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ORIGINAL ARTICLE

Isolation and identification of plant growth promoting rhizobacteria from maize (*Zea mays* L.) rhizosphere and their plant growth promoting effect on rice (*Oryza sativa* L.)

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Abstract

The use of plant growth promoting rhizobacteria is increasing in agriculture and gives an appealing manner to replace chemical fertilizers, pesticides, and dietary supplements. The objective of our research was to access the plant growth promotion traits of *Pseudomonas aeruginosa*, *P. fluorescens* and *Bacillus subtilis* isolated from the maize (*Zea mays* L.) rhizosphere. *In vitro* studies showed that isolates have the potential to produce indole acetic acid (IAA), hydrogen cyanide, phosphate solubilisation, and siderophore. RNA analysis revealed that two isolates were 97% identical to *P. aeruginosa* strain DSM 50071 and *P. aeruginosa* strain NBRC 12689 (AK20 and AK31), while two others were 98% identical to *P. fluorescens* strain ATCC 13525, *P. fluorescens* strain IAM 12022 (AK18 and AK45) and one other was 99% identical to *B. subtilis* strain NCDO 1769 (AK38). Our gnotobiotic study showed significant differences in plant growth variables under control and inoculated conditions. In the present research, it was observed that the isolated strains had good plant growth promoting effects on rice.

Key words: genotyping, IAA, phytohormone, rhizosphere, rice

Introduction

The rhizospheric region of plants has a great diversity of microbes i.e. bacteria, fungi, cyanobacteria, which impact plant development both positively and negatively (Berg 2009; Souza *et al.* 2015; Kim *et al.* 2016). This region includes various rhizospheric bacterial species and strains e.g. *Klebsiella pneumonia*, *Pseudomonas putida, Bacillus amyloliquefaciens*, *Azospirillum fluorescens, B. subtilis*, *B. spharricus*, *Azospirillum lipoferum, B. circulans*, and *Paenibacillus azotofixans.* These have plant growth promoting propertiesand enhance the growth of native plants either by the production of phytohormones (Arzanlou *et al.* 2016; Asari *et al.* 2016), metabolites (Kurepin *et al.* 2014), phosphate solubilisation (Altomare *et al.* 1999; Das *et al.* 2003), or by suppression of deleterious organisms by the production of hydrogen cyanide (HCN) and siderophore (a high-affinity iron-chelating compound that enhances plant growth by scavenging iron from the environment and making the mineral available to the cells near the root) as well as antimicrobial enzyme activity (Kejela *et al.* 2016). These plant growth promoting bacteria (PGPB) are termed as plant growth promoting rhizobacteria (Kloepper *et al.* 1989; Glick 1995).

Plant growth promoting rhizobacteria (PGPR) have been used by many researchers (Fatnassi *et al.* 2015; Adediran *et al.* 2016; Huang *et al.* 2016) to analyse their beneficial effects in different areas e.g. bioremediation, biopesticides, biofertilizers, probiotics, antibiotics. Many bacterial genera such as *Pseudomonas*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Bradyrhizobium*, and *Xanthomonas* have been reported as potential phytohormones producing rhizobacteria (Patten and Glick 1996; Karnwal 2009) to help plant growth. *Pseudomonas* and *Bacillus* spp. have been the most studied bacteria for their plant growth promotion (PGP) activity and ability to produce beneficial substances (Kejela

et al. 2016; Pham *et al.* 2017). These bacterial genera have potent PGP characters which include phosphate solubilisation, the production of siderophore, auxins, cytokinin, HCN and antibiotics (Mehta *et al.* 2015; Scagliola *et al.* 2016).

To analyse the plant growth promoting bacterial effect, different research approaches have been used to target the final yield of the plants by short-term *in vitro* studies and medium-term gnotobiotic studies. *In vitro* and gnotobiotic studies typically analyse the effect of PGP variables on plant growth, including shoot and root fresh weight, dry weight or both (Ji *et al.* 2014).

