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A N I N T E R N A T I O N A L J O U R N A L

Assessment of Rice Associated Bacterial Ability to Enhance Rice Seed Germination and Rice Growth Promotion

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ABSTRACT

The application of beneficial bacteria has recently been used for sustainable agriculture. In current research, 71 bacterial isolates were obtained from rice plant and the rhizosphere soil of different paddy fields in Guilan province, Iran. After primitive investigation, 40 bacteria with typical predominant characteristics were selected. By PCR-RFLP of their 16S r-DNA gene, 8 Operational Taxonomic Units (OTUs) totally consisted of 33 isolates were obtained. From all of them, 8 isolates were selected for rice seed germination experiment, then, effective isolates were used for pot experiment to evaluate their ability for promoting rice growth. All of them were able to increase rice growth and yield, but in different potential. These tested isolates were identified as Alcaligenes faecalis *(DEp_8, O_1R_4),* Pantoea ananatis *(AEn_1),* Bacillus vietnamensis *(MR_5),* Bacillus idriensis *(MR_2) and* Stenotrophomonas maltophilia *by partial sequencing of their 16S r-DNA gene. Among them, AEn_1 and MR_5 produced indole-3- acetic acid (IAA) in larger amounts than the other isolates and the isolates AEn_1 and O_1R_4 were able to solubilize phosphate in higher amounts. According to the results obtained, it can be concluded that AEn_1, O_1R_4 and MR_5 can be considered as bacterial inoculants to use as alternatives for chemical fertilizers.*

Key words: Rice associated bacteria, Plant growth promoting bacteria, PCR-RFLP of 16S r-DNA, IAA production, Phosphate solubilizing bacteria.

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INTRODUCTION

Rice, which is the most important cereal crop and the main food in many countries, provides more than 20% of the consumers' daily calories. About 90% of rice is cultivated in 200 million paddy fields in Asia [19]. In Iran, most rice farms are in the Northern provinces including Guilan and Mazandaran.

Due to the growing demand for rice, one of the increasing concern about rice production in the world especially in Iran is the extra use of chemical fertilizers, which have negative effects on human health and environment [9]. So, there is an urgent need to use the substitutes without or with less harmful side effects. Thus, recently, microorganisms as an inoculants to reduce the use of chemicals are being a matter of interest [13]. For stimulating rice seed germination and rice growth promotion biologically, there are many attempts to find successful bacteria [3, 5, 21, 42].

Rice seed priming with plant growth promoting bacteria (PGPB), as an alternative for chemicals, can be considered to enhance seed germination, vigor index and germination speed [11]. Because germination stage is the most susceptible period in rice life cycle [39]. Rice growth promotion can be achieved by beneficial bacteria but the exact mechanism by which PGPB induce their effects is not clearly understood, however, several hypotheses such as producing phytohormones, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually considered to be involved [8, 14, 26, 29]. Production of phytohormones is one of the important mechanism and indole-3 acetic acid (IAA), which is a member of native auxin, is a common metabolism produced by several beneficial bacteria [31]. Indole-3 acetic acid stimulates cell elongation through enhancement of cell osmotic contents and cell wall synthesis, permeability of water into cell, decrease in wall pressure and inducing protein synthesis, flowering and fruiting [2]. Solubilization of phosphate is one of the other important mechanism the PGPB uses to enhance growth of host plant. A great part of soil phosphorus is in the form of insoluble phosphate and cannot be used by plants [22] and phosphorus deficiency results in small leaves, weak stem and slow development [40]. By considering these facts, the aim of current study is to isolate rice associated bacteria from Guilan's paddy fields, the evaluation of their ability to enhance rice seed germination and plant growth, and investigation of the mechanisms such as IAA production and phosphate solubilization in order to introduce any possible native beneficial bacteria for rice growth promotion as a biological inoculant to use in paddy fields instead of hazardous chemical fertilizers.

MATERIAL AND METHODS

Sample Collection and Isolation of Bacteria

Rice plants (variety Hashemi) including stem, leaf, root and rhizosphere soil were collected from different paddy fields of 16 sites in Guilan province, (37.268° N, 49.589° E, 2 m a.s.l), Iran, including Astara, Talesh, Rezvanshahr, Masal, Fooman, Shaft, Anzali, Rasht, Astaneh Ashrafieh, Lahijan, Langerood, Roodsar, Amlash, Rostam Abaad, Some Sara and Siahkal from June to July 2015. The condition of rice cultivation in paddy fields were continuous irrigation or continuous flow of water (ponding) and the soil type was clayey with the average pH of 6.7. Samples were kept in plastic bags and immediately brought to the laboratory of Rice Research Institute of Iran, Guilan. Samples were kept at 4℃ in refrigerator.

