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Identification of predominant epiphytic and endophytic bacterial isolates in rice seeds effective for enhancement of seed germination and plant growth

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ABSTRACT- During rice growing season, the symptomless rice seeds from different paddy fields in Guilan province, Iran, were collected. After isolation of epiphytic and endophytic bacteria, 39 isolates including 19 epiphytes and 20 endophytes were selected based on the predominant characteristics. Five Operational Taxonomic Units (OTUs) were obtained based on PCR-RFLP of 16S r-DNA in these isolates. According to biochemical tests and partial sequencing of 16S-rDNA, in both epiphyte and endophyte bacteria of rice seeds, the most populated OTUs (V and II) were identified as *Pantoea ananatis* and *Pseudomonas oryzihabitans*, respectively. Six representative isolates from these two OTUs were selected to be evaluated for their abilities for rice seed germination and growth enhancement. Among them, *P. oryzihabitans* was not beneficially effective. However, JpB_1 isolate of *P. ananatis* was considered to be the most effective plant growth promoting isolate, since it showed stable beneficial effects on most surveyed characteristics in both rice seed germination and growth enhancement experiments. Although, OpB_3 isolate of *P. ananatis* produced IAA in higher amount and solubilized phosphate more than the other isolates followed by JpB_1 and *P. ananatis* L_3pB_3, it was not beneficially effective on rice seed germination.
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INTRODUCTION

Host plants harbor different bacterial species. Plant treatment with beneficial bacteria in order to replace or reduce the amount of chemical compounds has been endorsed in several studies (Glick, 1995; Glick et al., 1999; Burdman et al., 2000; Dobbelaere et al., 2003). These microorganisms can perform a pivotal role in this challenge since they have important ecological potentials for plants and soils (Hermosa et al., 2011). Selective application of these potentials can lead to more valuable improvement in agricultural productions. Endophytic bacteria which are considered as non-pathogens are found in different parts of plant internal tissues (Surette et al., 2003). Since they colonize the same niche to that of phytopathogens, they act as useful biocontrol agents and have positive effects on their hosts (Long et al., 2008; Ryan et al., 2008). Moreover, they have been reported to induce resistance in plants against pathogens (Surette et al., 2003). While few bacterial species can be isolated from internal tissues, far more can be found on phyllosphere (Lindow and Brandl, 2003). The large variation in physical and nutritional conditions of phyllosphere explains the considerable variation in bacterial communities (Lindow and Brandl, 2003). Epiphytic bacterial communities of plant vegetative surfaces have been studied numerously, but little has been studied of seeds (Andrews and Hirano, 1991; Hirrano and Upper, 2000; Vacher et al., 2016). Seeds as sources of bacterial transmission to the next generation are of high importance (Cottyn et al., 2001). Seed priming with beneficial bacteria can result in better establishment of seedlings and further improvement in plant growth. In order to decrease the risk of inefficiencies of plant growth promoting bacteria (PGPB) introduced to a new environment, characterization and evaluation of the bacteria associated with the native plants are essential. Most of the studies have so far been limited to rice seed bacterial pathogens or to the bacterial microbiome of

rhizosphere and vegetative parts. In the current study, we aimed to investigate rice seeds for the predominant epiphytic and endophytic bacterial population in Guilan province, Iran, and evaluate them for their abilities of promoting rice seed germination and plant growth through laboratory and greenhouse experiments in order to introduce indigenous PGPBs as rice biofertilizers.

