J. Agr. Sci. Tech. (2016) Vol. 18: 775-788

Molecular Identification and Characterization of Phosphate Solubilizing *Pseudomonas* sp. Isolated from Rhizosphere of Mash Bean (*Vigna Mungo* L.) for Growth Promotion in Wheat

M. Ehsan[1, 2, 3], I. Ahmed[1,*], R. Hayat[2], M. Iqbal[1], N. Bibi[1, 2], and N. Khalid[4]

ABSTRACT

Bio-inoculants have potential role in plant growth promotion. The present study evaluated the potential of *Pseudomonas* strains as bio-inoculants in wheat on the basis of plant growth promotion and physiological characterization. The 16S rRNA gene sequencing and phylogenetic analysis revealed that four isolated strains belonged to genus *Pseudomonas*. These strains were positive for phosphorus solubilization and indole acetic acid production, whereas only two strains were positive candidate for their nitrogen fixing ability as determined by presence or absence of *nifH* gene through amplification from polymerase chain reaction. The pot experiment showed that the integrated use of *Pseudomonas* strains as co-inoculant and 50% applied mineral fertilizers enhanced the maximum wheat growth and development from 58 to 140% for different shoot and root growth parameters. The strain NCCP-45 and NCCP-237 were closely related to *Pseudomonas beteli* and *Pseudomonas lini,* respectively. These isolated strains can be used to increase crop productivity by using as a bio-fertilizer inoculum.

Keywords: Bio-inoculant, PCR, PGPR, Phylogenetic analysis, 16S rRNA gene.

INTRODUCTION

Microorganisms are abundant in various ecologies. For instance, at least 10^4 bacterial taxa and 10^{10}-10^{11} different bacterial copy numbers exist per gram of soil (Torsvik *et al.*, 1990). In rhizosphere, the interaction between bacteria and roots has been reported to be beneficial, neutral, or detrimental depending upon soil and plant type (Hayat *et al.,* 2010). Plant Growth Promoting Rhizobacteria (PGPR) have historically received global attention in the context of increasing food production and improving environment. PGPR have been applied to different crops for enhancing plant growth, seed emergence, crop yield, and as bio-control agents against certain plant pathogens and pests (Dey *et al.,* 2004; Herman *et al.,* 2008). PGPR play their role in the uptake of nitrogen, solubilization of minerals such as phosphorus (inorganic and/or organic phosphates), synthesis of phytohormones and production of siderophores that chelate iron and make it available to plant roots for improving plant growth and development (Glick, 1995; Bowen and Rovira, 1999).

Phosphorus (P) is the second major essential macronutrient after Nitrogen (N)

[1]Institute of Microbial Culture Collection of Pakistan (IMCCP), National Agricultural Research Center, Islamabad-45500, Pakistan.
* Corresponding author; e-mail: iftikharnarc@hotmail.com
[2]Department of Soil Science and SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan.
[3]Soil and Water Testing Laboratory, P. O. Box No. 35, Talagang Road, Chakwal, Pakistan.
[4] Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

for plants. To overcome its deficiency, different phosphatic fertilizers are applied to soil to improve its nutritional status. However, a large proportion of soluble inorganic phosphate becomes unavailable to plants due to immobilization and the plants can uptake only 45% of phosphatic fertilizers (Mózner *et al.,* 2012). The remaining P is converted to less available forms due to various chemical reactions in the soil depending upon the soil pH. Legumes and rhizospheric soil are very rich in rhizobacteria and, at present, *Bacilli, Rhizobia* and *Pseudomonas* are the most studied phosphate solubilizers (Rodríguez and Fraga, 1999). Among these, *Pseudomonas* strains are considered as one of the most important PGPRs. These are diverse group of aerobic, heterotrophic, and gram-negative bacteria isolated from different habitats (soil, animals, plants and aquatic) (de Lorenzo, 2000). *Pseudomonas* has been reported for activities such as phytostimulation, bio-fertilization, and biocontrol against pathogens. *Pseudomonas* also promote plant growth directly through the production of siderophore, Indole Acetic Acid (IAA), phosphate solubilization, nitrogen fixation, ACC deaminase, root elongation, and degradation of toxic compounds (Wu *et al.,* 2005). Biological Nitrogen Fixation (BNF) shares almost half of the annually used nitrogenous inputs in the agricultural system. The nitrogenase catalyses the BNF and dinitrogenase reductase encoded by *nifH* gene converts N_2 into NH_3 (Gaby and Buckley, 2014). IAA produced by several bacteria is also useful for the development of vascular tissues, cell elongation, lateral root growth, and yield increase (Abdoli *et al.,* 2013).