The epiphytic, endophytic and rhizospheric culturable bacteria were isolated according to Lindow *et al*. (1978), Sturz *et al*. (1997) and Mohite (2013), respectively. For isolation of epiphytic bacteria, rice samples of leaves and stems were cut in to small sizes and separately put in 500 ml flasks with 200 ml of 0.1 M

phosphate buffer (pH:7.0) and 1 g/l peptone. Flasks were shaken for about 2h at 150 rpm. For endophytic isolation, stems, leaves and roots of rice were washed thoroughly under tap water and rinsed with deionized water. Surface sterilization of samples was done with 5% sodium hypochlorite solution for 5 minutes followed by 3 times washing with sterile distilled water. Then samples were cut into small pieces with sterile scalpel and were ground well with a sterile pestle and mortar. Rhizospheric bacteria were isolated by keeping the clay firmly adhering the roots and removing the rest. Ten grams of this clay from each sample were suspended in 90 ml of sterile distilled water in a 250 ml flasks and shaken for about 20 min at 120 rpm. Serial dilutions of all suspensions were made up to $1/10^5$ and an aliquot of 100 µl from each dilutions were spread on Nutrient Agar (NA) medium. Single colonies were picked up after 3 days of incubation at 28℃ and streaked on fresh plates to get pure colonies.

Colony Characteristics

Colony characteristics such as shape, color, margins and gram staining were carefully studied. The final isolated bacteria were selected from the bacteria with typical predominant characteristics on NA medium. Thus, the redundant isolates with different characteristics but in few population were discarded.

DNA Extraction and Amplification of 16S rDNA

Total genomic DNA of bacteria in each group was extracted from freshly grown cultures on NA medium according to the method described by Murry & Thompson (1980). Quantification of extracted DNA was done on 1% agarose gel electrophoresis. Universal primers 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492r: 5′- GGTTACCTTGTTACGACTT- 3′ were used for amplification of 16S rDNA region. The final reaction mixture was 10 µl, contained of 0.5 µl of magnesium chloride [$MgCl_2$], 1 µl of PCR buffer, 1.2 µl of dNTPs, 0.6 µl of each forward and reverse primers, 2.96 µl sterile deionized water and 0.14 µl of Taq DNA Polymerase (Cinnagen, Iran). Polymerase Chain Reaction (PCR) amplification were carried out on 3 µl of DNA in an automated thermal cycler (T Gradient-Biometra, Germany) with following program: an initial denaturation in 94℃ for 4 min, 35 cycles of denaturation (94℃ for 45s), annealing (60℃ for 1 min), extension (72℃ for 2 min) and final extension in 72℃ for 7 min. PCR product size was confirmed on 1.5% agarose gel electrophoresis and photographed under UV light.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of 16S rDNA

This method was used to cluster bacterial isolates in groups with most similarity according to their restricted bands. Digestion of the amplified 16S rDNA was carried out by two recognizing enzymes: Mse I and Hinf I. The final reaction mixture was 15 µl, contained of 8 µl of sterile deionized water, 1.5 µl of 10X buffer with 5 µl of the PCR products subjected to digest with 5 units (5 µl) of each enzyme at 37˚C for 30 to 60 min. The restricted DNA fragments were separated on 10% polyacrylamide gel (140 V for 2 h) stained in ethidium bromide and photographed under UV light. The results were converted into a binary matrix indicating the presence and absence of restricted bands. The analysis of this matrix was done using SM (Simple Matching) correlation index and clustering analysis was performed using Complete method. The correlation index and clustering analysis method was performed according to the highest cophenetic correlation coefficient (0.8486). The software NTSYSpc Ver2 was used for these analyses.

Sequencing of 16S r-DNA

The 16S r-DNA gene of tested bacterial isolates, were partially sequenced using forward or reverse universal primers 27f and 1492r (used in PCR above) to identify the isolates. The sequencing reaction were done at Macrogen Sequencing Service, Republic of Korea and sequences were submitted to the National Center for Biotechnology Information database.

Rice Seed Germination Test

Rice seed (Variety Hashemi), obtained from Rice Research Institute of Iran, Guilan, were surface sterilized by 5% sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. Then, they were soaked in different bacterial suspensions including 4 bacteria isolated from rhizosphere (O_1R_4, MR_5, MR_2 and KR_1), 3 from rice endophyte (AEn_1, AEn_4 and FEn_1) and 1 from rice epiphyte (DEp_8), with sufficient population density of 10^6 to 10^7 cfu/ml and shaken overnight in 150 rpm. Seeds were soaked in sterilized distill water considered as control. For each isolate, 45 seeds in 3 replications were allocated and in each replication, 15 seeds were put on sterile filter paper in 100 mm petri dishes. They were irrigated by sterilized distill water during 6 days of incubation at 28±2℃.