Although cereal crops serve as a part of a nutritious diet malnutrition is stilla widespread issue in under developed countries (Mäder *et al.* 2011). According to the Food and Agriculture Organization of the United Nations (FAO), currently 795 mln people are undernourished globally (FAO 2008). Rice is the chief staple food crop consumed by the world's human population (Gopalakrishnan *et al.* 2013). China and India produce 26% and 20%, respectively, of the world's rice. It has been reported that the world's rice production should be increased by 65% by 2020 in order to keep pace with the projected human population growth (IRRI 1989). This creates an urgent need to explore new agricultural engineering practices in order to produce rice of both sufficient quantity and quality (Chung *et al.* 2015).

The aim of the present study was to analyse PGP traits [the production of HCN, indole acetic acid (IAA), and siderophore as well as phosphate solubilisation] of maize rhizobacteria *in vitro* and their effect on rice growth under gnotobiotic conditions.

Materials and Methods

The present study included two parts: an *in vitro* study and a gnotobiotic study with a target plant (rice).

In vitro study

Isolation of bacterial isolates

For the present study, bacterial species were isolated from the maize (*Zea mays* L.) rhizosphere by following the serial dilution agar plate method of Karnwal (2012). To do so, 10 g maize rhizospheric soil was aseptically collected and transferred to 90 ml sterilized distilled water with proper mixing on a rotary shaker for 5 min (Kumar *et al.* 2012) and 0.1 ml of suspension was transferred to nutrient agar plates in triplicate and incubated at 28±1°C for 24 h. After incubation, isolated colonies were further transferred to pre-sterilized nutrient agar plates for the isolation of pure cultures of bacteria. A total of 80 bacterial colonies were isolated in their pure form and further analysed for morphological, biochemical and PGP activities.

Morphological characterization

All 80 isolates were morphologically and biochemically characterized as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994). Gram staining, urease production, lipolysis activity, gelatine liquefaction, starch hydrolysis, citrate utilization, casein hydrolysis, catalase test, indole, and the production of H_2S, and HCN as well as oxidative-fermentative (OF) reaction were used for characterization of isolates.

IAA production

Indole acetic acid (IAA) production was assayed as described by Patten and Glick (1996). Dworkin and Foster (DF) medium enriched with different concentrations of L-tryptophan (0, 50, 100 and 500 $\mu g \cdot ml^{-1}$) was used for indole production at 28±1°C for 48 h. After appropriate incubation, the bacterial supernatant was isolated by removing bacterial cells from the DF broth by centrifugation at 4,000 rpm for 20 min at 4°C (Karnwal 2009).

One millilitre of preserved supernatant was thoroughly infused in 4 ml of Salkowski's reagent and incubated at room temperature for 20 min until pink (an indicator of indole production). The quantity of indole was measured by comparison with the IAA standard graph at 535 nm. Enzyme-linked immunosorbent assay (ELISA) was used to estimate the production of IAA by bacterial isolates as described by Karnwal (2009).

HCN determination

A modified method of Bakker and Schippers (1987) was used for the production of HCN by bacterial isolates. Nutrient agar medium (NAM) plates enriched with 4.4 g glycine $\cdot l^{-1}$ and Whatman-1 filter paper soaked in 0.5% picric acid in 1% Na_2CO_3 in the upper lids of Petri plates along with uninoculated control were used for the detection of HCN production. Parafilm-sealed Petri plates were incubated at 28±1°C until light, moderate or dark brown which indicated the production of HCN.

Siderophore production

Siderophore production was determined on chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). Twenty-four hour old bacterial culture was streaked on CAS agar and incubated at 28±1°C for 48–72 h. When the medium changed colour from blue to the appearance of an orange or yellow halo around the colonies it was an indication of siderophore production.

Phosphate solubilisation

Phosphate solubilisation was performed by doing spot inoculation of individual bacterial isolates on Pikovskaya's medium (Pikovskaya 1948). The plates were

incubated for 4–5 days at 28±1°C. A clear zone around the bacterial colony was considered as a positive indication of phosphate solubilisation.

16S rRNA sequencing and phylogenetic analysis

On the basis of higher PGP activity a total of five bacterial isolates was selected for the genotypic study. Isolates AK18, AK20, AK31, AK38, and AK45 were completely identified by using the 16S rRNA sequence technique. Selected isolates were grown in nutrient broth on a rotary shaker at 120 rpm for 24 h at 28±1°C. Bacterial genomic DNA was isolated as described by Sambrook and Russel (2001). The universal 16S rRNA primers (8F 5'AGAGTTTGATCCTGGCTCAG3' and U1517R 5'ACGG(A/C)TACCTTGTTACGACTT3') were used for 16S rRNA amplification of bacterial isolates under polymerase chain reaction (PCR). Primers were checked for specificity using the probeBase online utility and the BLAST search facility at the National Center for Biotechnology Information (GenBank database).

