selective with their associations with bacteria that are beneficial for growth, nutrient uptake, exhibit induced resistance or antagonism towards pathogens. Cyanobacteria as bioinoculants are known to promote the growth and health of rice plants. The present investigation was aimed at understanding whether and how cyanobacterial (*Calothrix elenkinii*) inoculation influenced the rice plant growth and the culturable bacterial populations and identifying the dominant culturable "microbiome" members. The plant tissue extracts were used to enumerate populations of the culturable microbiome members using selected enrichment media with different nutrient levels. About 10-fold increases in population densities of culturable microbiome members in different media were recorded, with some isolates having metabolic potential for nitrogen fixation and phosphorus solubilization. Fatty acid methyl ester (FAME) analysis and 16S rRNA sequencing of selected microbial morphotypes suggested the predominance of the members of *Bacillaceae*. Significant increases in plant growth attributes, nitrogenase activity and indole acetic acid production, and activities of hydrolytic and defense enzymes were recorded in the *Calothrix* inoculated plants. The PCR-based analysis and scanning electron microscopic (SEM) observations confirmed the presence of inoculated cyanobacterium inside the plant tissues. This investigation illustrated that cyanobacterial inoculation can play significant roles in improving growth and metabolism of rice directly and interact with the beneficial members from the endophytic microbiome of rice seedlings synergistically.

1. Introduction

Plants interact with microorganisms from seed to maturity and their relationships range from transient (casual) to symbiotic associations that are either beneficial (mutualistic) to detrimental (pathogenic) (Glick et al. 2007; Mano and Morisaki 2008; Hardoim et al. 2011, 2012; Lucas et al. 2014). Most of these interactions influence growth and development of plants, by altering nutrient uptake dynamics and susceptibility to pathogens (Dobbelaere et al. 2001). The microbial members which can interact with plants depend not only on their functional capabilities, but also the plant attributes which leads to the selection of a beneficial microbiome. Certain traits such as metabolic capabilities and cell wall features play critical roles in the host plant's selection of soil bacteria for colonization (Bulgarelli et al. 2012). Recent evidence suggests that plants and microorganisms co-evolve and, probably, no plant, even under tissue culture conditions, is "microbe-free" (Leifert et al. 1989; Mano and Morisaki 2008; Partida-Martinez and Heil 2011).

Cyanobacteria in rice fields are important microbial members that are employed as bioinoculants for enhancing fertility, improving structure of soils and crop yields (Venkataraman 1972; Kaushik 1998; Prasanna et al. 2012a). These evolutionarily significant photosynthetic bacteria are the primary colonizers in various environments and form associations with other organisms, including higher plants (Rai et al. 2000). Even in rice, colonization of roots and elicitation of defense responses due to cyanobacterial inoculation have been reported (Gantar et al. 1991; Nilsson et al. 2002; Prasanna et al. 2009a, 2013a; Babu et al. 2014; Bidyarani et al. 2014). However, what is not known are the influences of cyanobacterial inoculation on other naturally associated microbial members of rice seedlings. Certain members of microbial genera are found to be specific even to the inside or the surface of rice seeds (Mano et al. 2006). The changes in the diversity and functions of rice microbiome members due to cyanobacterial inoculation are hitherto less studied. This information may help to identify those beneficial microbial members which can be utilized for growth promotion or biological

* Corresponding author. Tel.: +91 11 25847649; fax: +91 11 25846420.
E-mail address: radhapr@gmail.com (R. Prasanna).

http://dx.doi.org/10.1016/j.micres.2014.12.011
0944-5013/

control along with cyanobacteria. The main goal, therefore, was to identify the culturable bacteria which have the potential to act synergistically with the inoculated cyanobacterium.

The present investigation was aimed at analyzing the enrichment samples of cultivable bacterial populations of plant microbiome bacteria and the changes in the plant growth and biochemical parameters, due to cyanobacterial inoculation in rice under the controlled conditions.

2. Materials and methods

2.1. Growth, maintenance and characterization of cyanobacterium

The pure cyanobacterial culture obtained from the Division of Microbiology, IARI, New Delhi, was identified as *Calothrix elenkinii* (RPC1), using both taxonomic keys (Desikachary 1959) and 16S rDNA sequencing (GenBank Accession No. GU292083; Natarajan et al. 2012). The strain was grown and maintained using BG-11(-N) medium (Stanier et al. 1971), at 27 ± 2 °C under light:dark cycles (16:8 h) and intensity of 50–55 μmol photons m^{-2} s^{-2} using cool white fluorescent tubes.

The protein concentrations in the extracellular culture filtrates were determined as described by Herbert et al. (1971) with bovine serum albumin (BSA) as standard. The activity of enzymes such as chitosanase, endoglucanase (β-1,3-glucanase) and carboxymethyl cellulase (CMCase/β-1,4-endoglucanase) were assayed spectrophotometrically using glycol chitosan, laminarin and carboxymethyl cellulose respectively, as the substrates using protocols optimized earlier (Prasanna et al. 2008). One unit (IU) represents one μmol of end product released ml^{-1} min^{-1} under the assay conditions. The concentration of indole acetic acid (IAA) in the culture filtrates was determined spectrophotometrically by the standard method (Gordon and Paleg 1957).