In the stage of imperfect leaf emergence (code 09) [6], shoot and root length of all seedlings in each replication were measured in mm scale. For weight measurements, shoot and root were separated and weighted as fresh weight and dry weight after keeping at 48℃ for 72 h in an electric oven. Weight measurements was done by digital weight scales and noted in gram.

Germination rate or seedling emergence was calculated using the following formula proposed by International Rice Research Institute (2011) on last day of experiment:

$$\%germination = \frac{\text{number of seeds that germinated}}{\text{number of seeds on the tray}} \times 100$$

Vigor index of seedling were measured on last day of experiment (day 6) according to the formula proposed by Abul-Baki & Anderson (1973):

$$seed\ vigor\ index = germination\ (\%) \times seedling\ length$$

The speed of germination was calculated by counting the number of emerged seedlings every day of experiment according to Gupta (1993):

$$speed\ of\ germination = \frac{\text{number of seedling emerging on day}}{\text{day after planting}}$$

The experiment was performed in a completely randomized design. Data were statistically analyzed with SAS version 9.0 and the comparison of means was done by LSD.

Growth Promotion in Pot Experiment

More effective isolates on enhancement of rice seed germination were selected for evaluation of their effects on growth and yield of rice. Seedling trays were sterilized with 5% sodium hypochlorite and washed thoroughly by sterile distilled water. They were filled with autoclaved soil and surface sterilized rice seed (Variety Hashemi) were sown for nursery raising. Seedling trays were irrigated by sterile distilled water until the stage of unfolded 3 leaves (code 13) [6]. Rice pots sterilized with 5% sodium hypochlorite were filled with autoclaved paddy field soil and irrigated by sterilized water. For each isolate 6 pots (replications) and for each pot, 3 seedlings were allocated. Before planting, rice seedling roots were dipped and shaken in selected rhizobacterial suspension (the bacteria isolated from rice rhizosphere) with a population density of 10^6 to 10^7 cfu/ml for one hour in 150 rpm to let bacteria colonize roots (Sharma *et al*. 2014) and the phyllosphere spraying of bacterial suspension (the epiphytic and endophyte bacteria isolated from rice leaf and stem)

was done after planting for epiphyte and endophyte bacterial colonization. Sprayed inoculated seedlings were wrapped up with plastic bags for 24 hours to make a humid condition for better colonization of bacteria. Seedlings without bacterial inoculation considered as control. Data collection of rice plant growth including stem and root length and weight were done by tillering stage (code 29) [6], for 3 pots (replications) and the other 3 pots were remained to evaluate the bacterial effects on rice yield. Yield data collection including plant height and weight, flag leaf area, number of tillers, number of panicle, panicle length, weight of 1000 grain and number of full grain were done according to standard evaluation system for rice [20] at ripening stage (code 89) [6].

The pot experiment was done in a randomized complete block design and the data obtained were subjected to analysis with SAS version 9.0. The significance of differences between mean values were evaluated by LSD.

Phosphate Solubilizing Ability

The phosphate solubilizing ability was studied by plate assay using the National Botanical Research Institute's Phosphate (NBRIP) growth medium [38]. The medium was consisted of 10g glucose, 5g calcium phosphate [$Ca_3(PO4)_2$], 5g magnesium chloride [$MgCl_2$], 0.25g magnesium sulfate [$MgSO_4$], 0.2g potassium chloride [KCl], 0.1g ammonium sulfate [$(NH_4)_2SO_4$] and 15 g/l agar in 1 liter. The pH was adjusted to 7.0 before autoclave. Plates were kept at 28℃ and formation of visible halo zones in NBRIP media around bacterial colonies after 7 days showed the ability of solubilizing phosphate .

Production of IAA

The presence of IAA in bacterial metabolites and the amount of IAA production was colorimetrically determined by Salkowski reagent [15]. Bacterial isolates were inoculated in broth containing 20g/l bacteriological peptone, 1.15g/l dipotassium phosphate [K_2HPO_4], 1.5g/l magnesium sulfate [$MgSO_4$.7H2O] and the same broth supplemented by tryptophan (0.5g/l). Bacterial grown cultures were centrifuged after 48 hours incubation at 28℃ and 1ml of supernatant was mixed with 1ml of Salkowski reagent (12 g/l ferric chloride [$FeCl_3$] in sulfuric acid [H_2SO_4, 7.9 M]). After 30 min of dark incubation at room temperature, the optical densities were measured at 530 nm by spectrophotometer (CECIL, England). The concentration of IAA production was estimated by a standard IAA curve.