Gnotobiotic study

The gnotobiotic study was performed as described by Lifshtiz *et al.* (1987). The results of isolated strains on plant development were investigated in pot experiments. The inoculation experiments were planned in a randomised style with six replications. Surface sterilization of rice (*Oryza sativa* L.) seeds was done by washing with 95% ethanol (v/v) for 20 s, followed by soaking in 20% bleach (v/v) for 10 min and rinsed with sterile distilled water seven times to remove extra bleach. After drying under laminar air flow, surface sterilized seeds were transferred onto half-strength tryptic soy agar (TSA) plates to check for any form of contamination at 28°C for 24 h. Surface sterilized seeds were coated with the selected isolates grown in tryptic soy broth (TSB) for 15 min by using 10% Arabic gum, and then left to dry for 15 min.

Plastic pots, 10 cm wide were filled with a mixture of 3 kg sterilized soil, 100 g sterilized Zonolite and 4 g rock phosphate. Bacteria coated rice seeds were sown in pots under the soil surface and irrigated weekly. Six seeds per pot were sown. The plants were irrigated with sterilized water from time to time to make rich water conditions required for rice cultivation. Thirty days after sowing rice plants were harvested and measured for different plant growth variables like root and shoot length, and root and shoot dry weight (Zafar-ul-Hye *et al.* 2015).

Statistical analysis

Analysis of variance (ANOVA) was used to analyse data results statistically and the differences between various treatments were compared using the Fisher's protected Least Significant Differences test (LSD) at 5% probability level by using SAS (Statistical Analysis System) software.

Results and Discussion

Characterization of bacterial isolates

Morphological and biochemical characterization results of bacterial isolates AK18, AK20, AK31, AK38, and AK45 are given in Table 1. Morphological characterization revealed that all selected five isolates were rod-shaped. AK20, AK31, AK18, and AK45 were Gram-negative while AK38 was Gram-positive. It was observed that AK38 was endospore former and oxidase negative while the other four isolates were endospore negative and oxidase positive. All isolated bacteria produced catalase which was used by all aerobic microorganisms (Elbeltagy *et al.* 2000).

IAA production

Indole acetic acid (IAA) is considered to be the most important auxin that possesses a regulatory effect on plant growth and development. The amino acid, tryptophan played a major role in the production of IAA by rhizobacteria (Aldesuquy *et al.* 1998; Stachecki *et al.* 2004;

Table 1. Morphological characteristics of bacterial isolates from the maize rhizosphere

Isolate	Shape	Gram stain	Spore	Motile	Catalase	Oxidase	OF	Citrate	Indole	Urease	Nitrate reduction	H_2S	Gelatinase	HCN	Starch hydrolysis	Lipolysis
AK18	Rod	–ve	–	+	+	+	+	+	+	–	–	–	+	+	+	+
AK20	Rod	–ve	–	+	+	+	+	+	+	–	+	–	+	+	+	+
AK31	Rod	–ve	–	+	+	+	+	+	+	–	+	–	+	+	–	+
AK38	Rod	+ve	+	+	+	–	+	+	+	–	+	–	+	+	+	+
AK45	Rod	–ve	–	+	+	+	+	+	+	–	+	–	+	+	+	+

OF – oxidative-fermentative reaction

Table 2. Production of Indole acetic acid (IAA) (μg · ml^{-1}) by bacterial isolates at different L-tryptophane concentrations

Isolates	L-tryptophane concentration [μg · ml^{-1}]			
	0	50	100	500
Ak18	0.2	1.4	4.0	9.9
Ak20	0.7	0.7	3.8	8.6
AK31	0.1	1.9	3.2	8.2
AK38	0.2	2.6	6.0	10.0
AK45	0.15	0.45	4.3	9.1