MATERIALS AND METHODS

Isolation and Identification of Bacteria

Bacterial strains were isolated from mature rice seed samples (Hashemi variety) from paddy fields of Guilan province, Iran. Culturable epiphytic and endophytic rice seed associated bacteria were isolated according to Lindow et al. (1978) and Sturz et al. (1997), respectively. Bacteria with prevalent colony features were selected and total DNA from 39 selected bacterial isolates were isolated according to Dellaporta et al. (1983). The universal forward 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 1492r: 5′- GGTTACCTTGTTACGACTT- 3′ primers were applied to amplify the 16S r-DNA in bacterial isolates (Loaces et al., 2011). The final 10 µl reaction mixture contained 3 µl of template DNA, 0.5 µl of $MgCl_2$ (50 mM), 1.2 µl dNTPs (10 mM), 0.6 µl of each forward and reverse primers (10µM), 2.96 µl sterile deionized water and 0.14 µl of *Taq* DNA Polymerase (5 U/µl) (Cinnagen, Iran) in 1X reaction buffer supplied by the same source. The Polymerase Chain Reaction (PCR) procedure was performed in an automated thermal cycler (T Gradient-Biometra, Germany) with the below-mentioned program: an initial denaturation at 94˚C for 4 min, 35 cycles of denaturation (94˚C for 45s), annealing (60˚C for 1 min), extension (72˚C for 2 min) and a final extension at 72 ˚C for 7 min. PCR products electrophoresed on 1.5% agarose gel and visualized under UV light. Amplified 16S r-DNA products were digested by two digestion enzymes *Mse I* and *Hinf I* and the digested PCR products were dispersed on 10% polyacrylamide gel electrophoresis and visualized under UV light. PCR-RFLP (Restiction Fragment Length Polymorphism) profiles were scored as "1" and "0" for the presence and absence of digested bands, respectively, in a binary matrix. Dice correlation index was used to analyze this matrix and clustering analysis was done using UPGMA method with the highest cophenetic correlation coefficient (0.97). The software NTSYSpc Ver2 was used for these analyses (Rohlf, 2009). Identification of bacterial isolates was done through both biochemical and molecular (partial sequencing) methods. Phenotypic characteristics of selected isolates were studied according to Schaad et al. (2001) and Bergey's Manual of systematic Bacteriology (Brenner et al., 2005). The amplified products of 16S r-DNA region were partially sequenced using forward or reverse universal primers 27f and 1492r. Sequencing was performed at Macrogen Sequencing Service, Republic of Korea. Sequence data were submitted to the National Center for Biotechnology Information database (NCBI) with related accession numbers.

Seed Germination Experiment

Surface sterilized rice grains (Variety Hashemi) achieved from Rice Research Institute of Iran (Rasht) were soaked in isolated bacterial suspensions of four representative epiphytes and two endophytes with population density of 10^6 to 10^7 CFU/ml and shaken overnight with 150 rpm. Inoculated grains with sterile distilled water were considered as control. For each isolate, 15 grains in three replications were allocated and were put on sterile filter papers in 100 mm petri dishes and incubated at 28±2 ˚C for six days. Grains moisture was supplied by sterile distilled water during the experiment. At the end of the sixth day, when seedlings were in their imperfect leaf emergence stage (Meier, 1997), seedling shoot and root length in each replication were measured. The fresh weight of separated shoots and roots was measured. After keeping the shoots and roots at 48˚C for 72 h in an electric oven, their dry weights were measured. Seedling emergence was computed according to the following method presented by International Rice Research Institute (2011) at the end of the test:

%Germination= (number of seeds that germinated)/ (number of seeds on the tray) × 100

Germination speed was recorded by counting the number of germinated seedlings each day of the test according to Gupta (1993):

Germination speed= (number of seedling emerging on day)/ (day after planting)

A completely randomized design was used for this experiment and the data were analyzed in the SAS statistical software version 9.0. Comparison of means was done by LSD.

Phosphate Solubilizing Experiment

The ability of bacterial isolates to solubilize phosphate was assayed in National Botanical Research Institute's Phosphate (NBRIP) growth medium (Nautiyal, 1999). After seven days of bacterial incubation at 28 ˚C, bacterial ability to solubilize phosphate was measured according to the visible halo zone around the colonies.

IAA Producing Experiment

To evaluate bacterial ability to produce IAA, Salkowski reagent was used in a colorimetrical method (Glickmann and Dessaux, 1994). The medium consisted of 20 g/l bacteriological peptone, 1.15 g/l K_2HPO_4, 1.5 g/l $MgSO_4.7H_2O$ and another medium with the same contents and supplemented with tryptophan (0.5 g/l) were used for bacterial culture. They were incubated at 28˚C for 48 hours and then centrifuged. One milliliter of supernatant was added to 1 ml of Salkowski reagent (12 g/l ferric chloride [FeCl3] in sulfuric acid [H_2SO_4, 7.9 M]) and kept for 30 min in dark at room temperature. Optical density of each sample was measured at 530 nm by a spectrophotometer (CECIL, England) and the concentration of IAA was compared to a standard IAA curve.