RESULT

Isolation and Colony Characteristics

A total of 71 pure bacterial isolates were obtained and their morphological study showed typical bacterial characters. Their color varied from yellow to pink, milky and orange. Their shape was mostly rounded with some smooth or undulate margins and gram test separated them in to two major groups. Due to these typical predominant characteristics the total number of bacteria reduced to 40 and the rest with many diverse characteristics but in fewer population were removed.

Amplification of 16S r-DNA and PCR-RFLP

Amplification of all 40 bacterial 16S r-DNA genes produced single band around 1500 bp. After digestion of this amplified genes with restriction enzymes, the bacterial isolates were grouped according to their restricted patterns. Eight Operational Taxonomic Units (OTUs) including 33 bacterial isolates were found, whereas 7 isolates showed unique patterns (Fig 1). According to PCR-RFLP results,

from OTU (I), with higher number of isolates, two representatives (DEp_8 and OR_4) and from each one of II, III, IV, V, VI and VIII OTUs, one representative isolate including (FEn_1, AEn_4, MR_5, MR_2, KR_1 and AEn_1) were selected respectively for evaluation of their ability to enhance rice seed germination.

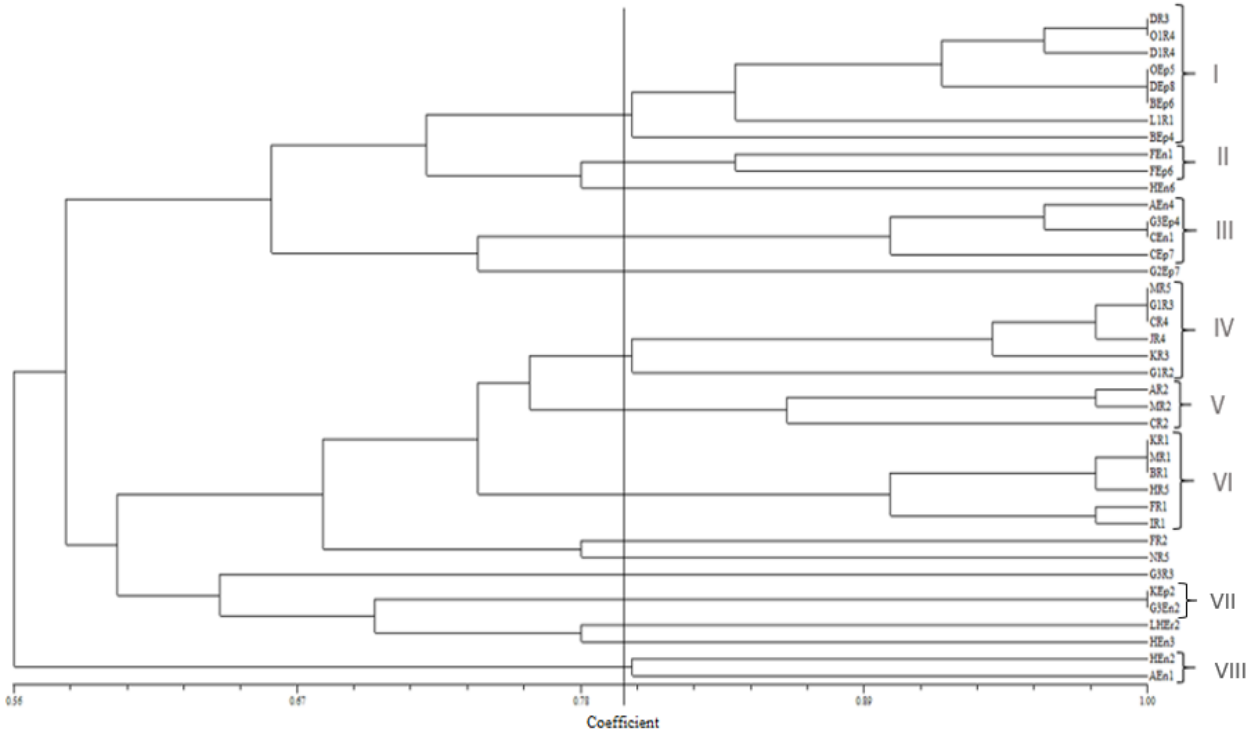

Figure 1. Dendrogram based on UPGMA cluster analysis of PCR-RFLP pattern of 16S r-DNA profiles showing the relationships among the isolated bacteria.