De La Torre-Ruiz *et al.* 2016). All five of the isolates were able to produce IAA in significant quantities with or without L-tryptophan (Table 2). Islam *et al.* (2015) also reported IAA production by cucumber rhizospheric isolates with or without L-tryptophan. All five isolates produced detectable IAA concentrations in medium. In the presence of 50 μg · ml^{-1} of L-tryptophan AK18, AK31 and AK38 produced higher concentrations of indole (range from 1.4–2.6 μg · ml^{-1}) than AK20, AK45 (0.7––0.45 μg · m^{-1}). It was observed by Aldesuquy *et al.* (1998) and Khamna *et al.* (2010) that different concentrations of L-tryptophane play an important role in deciding the concentration of IAA production of microorganisms under *in vitro* conditions. When 100 μg · ml^{-1} L-tryptophan was added to the medium AK38, AK20 and AK45 produced three (4.0 μg · ml^{-1}), five (3.8 μg · ml^{-1}) and eight times (4.3 μg · ml^{-1}) higher concentrations of indole than that produced at the 50 μg · ml^{-1} L-tryptophan concentration while AK31 and AK38 produced two times higher concentrations than 50 μg · ml^{-1} L-tryptophan concentration. A significantly higher concentration of IAA production by AK38 and AK18 was recorded when 500 μg · ml^{-1} L-tryptophan was provided to the isolates. The same pattern of IAA production was recorded in AK20, AK31 and AK45 (Table 2). Similarly, our results were in agreement with previous research reports, that different L-tryptophan concentrations, ranging from 0 to 500 μg · · ml^{-1}, are suitable for growth and IAA production by rhizospheric bacterial species (Karnwal 2009; Karthik *et al.* 2016; Damam *et al.* 2016).

Siderophore production and phosphate solubilisation

Iron is an essential growth element for all living cells. The bioavailability of iron on plant root surfaces or in the soil stimulates competition between soil micro--organisms (Persmark *et al.* 1990). Researchers (Kloepper *et al.* 1989) described the role of bacterial siderophores in stimulating plant growth by increasing the iron availability in the rhizosphere region. It was reported that siderophores could be a source of soluble iron for the host plant that is synthesized by root colonizing microorganisms and help in plant growth.

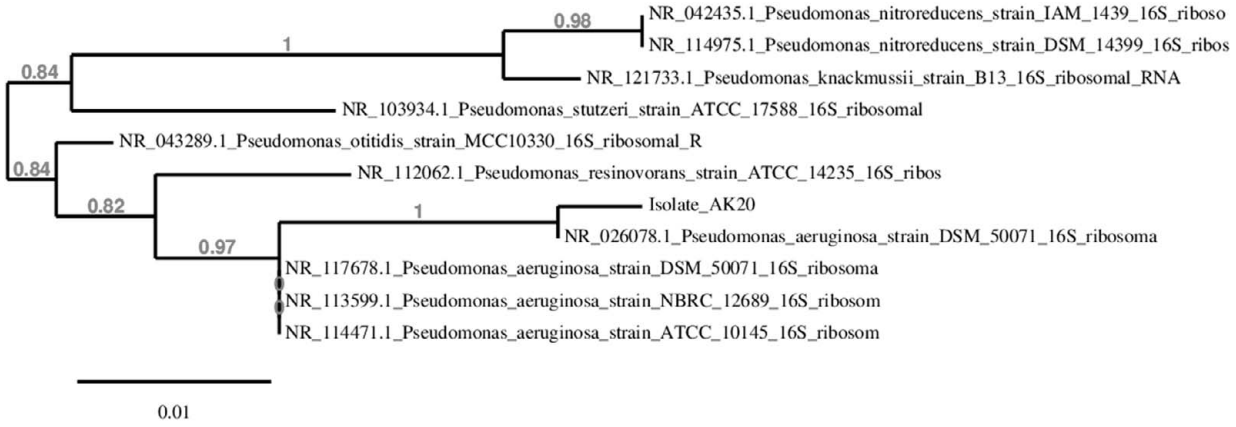
Many rhizobacteria have the ability to produce siderophore and improve the iron uptake ability of plants (Persmark *et al.* 1990; Rajendran *et al.* 2007; Zhao *et al.* 2013; Singh and Jha 2016). In the present study, all five isolates developed an orange halo zone around the bacterial colonies on CAS agar.