Nitrogenase activity was assayed using 5 ml samples of 2-week old cultures in air-tight glass tubes by the acetylene reduction assay. In each tube, the head space (10% v/v) was replaced with acetylene as the substrate for nitrogenase. The tubes were incubated statically for 24 h at 27 ± 1 °C with light:dark cycles of 16:8 h (white light, 50–55 μmol photons m^{-2} s^{-1}). Three replications per treatment with appropriate controls (without acetylene) were maintained. After incubation, the gas sample (0.1 ml) was removed for the GC analysis in a Bruker 450 Gas Chromatograph with FID. Standard ethylene gas was used for calibration; acetylene reduction to ethylene was calculated and expressed as nmol ethylene produced mg $chlorophyll^{-1}$ h^{-1}.

2.2. Experimental set up with rice plants under controlled conditions

Seeds of rice cv. *Pusa Sugandh 5* were obtained from the Division of Agronomy, IARI, New Delhi. They were surface sterilized using alcohol (70%) for 30 s and then mercuric chloride (0.1%) for 5 min, and later, rinsed several times with sterile distilled water. Surface-sterilized seeds were kept for germination on the sterilized wet blotting sheets. The rice seedlings (4-d old) were transferred into glass containers containing sterilized water agar medium (0.8%) (Fig. S1).

The concentration of chlorophyll in the cyanobacterial culture was determined by the standard method (Mackinney 1941). For the inoculation with rice seedlings, the culture was centrifuged, washed twice with sterile water, diluted to the level of chlorophyll concentration at 5.0 μg ml^{-1} and then used. In the control treatment, the equivalent amount of sterile water was used (Supplementary Fig. 1). The entire setup, separately for two treatments (with or without the cyanobacterial culture) in several replicates, was kept in a growth chamber, maintained at 27 ± 2 °C and illumination of 50–55 μmol photons m^{-2} s^{-2} light intensity (16:8 h of light:dark cycles).

2.3. Analyses of plant and microbiological parameters

Plant growth parameters such as root- and shoot length, fresh and dry weight were measured on the 10-day old seedlings from all the treatments, by sampling three seedlings randomly from each treatment. The chlorophyll content of plant tissues were determined spectrophotometrically at 650 and 665 nm, after extracting with dimethyl sulphoxide (DMSO) as described by Jeffrey and Humphrey (1975) and Sood et al. (2011).

The method of Gordon and Paleg (1957) was used to determine the concentration of IAA in roots and shoots using methanol as an extractant and the concentration of IAA was measured at 535 nm. Acetylene reduction assay (ARA), an index of nitrogenase activity, was measured for the whole plants following the methodology described in Prasanna et al. (2013b). A single plant was placed in a glass vial (15 ml) and sealed with air-tight rubber stoppers. The concentration of ethylene after acetylene reduction was measured gas chromatographically and expressed as nmol of ethylene produced $plant^{-1}$ h^{-1}.

The activity measurements for different defense enzymes were made using the modified procedures, as described in Prasanna et al. (2008, 2013b) using fresh tissues after washing in running tap water. Polyphenol oxidase (PPO) activity was measured using the catechol as the substrate and the enzyme activity was determined at 546 nm. The changes in absorbance were recorded at 30 s intervals for 3 min. One unit of enzyme is expressed IU g^{-1} fresh weight. Phenylalanine ammonia lyase (PAL) activity was assayed in leaf and root extracts (100 μl) and the amount of trans-cinnamic acid formed from L-phenylalanine was measured at 290 nm against the blank. For the blank, distilled water (0.1 ml) was used in place of test sample, and the activity was expressed as nmol of cinnamic acid h^{-1} g^{-1} fresh weight. Peroxidase (PO) activity was also measured using guaiacol (molar extinction coefficient 26.6 mM^{-1} cm^{-1}) as a hydrogen donor. The changes in absorbance at 470 nm were recorded at 30 s intervals for 3 min. One unit of enzyme is expressed as IU g^{-1} fresh weight. The activities of chitosanase, endoglucanase and carboxymethyl cellulase were assayed using glycol chitosan, laminarin and carboxymethyl cellulose as the substrates, respectively as given earlier.

The scanning electron microscopic (SEM) analysis of plant tissues was performed after surface sterilization. Triplicate tissue samples were observed after being cut, prefixed overnight in 2.5% (v/v) glutaraldehyde, washed using 0.1 M sodium phosphate buffer of pH 6.9 for 10–15 min (3 times), and post-fixed using osmium tetraoxide (OsO_4, 1% (w/v)). The fixed cells were dehydrated by sequential passage through increasing concentrations of acetone 30–100% (v/v) with 20% increments up to dry acetone. The samples were dried in a critical point dryer (saturated with CO_2 at a temperature of 40 °C under the pressure of 70 atm), coated with gold palladium for 60 s in a Pelco 3 sputter coater, and visualized under Scanning Electron Microscope (Evo Maio Zeiss, Germany).

2.4. Enumeration and diversity analyses of culturable bacteria from root and shoot tissues

Root and shoot tissue samples were ground using pestle and mortar using normal saline (0.8% NaCl) and suitably diluted aliquots of macerated tissues in sterile saline were plated on to eight different media using the standard spread plate technique. The different media used were: yeast extract mannitol agar, Pikovskaya's agar, soil extract agar, R2A (Reasoner's 2A agar) medium, Jensen's agar,