Rice Seed Germination

Seed inoculation with 8 bacterial isolates from 8 groups resulted in increased seedlings' length and vigor index compared to un-inoculated control. In all treatments, the increase in shoot length were higher than control but in root elongation, just the isolate KR_1 showed similar effect with control. The highest effect on shoot and root length obtained from the seedlings inoculated with isolates DEp_8 and AEn_1 respectively followed by O_1R_4 in shoot length and FEn_1 in root length. Isolates KR_1, MR_2 and AEn_4 could not increase the shoot fresh and dry weight more than control and O_1R_4 was the isolate which had the most effect on enhancement of shoot fresh and dry weight. The root fresh and dry weight of seedlings inoculated by MR_2 were the highest but AEn_4 could not increase fresh weight of roots. Comparing the treated seeds and non-treated seeds, there was not a considerable difference in germination rate and the result of data analysis showed that MR_5, MR_2, O_1R_4, DEp_8 and FEn_1 treated seeds had the same germination rate but some of the seeds in KR_1, AEn_1 and AEn_4 treatments did not germinate. Speed of germination which computed by counting the number of germinated seeds at an interval of 24 h to the day number of counting from the beginning day of experiment, were faster in seeds inoculated by isolates MR_2, OR_4 and FEn_1 respectively than un-inoculated seeds and vigor index of all seeds were enhanced by all bacterial treatments except the isolate KR_1 (table 1).

Table 1. Mean comparison for the effect of different bacterial isolates on enhancement of rice seeds

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	seed vigor index
OR_4	96.8b	53.6a	0.330bc	0.427a	0.039cd	0.051a	100a	7.40ab	15040ab
MR_5	89.8d	47.5bc	0.349ab	0.353bc	0.040bc	0.046bc	100a	6.64c	13730cd
MR_2	95.6bc	47.7bc	0.366a	0.327cd	0.044a	0.039e	100a	7.55a	14330bc
KR_1	84.4e	46bc	0.326bcd	0.314d	0.038d	0.042de	97.76ab	6.80c	12758e
AEn1	103a	48.7b	0.348ab	0.362b	0.042ab	0.044cd	95.53b	6.69c	14503bc
AEn4	89.2de	45.5bc	0.252e	0.312d	0.033e	0.039e	95.53b	6.61c	12866ed
DEp8	92cd	554a	0.300cd	0.376b	0.037d	0.048ab	100a	7.12b	14740ab
FEn1	101.8a	53.3a	0.350ab	0.409a	0.042abc	0.049ab	100a	7.26ab	15510a
control	84.4e	44.1c	0.291d	0.350bc	0.028f	0.043cd	100a	7.13b	12860ed
L.S.D*	4.745	4.148	0.035	0.031	0.002	0.003	3.831	0.288	914.42
StandardError	±1.597	±1.396	±0.011	±0.010	±0.0008	±0.001	±1.28	±0.096	±307.7

The abbreviations given to isolates represents respectfuly from left to right: the name of sampling site (O for Langerood, M for Fooman, K for Shaft, A for Astara, D for Amlash and F for Rostam Abaad),, the part of rice plant the bacteria isolated (R for rhizosphere, Ep for epiphyte and En for Endophyte) and the number at the end shows the number of isolate

*Least Significant Differences

Growth Promotion in Pot Experiment

From 8 isolates studied in seed germination test, the isolates KR_1 and AEn_4 which had less effects on evaluated traits, were discarded and the rest were used for pot experiment. At tillering stage (code 29) [6], 3 pots were collected and the other 3 pots were left for yield increasing evaluation. The results of seedling inoculation with selected bacteria showed significant increase in all investigated characters including root and stem length and weight compared to un-inoculated seedlings. The isolate O_1R_4 showed the highest consistency in ability to enhance growth in almost all traits except the root length which the highest was for isolate MR_2 followed by FEn_1. The isolates MR_2, DEp_8 and AEn_1 were in second place after O_1R_4 in increasing each evaluation of root and stem fresh and dry weight (table 2).