Phosphorus is the second most important macronutrient after nitrogen, required by plants for growth. In the environment, most phosphorus is available in an insoluble form that cannot be directly utilized by plants. Various soil bacteria are capable of solubilising mineral phosphates into a plant utilizable form that represents a possible mechanism of plant growth promotion under field conditions (Verma *et al.* 2001; Ashrafuzzaman *et al.* 2009). The primary mechanism involved in phosphate solubilisation is acidification, due to organic acid production by PGPR (Puente *et al.* 2004). All five isolates used in this study formed clear halos around the colonies on Pikovskaya's agar plates supplemented with calcium phosphate as a source of inorganic phosphate. This confirms the solubilisation of inorganic phosphate by bacteria.

16S rRNA sequencing and phylogenetic analysis

BLAST search of the 16S rRNA gene sequence of the isolates showed the maximum sequence similarity with *P. aeruginosa* strain DSM 50071 (98% identical) for isolate AK20 and *P. aeruginosa* strain DSM 50071 (97% identical), ATCC 10145 (97% identical), NBRC 12689 (97% identical) for isolate AK31. This was recorded by phylogenetic tree analysis using MUSCLE algorithm (Fig. 1). The 16S rRNA data of AK18 and AK45 had maximum sequence similarity with *P. fluorescens* strain ATCC 13525 (97% identical) and *P. fluorescens* strain IAM 12022 (98% identical), respectively, as shown in Figure 1. The fifth isolate AK38 showed maximum sequence similarity with *B. subtilis* strain NCDO 1769 (99% identical).

A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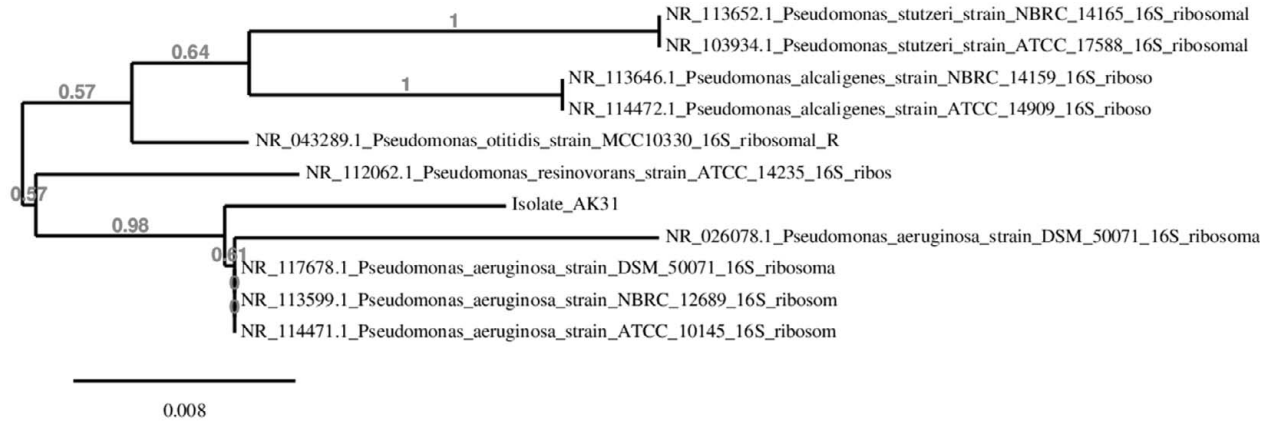
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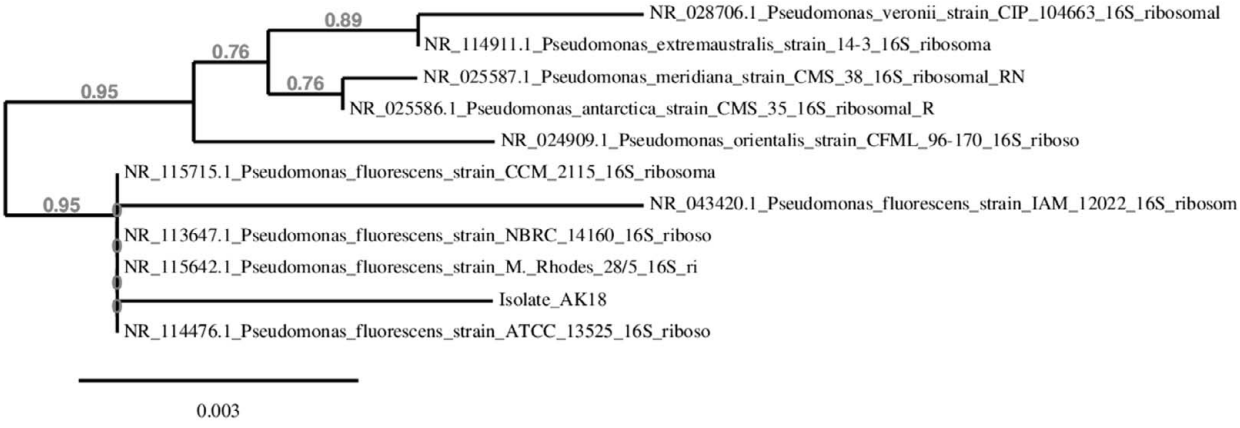
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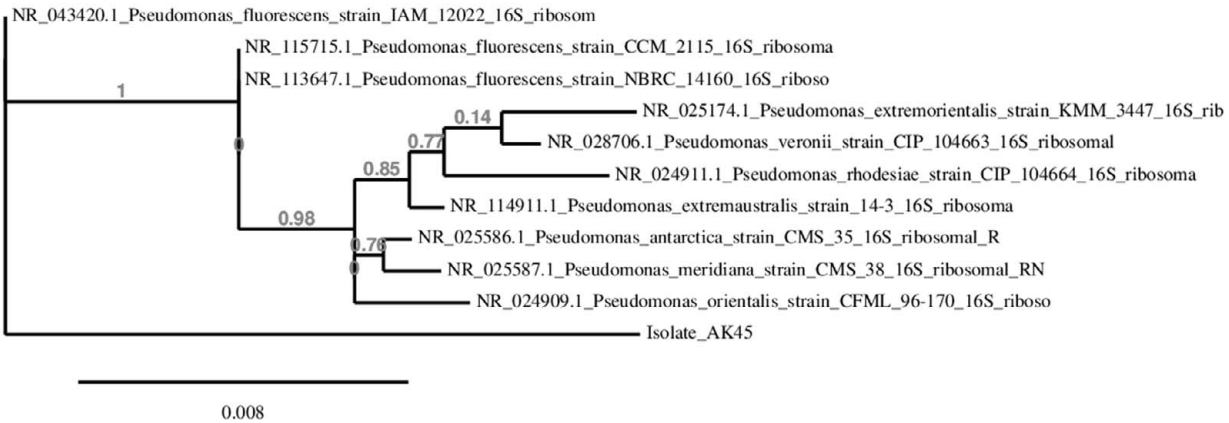
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E