Pot Experiment of Growth Promotion

In pot experiment, surface sterilized rice seeds (Variety Hashemi) were sown on sterile soil in sterile seedling trays and kept at a greenhouse. Irrigation was done using sterile distilled water until the stage of unfolded three leaves (Meier, 1997). Then, seedling roots were put and shaken in selected bacterial suspensions with a population density of 10^6 to 10^7 CFU/ml for one hour at 150 rpm (Sharma et al., 2014) and planted in sterilized pots which were filled by sterile paddy soil. In each pot (replication), three seedlings were planted and three pots were allocated to each isolate. Seedlings without bacterial inoculation treatment were considered as the control. Data collection of the experiment including stem and root length and weight were noted at tillering stage. A randomized complete block design was used for these experiments and the data obtained were analyzed in the SAS statistical software version 9.0. Comparison of means was done by LSD.

RESULTS AND DISCUSSION

From all isolated colonies, a total of 39 bacterial isolates including 19 epiphytes and 20 endophytes with prevalent characteristics of colonies with yellow color and smooth to round margins were selected to be evaluated for their potential for growth promotion. Since a successful PGPB was considered to be able to colonize well and increase its population density in order to effect beneficially on host plant (Ashrafuzzaman et al. 2009), colonies with various characteristics but in less population were discarded and to have some representative isolates from populated species for further experiments, digestion of amplified 16S-rDNA was used as a molecular-based method. This technique has been used by many researchers to differentiate bacterial species (Biswas et al., 2000; Tripathi et al., 2000; Loaces et al., 2011). The amplified 16S r-DNA showed an approximate 1500 bp band in the agarose gel after electrophoresis and a total of five Operational Taxonomic Units (OTUs) resulted from the digestion of this DNA part (Fig. 1).

Based on the combined dendrogram, many isolates from different regions shared the same digestion patterns showing the wide distribution of theses bacteria in rice plants in Guilan province. The most population of bacterial isolates were in OUT V and II with 27 and 8 isolates, respectively. Based on biochemical characteristics and partial sequencing of 16S-rDNA, the bacterial isolates in OUT II were identified as *Pseudomonas oryzihabitans* and the most predominant bacterial community, i. e. OUT V, was identified as *Pantoeaananatis*. Six representative isolates from these two species were selected for further studies. Biochemical test results are presented in Table 1, and Table 2 shows the results of 16S r-DNA sequencing of these six isolates.

Isolation of endophytic and epiphytic *P. ananatis* has been reported from rice seeds (Watanabe et al., 1996; Okunishi et al., 2005; Mano et al., 2006). In the study carried out by Mano et al., (2006), 56% from total of isolated endophytic bacteria in rice seed were isolated from seed epiphytes, as well. In the current survey, *P. ananatis* was isolated both endophytically (40%) and epiphytically (60%) from rice seeds. The next most populated bacteria, *P. oryzihabitans*, was isolated mostly from epiphyte (87.5%) and in less population from endophyte (12.5%). In a survey conducted by Cottyn et al. (2001), most rice seed associated bacteria were identified as *P. oryzihabitans* from epiphyte. The results of seed germination experiment in current research showed that the isolate *P. ananatis* JpB_1 was the most capable isolate in growth promotion which showed higher significant effects on most surveyed characteristics (Table 3).