Table 2. Mean comparison for the effect of different bacterial isolates on rice growth promotion

Bacterial isolates	root length (mm)	stem length(mm)	root fresh weight(g)	stem fresh weight(g)	root dry weight(g)	stem dry weight(g)
OR_4	133.3bcd	500a	19.1a	18.7a	4.11a	3.12a
MR_5	130cd	463.3cd	15b	14.1c	1.68c	2.32bc
MR_2	155a	475bc	15.1b	15.7b	2.03c	2.63ab
AEn_1	138.3bc	481.6b	11.9d	16b	2.24bc	2.58ab
DEp_8	130cd	458.3d	13.2c	13.8c	2.79b	2.12bcd
FEn_1	143.3ab	473.3bc	9.58e	12.3d	0.97d	1.88cd
control	125d	433.3e	6.2f	10.4e	0.65d	1.55d
L.S.D*	11.88	14.88	0.90	1.40	0.65	0.61
StandardError	±3.85	±4.83	±0.29	±0.45	±0.21	±0.19

*Least Significant Differences

The evaluation of rice yield in 3 left pots were studied at the end of ripening stage. Many traits were measured and the inoculated bacteria were different in their potential to enhance rice yield. Plant height, which were measured from the soil surface up to the end of the longest panicle, were the highest in plants inoculated by the isolates AEn_1 and DEp_8 followed by MR_5. The isolate AEn_1 also produced the most number of full grains in inoculated rice plants compared to other isolates and un-inoculated control. Plants inoculated by MR_5 produced the highest weight of 1000 grain and also the highest plant and panicle fresh and dry weight but in the evaluation of increasing flag leaf area, MR_5 could not increase the area of flag leaf more than control. The number of panicles and tillers were significantly the highest for isolate DEp_8. The isolate O_1R_4 showed the highest consistency in ability to increase panicle length and flag leaf area, but O_1R_4 with 3 other isolates, AEn_1, FEn_1 and DEp_8 could not increase plant and panicles fresh and dry weight more than control (table 3).

Table 3. Mean comparison for the effect of different bacterial isolates on rice growth yield

Bacterial isolates	Plant height(mm)	Flag leaf area(mm^2)	Number of tillers	Panicle number	pa.l(mm)	p.f.w	p.d.w	pa.f.w	pa.d.w	w.1000.g(g)	n.f.g
OR_4	1128bc	243.6a	17.6cd	16.3c	276.6a	81.3b	28.7ab	26.3d	24.5d	23.5ab	51.6cd
MR_5	1145a	152.6e	20b	18.6b	271.6ab	88.9a	29.3a	32.7a	29.4a	24a	53.6bc
MR_2	1143ab	181.6b	19.6bc	18.6b	250d	79.9b	29.1ab	30.3b	27.9abc	23.5ab	54.6b
AEn_1	1146a	186.1b	20b	18.6b	251d	70.3c	26.2cd	28.9bc	26.3cd	23.9ab	58.3a
DEp_8	1146a	178.5bc	22.6a	22a	266.6abc	71.7c	27.3bc	27.3cd	21.7e	22.4b	54.3bc
FEn_1	1116c	167.3cd	20b	18.3bc	255cd	79.4b	25.3d	30.4ab	28.8ab	23.9ab	50.6d
control	1113c	155.6de	16d	16.3c	260bcd	78.9b	26.6cd	30.2b	26.6bcd	19.8c	30.6e
L.S.D*	16.06	13.57	2.17	2.29	12.50	2.68	1.84	2.28	2.39	1.58	2.68
Standard Deviation	±5.21	±4.4	±0.7	±0.74	±4.05	±0.87	±0.59	±0.74	±0.77	±0.51	±0.86

*Least Significant Differences

Identification of Bacterial Isolates

Identification of tested bacteria in pot experiment were done through partial sequencing of their 16S r-DNA genes. They were identified as *Alcaligenes facealis* (O_1R_4 and DEp_8 from the same OTU), *Stenotrophomonas maltophilia* (FEn_1), *Pantoea ananatis* (AEn_1), *Bacillus vietnamensis* (MR_5) and *Bacillus idriensis* (MR_2). The information of these isolates with related accession numbers are presented in table 4.

Table 4. Identification of the tested isolates based on partial sequencing of 16S r-DNA gene

Bacterial isolates	Source	OTU	Accession number	Closest relative	Similarity
DEp_8	rice epiphyte	I	KX118704	*Alcaligenes faecalis* SY1	95%
AEn_1	rice endophyte	VII	KX257397	*Pantoea ananatis* Hb-9	98%
MR_5	rice rhizosphere	IV	KX247387	*Bacillus vietnamensis* KNUC511	96%
O_1R_4	rice rhizosphere	I	KX118706	*Alcaligenes faecalis* SK12	95%
MR_2	ricr rhizosphere	V	KX247386	*Bacillus idriensis* M50	98%
FEn_1	rice endophyte	II	KX247388	*Stenotrophomonas maltophilia* DD73	95%

Phosphate Solubilizing Ability

All of six bacterial isolates were able to solubilize phosphate and produced clear halo zones around bacterial colonies on NBRIP medium. According to the halo zone size

the isolates AEn_1 (*P. ananatis*), O_1R_4 and DEp_8 (*A. facealis*) were high solubilizer, but FEn_1 (*S. maltophilia*), MR_2 (*B. idriensis*) and MR_5 (*B. vietnamensis*) solubilize phosphate in lower amount (table 5).