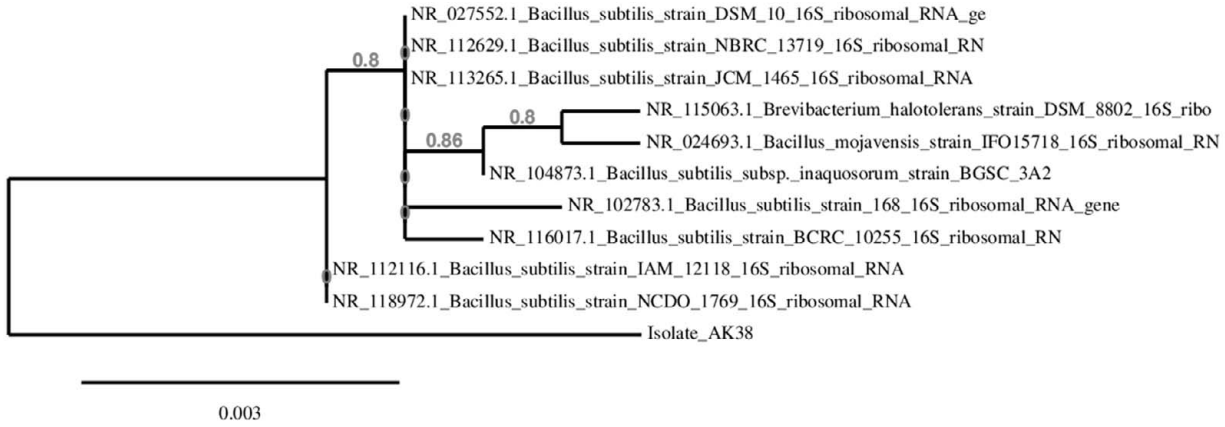

Fig. 1. Phylogenetic tree of bacterial isolates created by using: MUSCLE alignment (A) BLAST similarity search results and phylogenetic tree for isolate AK20; (B) BLAST similarity search results and phylogenetic tree for isolate AK31; (C) BLAST similarity search results and phylogenetic tree for isolate AK18; (D) BLAST similarity search results and phylogenetic tree for isolate AK45; (E) BLAST similarity search results and phylogenetic tree for isolate AK38

Strains of *P. aeruginosa* and *P. fluorescence* were previously reported as plant growth promoting bacteria from wheat, okra, tomato and African spinach (Adesemoye and Ugoji 2009; Islam *et al.* 2014). *Bacillus subtilis* release a number of metabolites which increase the availability of nutrients to plants (Erturk *et al.* 2010).

Gnotobiotic study

Thirty days after sowing, all plants were harvested for analysis and the average of six replicates for each isolate was used for statistical analysis. Results of the gnotobiotics study showed that all five isolates significantly ($p \leq 0.05$) increased the growth of rice compared to the control (Table 3). The shoot length was the longest in AK31 and AK38 inoculated treatments while root length was longest in AK20 and AK31 inoculated treatments. Root dry weight was significantly ($p \leq 0.05$) higher in AK20 and AK18 inoculated treatments while shoot dry weight was significantly higher in AK31 and AK18 inoculated treatments (Table 3). Araujo (2008) observed that *B. subtilis* has the potential to synthesise indole acetic acid which favours plant growth by increasing the number of root hairs. However, there are different mechanisms involved in the beneficial effect on plant growth and development by PGPR. To provide a beneficial effect by PGPR, the colonization of bacteria in the plant rhizospheric zone is the most important aspect. A sufficient population density is another important factor needed to produce beneficial effects under field conditions. The specific mechanism by which PGPR spark plant growth has not been clearly established. However different elements such as phytohormone production, the elimination of pathogenic organisms (Błaszczyk *et al.* 2014), phosphate solubilisation, and favouring the inorganic nutrient uptake are usually believed to be involved (Kloepper *et al.* 1989; Glick 1995; Gopalakrishnan *et al.* 2013).

Table 3. The effects of bacterial isolates on the growth of rice grown in pots under greenhouse conditions

Bacterial isolates	Shoot length [cm]	Root length [cm]	Shoot dry weight [mg]	Root dry weight [mg]
AK18	26.2 e	4.7 de	504.8 d	83.6 b
AK20	24.0 h	6.1 a	403.0 h	97.5 a
AK31	35.2 a	5.1 d	610.1 a	72.6 e
AK38	29.4 d	3.5 ef	421.2 f	77.7 c
AK45	20.1 ij	3.8 e	447.3 e	69.4 ef
Zero control	18.3 j	2.7 f	189.4 i	25.4 f
LSD value	1.80	0.31	5.22	3.78

Means sharing the same letter(s) within a column did not differ significantly ($p \leq 0.05$)

Conclusions

The *P. aeruginosa* strain (AK20 and AK31), *P. fluorescens* strain (AK18 and AK45) and *B. subtilis* strain (AK38) showed potential as PGPB due to their ability to produce IAA and siderophore as well as phosphate solubilisation. Plant growth feedbacks were uncertain and completely dependent on the inoculant bacterial species, plant variety and growth parameters evaluated. Also, the PGPR effect is varied and depends on the bacterial species, its population, plant-bacterial interaction, genotype, growth variables evaluated and environmental factors (Adesemoye *et al.* 2009; Karnwal 2012). In the present study, PGPR applications had desirable effects on plant growth. It revealed that the PGP effect of the tested isolates, including *P. aeruginosa* strain (AK20 and AK31), *P. fluorescens* strain (AK18 and AK45) and *B. subtilis* strain (AK38) in rice, caused significant variations between weight and length of shoot and root in the gnotobiotic study. Further studies under field conditions are necessary to evaluate potential commercial application in rice to improve its growth and development since field conditions are complex, and various biotic and abiotic factors may modify the behaviour of a particular PGPR strain.

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