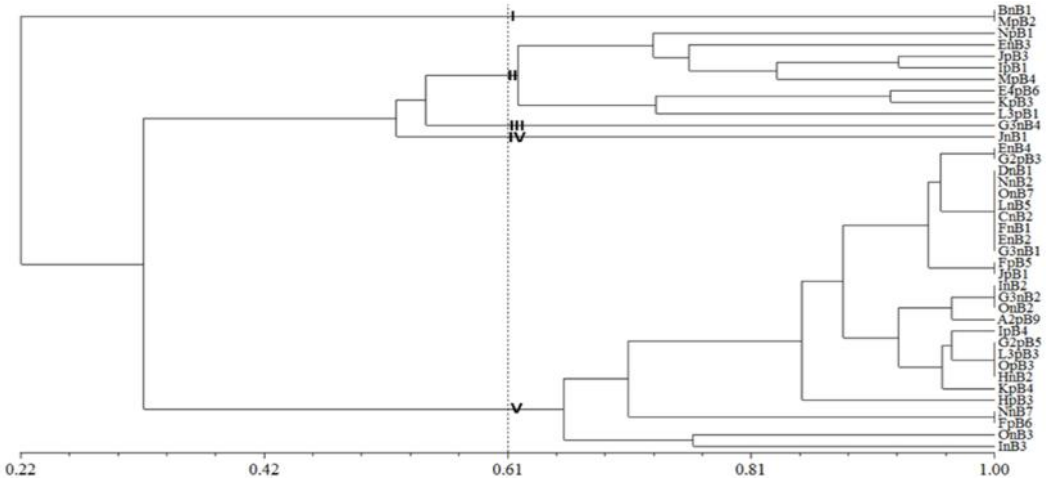

Fig. 1. Dendrogram based on UPGMA cluster analysis of PCR-RFLP pattern of 16S r-DNA profiles. The abbreviations given to isolates represents respectively from left to right: the name of sampling region (A: Astara, B: Astaneh Ashrafieh, C: Anzali, D: Amlash, E: Talesh, F: Roudbar, G: Rasht, H: Rezvanshahr, I: Roudsar, J: Siahkal, K: Shaft, L: Somesara, M: Fooman, N: Lahijan and O: Langeroud), "pB" for seed epiphyte, "nB" for seed endophyte, and the number at the end of each abbreviation shows the number of a special isolate which were selected from the collected isolates from that special regions. I, II, III, IV and V represent the Operational Taxonomic Units 1, 2, 3, 4 and 5, respectively.

Based on the results, it was concluded that different isolates of *P. ananatis* had different potentials to enhance rice seed germination which is due to the difference in their functional interactions with host plant. The same results were reported by Sheibani-Tezerji et al. (2015), where three genetically closely related isolates of *P. anantis* showed different plant growth promoting effects on the host plant. According to our results, *P. oryzihabitans* was less effective than *P. ananatis*, since *P. oryzihabitans* just showed significant beneficial effects on root weight (fresh and dry) and shoot length. Growth promoting potential of some isolates such as *P. ananatis* OpB_3, was not stable. Although it showed high significant beneficial effects in one or two features, it was not effective in other surveyed characteristics, but *P. ananatis* JpB_1 showed a rather stable growth promoting effects in most features which can be considered as an important trait for a plant growth promoting bacteria. None of the isolates were able to enhance seed germination significantly. This was in line with Baset Mia et al. (2012), where no significant difference was observed in seed germination of control and rhizobacterial inoculated rice seeds. Vegetative stage in rice plant is of high value, as it contributes to the establishment of host plant through better tillering (Sharma et al., 2014). In pot experiment, again *P. ananatis* isolates showed considerable positive effects on rice plants growth in different potentials (Table 4).

Beneficial effects of *P. anantis* on rice (Megias et al., 2016) and pepper (Kim et al., 2011) have previously been reported. Production of phytohormones by bacteria is one of the mechanisms that PGPBs apply to increase host plant growth. In this study, in seed germination experiment, the isolate *P. ananatis* OpB_3 which produced the highest amount of IAA was not able to enhance seedling growth as much as *P. ananatis* JpB_1. It is reported that higher amount of the effective concentration level of IAA has no effect on growth or even sometimes suppress it (Peck and Kende, 1995). It is identified that L-tryptophan is the main precursor of IAA biosynthesis in bacteria. All the surveyed bacteria were able to produce IAA in both non-L-tryptophan and L-tryptophan supplemented media, which explains their ability to produce IAA in both pathways (Table 5). However, IAA was produced in higher amount in the media supplemental with L-tryptophan.