Production of IAA

The bacterial ability to produce IAA was determined by changing the color of bacterial broth after addition of Salkowski reagent and this color change was measured by spectrophotometer. All tested bacteria produced IAA in both media, but, tryptophan supplemented media had higher readings than the media without tryptophan. The amount of IAA was the highest in *B. vietnamensis* (MR_5) which followed by *P.ananatis* (AEN_1). The results are shown in table 5.

Table5. Phosphate solubilizing and IAA production ability of bacterial isolates

Bacterial isolates	Phosphate solubilizing	IAA production without tryptophan	IAA production with tryptophan*
DEp_8	+++	0.25	3.75
AEn_1	+++	1.43	7.5
MR5	+	1.51	8.75
O_1R_4	+++	0.06	1.25
MR_2	+	0.08	1.87
FEn_1	+	0.20	2.5

low solubilizer (+),high solubilizer (+++)

*IAA concentration (µg/ml)

DISCUSSION

Different bacteria can be found in association with rice plant. They have the ability to enhance rice growth and because of this potential they can be used as biofertilizers [33]. In current research, many bacterial isolates were obtained from rice plants including endophytes, epiphyte and rhizosphere bacteria. At first, primitive investigations of bacteria, which is a common way of partial bacterial characterization [4, 37, 42] was done. In this way, the bacterial population density with very different characteristics can be discarded and the total population density is consisted of some nearly homogeneous bacterial groups that share same color, shape or gram reaction.

The studied bacteria were from different geographical locations and from different parts of rice plants and this was reflected in 16S r-DNA digestion with restriction enzymes. The final phylogenetic tree showed that the isolates were rather divers and from 40 isolates, 7 OTUs representing 31 isolates at the similarity of about 80% were obtained. Fingerprinting of 16S r-DNA has been used by many researchers to discriminate bacterial isolates genetically [7, 12, 45]. In such a similar practice, Loaces *et al*. (2011), found 13 OTUs consisting of 55 isolates from 109 total isolates, whereas the rest showed unique restricted patterns indicating that the population density was quite variable .

To find bacteria with growth promoting effects on rice plants, one or two isolates from each OTU were selected for rice seed germination test. Seed priming or seed inoculation by beneficial bacteria has become a newly acceptable substitute for sustainable agriculture [11]. The result of seed germination in current study showed that seedling length and weight can be increased by the inoculation with most of tested bacteria. Two isolates including KR_1 and AEn_4 could not affect rice seed germination beneficially significant compared to control and the other isolates. In

many literatures, the increase in seedling length and weight is attributed to bacterial phytohormones [5, 11, 41]. Among different phytohormones, indole-3-acetic acid (IAA) is usually considered as the most important native auxin. Production of IAA can vary in different species and strains and it is influenced by culture condition, growth stage and substrate availability [34]. Tryptophan is considered as a precursor for IAA biosynthesis, because IAA producing bacterial culture supplemented by tryptophan produced more concentration of IAA [10]. Tryptophan dependent IAA biosynthesis had been also reported in many other bacteria. Tien *et al*. (1979) reported that *Azospirillum* was able to produce IAA when exposed to tryptophan or Karnwal (2009) tested fluorescent *pseudomonas* production of IAA in the absence and presence of tryptophan and showed that indole production enhanced by increasing tryptophan concentration. The assessment of IAA production in current research showed different concentration of IAA produced from all tested bacteria but the addition of tryptophan to the bacterial broth, increased IAA production considerably. It showed all of tested bacteria preferred tryptophan dependent way to produce IAA. There was not a significant differences between rhizospheric and epiphytic or bacterial endophyte tested, which is in agreement with issued publication of Lindow & Brandl (2003) who said that bacterial epiphytes are also known to produce phytohormnes like endophytes and rhizospheric bacteria .

Seed vigor is mainly determined based on the seedling length [18]. Vigor index reflects the seedlings health, establishment and the state of final productivity of the plant [32]. According to results obtained here, since the seedling length were significantly increased by bacterial inoculations, the seed vigor enhancement was anticipated, as it was. The speed of seed germination was not affected considerably by seed priming compared to control. In some bacterial treatments, the rate of seed germination was slowly during the experiment time. The isolates MR_2 (*B. idriensis*) and O_1R_4 (*A. faecalis*) were able to colonize rice seeds in a shorter time and therefore can be more successful biofertilizers. This has been approved by other authors who said that to be an effective plant growth promoting bacteria, they must be able to establish themselves and colonize plant to reach at an appropriate density sufficient for producing beneficial effects [4].