In addition to phytohormones, phosphorus is very important in different plant processes leading to growth enhancement. Phosphate solubilizing bacteria can solubilize phosphate from organic and inorganic compounds and make them available for plants to use (Lipton et al., 1987). All the isolates characterized in this study were able to produce a distinct halo zone around the bacterial culture and this clear halo zone was quite larger around the isolate *p. ananatis* OpB_3 (Fig. 2), which was considered as a high solubilizer, followed by *p. ananatis* L_3pB_3 and *p. ananatis* JpB_1 as medium solubilizers (Table 5).

Phosphate solubilizing effects of the isolates were clearly observed in pot experiment where they could increase the root and stem length and biomass of the rice plants.

Table 1. Biochemical characteristics of the most populated bacterial OTUs (II and V)

Biochemical tests	**OTU (II)** ***Pseudomonas oryzihabitans***	**OTU (V)** ***Pantoea ananatis***
Gram	-	-
Oxidase	-	-
Catalase	+	+
Urease	+	-
Indole	-	+
Oxidative growth	+	+
Fermentative growth	-	+
Spore production	-	-
Starch hydrolysis	-	-
Gelatin hydrolysis	+	+
Growth at 41 C	+	-
Fluorescent pigment on KB medium	-	-
Arginine dihydrolase	-	-
levan	-	-
Pectolytic activity	-	-
Yellow mucoid colony on YDC medium	-	-
Utilizing of Glucose	+	+
Lactose	-	+
Sucrose	+	+
Manitol	+	+
Sorbitol	+	+
Maltose	+	+

Table 2. Partial sequencing results of 16S r-DNA gene

Bacterial isolates	Source	Accession number	Closest relative	Similarity
G_3nB_1	endophyte	KX257398	*Pantoea ananatis*	95%
NnB_2	endophyte	KX257399	*Pantoea ananatis*	98%
JpB_1	epiphyte	KX118705	*Pantoea ananatis*	98%
L_3pB_3	epiphyte	KX118708	*Pantoea ananatis*	98%
OpB_3	epiphyte	KX118707	*Pantoea ananatis*	98%
JpB_3	epiphyte	KX611127	*Pseudomonas oryzihabitans*	100%

Table 3. Mean comparison of bacterial isolates effects on rice seed germination

Bacterial isolates	root length (mm)	shoot length (mm)	root fresh weight (g)	shoot fresh weight (g)	root dry weight (g)	shoot dry weight (g)	germination rate	speed of germination
JpB_1	104.2a	46.8bc	0.355a	0.419a	0.044a	0.053a	97.76ab	7.1a
G_3nB_1	93.7b	44.53c	0.33ab	0.351b	0.039ab	0.043b	100a	7.19a
OpB_3	93.16bc	50.13a	0.301bc	0.351b	0.032bc	0.044b	91.1b	6.66b
NnB_2	91.63bcd	48.6ab	0.328ab	0.361b	0.039ab	0.044b	100a	7.22a
JpB_3	89.06bcd	49.3ab	0.326ab	0.351b	0.038ab	0.44b	100a	7.3a
L_3pB_3	85.63cd	44.23c	0.305bc	0.358b	0.039ab	0.039b	100a	7.34a
control	84.46d	44.13c	0.291c	0.35b	0.038c	0.043b	100a	7.13a
L.S.D*	7.59	3.25	0.034	0.032	0.0072	0.0055	7.208	0.36
Standard Error of Means	±2.5	±1.07	±0.011	±0.01	±0.0023	±0.0018	±2.73	±0.118

*Least Significant Differences
*In each column, the means followed by different letters are significantly different at $p<0.05$

Table 4. Mean comparison of bacterial isolates effects on rice growth in sterile condition