Vegetative growth is an important growth phase in many crops as it determines the amount of biomass production and in rice it is important for development of tillers. A strong vegetative growth of rice plants reflects a higher plant height and greater plant biomass, larger number of tillers and panicles [42]. In current investigations, after seed germination experiment, more successful bacteria were selected for pot experiment to evaluate bacterial growth promoting effects on vegetative growth phase. At tillering stage (code 29) [6], rice growth in all measured traits including root and stem length and weight was higher than control and it showed the beneficial effects of bacteria treated. One reason of this enhancement can result from phytohormones which led to higher root and stem length. Production of IAA from all of treated bacteria in different concentrations can explain the different effectiveness of beneficial bacteria on rice growth. Other than phytohormones, solubilizing of phosphate can be other reason for growth enhancement of rice. Phosphorus is one of the major nutrient requirements for plant growth. Phosphate-solubilizing microorganisms, in addition to provide phosphorus for plants, provide growth promoting substances like hormones, vitamins and amino acids. It is reported that mechanism of solubilizing phosphate is associated with release of low molecular weight organic acids which chelate the cation bound to phosphate and convert it into soluble form [16, 24, 25]. According to our result from phosphate solubilizing ability of studied bacteria on NBRIP medium, almost all of them could solubilize phosphate but in different amount. The isolates ability of phosphate solubilizing was detected by measuring the halo zone size around bacteria. This halo zone was quite small in

isolates FEn_1 (*S. maltophilia*), MR_2 (*B. idriensis*) and MR_5 (*B. vietnamensis*) which was showed by a single (+) as low solubilizers, but the isolates AEn_1 (*P. ananatis*), O_1R_4 and DEp_8 (*A. facealis*), were considered as high phosphate solubilizers (+++). According to our results, there was not a significant differences between tested rhizospheric and epiphytic or bacterial endophyte for solubilizing phosphate which is not completely in agreement by Vazquez *et al*. (2000) who stated that high proportion of phosphate solubilizing bacteria are concentrated in the rhizosphere and they are metabolically more active than other sources. In current research, the isolate AEn_1 (*P. ananatis*) which isolated from rice as a bacterial endophyte or DEp_8 (*A. faecalis*) as a bacterial epiphyte were considered as high solubilizers since the diameter of the halo zone made by these isolates were more than the others, but this privilege was not reflected in the result of rice growth enhancement, because the rhizospheric isolates, MR_5 (*B. vietnamensis*) and MR_2 (*B. idriensis*) with low ability of phosphate solubilizing showed considerable enhancement of rice growth and yield. The same results obtained from the experiment conducted by Mwajita *et al*. (2013) in which over half of the bacterial phyllosphere isolates were able to solubilize phosphate, but generally the bacterial isolates from the rhizosphere and rhizoplane were more efficient in plant growth enhancement.

The increase in shoot and root dry matter directly effects on the plant higher productivity [42], which is clear in our results and the investigation of bacterial effects on rice yield at the end of growth season showed higher productivity than un-inoculated control including tiller number, panicle number, the weight of 1000 grain and the number of full grain. It is evident that many different species colonize the tissues of the plants, but their persistence, activity and their interaction in this microbial community is not completely cleared. The understanding of the interaction between bacteria-bacteria and bacteria-plant would help us to modify the beneficial effects that bacteria may have on host plants. In other words, introducing a PGPB and its inoculation to plant cannot guarantee the enhancement of plant yield. The success of a PGPB inoculation depends on different factors including soil and agricultural conditions, plant properties and the characteristics of the bacteria involved .

CONCLUSION

The current study showed that rice plants collected from different paddy fields in Guilan province, harbour diverse bacterial epiphytic, endophytic and rhizospheric population which some of them had the ability of plant growth promoting, but in different potential. All of tested bacteria were able to produce IAA and solubilize phosphate. Among them, the isolates *Alcaligenes faecalis*, *Pantoea ananatis* and *Bacillus vietnamensis* showed high potential of IAA production and phosphate solubilizing. From this study, it can be concluded that the above bacterial isolates can be promising as PGPB, but the final conclusion will be obtained when their potential of being a successful PGPB is investigated in paddy field conditions and when they are identified as safe microorganisms for human life in field trials.

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