Bacterial isolates	root length (mm)	stem length (mm)	root fresh weight (g)	stem fresh weight (g)	root dry weight (g)	stem dry weight (g)
JpB_1	145a	481.6ab	15.46ab	13.93bc	1.9bc	2.43abc
G_3nB_1	140ab	433.3c	11bc	12.56cd	1.76c	2bcd
OpB_3	131.6abc	508.3a	14.96ab	16.53ab	2.73ab	2.66ab
NnB_2	133.3abc	460bc	10.73bc	13.23cd	1.16cd	2bcd
JpB_3	126.6bcd	435c	9.9c	12.7cd	1.06cd	1.83cd
L_3pB_3	116.6d	483.3ab	16.23a	17.26a	2.96a	2.73a
control	125cd	433.3c	6.26c	10.41d	0.65d	1.55d
L.S.D*	14.59	38.1	4.89	3	0.95	0.69
Standard Error of Means	±4.73	±12.3	±1.58	±0.97	±0.31	±0.22

*Least Significant Differences
*In each column, the means followed by different letters are significantly different at $p<0.05$

Fig. 2. The halo zone produced around OpB_3 isolate of *P. ananatis*, a high solubilizer isolate, in NBRIP growth medium.

Table 5. The ability of bacterial isolates to solubilize phosphate and produce IAA

Bacterial isolates	Phosphate solubilizing	IAA production without tryptophan*	IAA production with tryptophan⋆
JpB_1	++	1.64	9.65
G_3nB_1	+	0.47	2.02
OpB_3	+++	1.73	10.86
NnB_2	+	1.11	4.48
JpB_3	+	0.1	0.81
L_3pB_3	++	1.58	8.93

CONCLUSIONS

The most populated bacterial isolates in rice seeds from Guilan paddy fields were identified as *P. ananatis* and *P. oryzihabitans*. The first one was the most predominant bacterium in both epiphyte and endophyte of rice seed. Different isolates of *P. ananatis* showed high potentials to produce IAA and solubilize phosphate. Moreover, they could enhance rice seed germination and plant growth. The isolate *P. ananatis* JpB_1 showed a stable growth promoting effect on most surveyed characteristics in both seed germination and pot experiment, but *P. oryzihabitans* did not show a stable significant beneficial effect on rice growth or seed germination. Therefore and based on the results, *P. ananatis* isolates studied in this survey have the potential to be considered as rice indigenous biofertilizers in order to apply for seed priming.

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شناسایی باکتری‌های شایع رورست و درون رست بذر برنج موثر در تقویت رشد و جوانه‌زنی گیاه برنج

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چکیده- در طول فصل زراعی، بذور برنج فاقد علائم آلودگی از شالیزارهای مختلف استان گیلان، ایران، جمع آوری شدند. از میان ۳۹ جدایه‌ی باکتریایی شامل ۱۹ اپی‌فیت و ۲۰ اندوفیت، پنج گروه تاکسونومی بر اساس هضم آنزیمی محصول PCR ناحیه‌ی 16S r-DNA به دست آمدند. بر اساس آزمون‌های بیوشیمیایی و توالی‌یابی ناحیه‌ی 16S r-DNA در مجموع اندوفیت و اپی‌فیت بذور برنج، پرجمعیت‌ترین گروه‌های تاکسونومی، گروه‌های V و II ، به ترتیب به عنوان *Pantoea ananatis* و *Pseudomonas oryzihabitans* شناسایی گردیدند. شش جدایه‌ی نماینده از این دو گروه جهت ارزیابی توانایی‌شان در تقویت رشد و جوانه‌زنی بذر برنج انتخاب شدند. در بین آنها، *P. oryzihabitans* فاقد اثر افزایشی موثر بود ولی جدایه JpB_1 باکتری *P. ananatis* به عنوان موثرترین جدایه‌ی تقویت کننده‌ی رشد در نظر گرفته شد، زیرا این جدایه اثرات رشدی با ثباتی روی اکثر صفات مورد بررسی در هر دو آزمون جوانه‌زنی بذر و رشد برنج داشت. اگرچه جدایه OpB_3 باکتری *P. Ananatis* میزان بیشتری اکسین تولید نمود و فسفات بیشتری نسبت به سایر جدایه‌ها حل نمود، اما در تقویت جوانه زنی بذر برنج اثرات رشدی موثری نداشت.