Wheat (*Triticum aestivum* L.) is the major cereal crop and receives lots of mineral fertilizers for enhanced production to fulfill the food requirement of increasing population. There is a need to improve the fertilizer use efficiency through different organic resources. In Pakistan, DNA-based identification of microbes is not common due to which several potential PGPR isolates have remained unknown and have not been preserved for future use. Therefore, the present study was designed with the objectives to examine the phosphorus solubilization, *nifH* gene determination, and IAA production of molecularly identified *Pseudomonas* strains. Furthermore, the study aimed to analyze the identified strains for their potential as bio-inoculants to promote wheat growth and development.

MATERIALS AND METHODS

Sample Collection and Isolation of Strains

The samples of rhizospheric soil and roots of mash bean were collected from the research farm of Pir Mehr Ali Shah-Arid Agriculture University, Rawalpindi, Pakistan. A composite sample of crushed roots and soil was suspended in 10 mL of 1X Phosphate Buffer Saline solution (PBS) and shaken at room temperature for 10 min. Bacterial strains were isolated by serially dilution method on Tryptic Soya Agar (TSA) plates by incubation at 28°C for 48 hours. Bacterial strains were sub-cultured to obtain pure and morphologically distinct colonies. The purified strains culture were maintained on TSA plates as well as stored in 35% glycerol stock (w/v) at -80°C for further characterization.

Molecular Identification of the Isolated Strains

The isolated strains were identified based on 16S rRNA gene sequence. The amplification of 16S rRNA gene was performed according to previously described protocol by Roohi *et al.* (2012) and using Universal forward 9F (5´-GAGTTTGATCCTGGCTCAG-3´) and reverse primer 1510R (5´-GGCTACCTTGTTACGA-3´). The purified PCR products were sequenced using commercial service of MACROGEN, Korea

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

(http://dna.macrogen.com/eng). Nearly complete sequence data received from MACROGEN for 16S rRNA gene sequencing was aligned and edited through Note pad, BioEdit, Clustal W and Mega version 5.2 was used for construction of phylogenetic tree (Roohi *et al.,* 2012). The unambiguous sequence data used for constructing phylogenetic tree was also used to obtain DDBJ GenBank accession numbers for isolated strains which are given in Table 1.

Phosphate Solubilization, IAA Production and *nifH* gene Analyses

The quantitative phosphorus solubilizing capacity was determined on Pikovskaya (PKV) broth medium containing 5 g L^{-1} insoluble tri-calcium phosphate. The broth medium flasks were inoculated with 500 µL of freshly grown bacterial culture and the pH of the media was recorded before and after 7 days of inoculation. The extract preparation and phosphorus solubilization analyses were performed as described previously (Hayat *et al*., 2013). Standard curve graph was used for determination of phosphorus solubilization and the maximum standard was 1.00 µg mL^{-1}. Three replications of each treatment were carried out to get reliable results.

For IAA production, the 100 µL of fully grown bacterial culture was inoculated in 5 mL Luria Bertani broth (LB) medium with and without adding tryptophan (200 µg mL^{-1} tryptophan). After 48 hours growth, the supernatant (2 mL) was mixed with two drops of orthophosphoric acid (10 mM) and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5M $FeCl_3$ solution). IAA production of bacterial strains was measured spectrophotometerically (Hayat *et al*., 2013). Standard curve graph was used for IAA quantification with maximum standard set at 3.00 µg mL^{-1}.

The *nifH* gene was used as screening tool for N_2-fixing bacteria. This gene was

Table 1. Identification of isolated strains based on 16S rRNA gene sequence and their accession numbers published in DNA database.

Strain ID	Strain Name/ Genus	Length of 16S rRNA gene (ntd)	Accession number of 16S rRNA gene	Closely related taxa identified by using the EzTaxaon Server Database (http://eztaxon-e.ezbiocloud.net)	Sequence similarity (%) of 16S rRNA gene with closely related taxa
NCCP-45	*Pseudomonas* sp.	1423	AB547226	*Pseudomonas bereli* ATCC 19861^T (AB021406)	99.150
NCCP-47	*Pseudomonas* sp.	1415	AB547227	*Pseudomonas bereli* ATCC 19861^T (AB021406)	99.575
NCCP-237	*Pseudomonas* sp.	1437	AB839885	*Pseudomonas lini* CFBP 5737^T (AY035996)	99.234
NCCP-245	*Pseudomonas* sp.	1433	AB665217	*Pseudomonas plecoglossicida* FPC951^T (AB009457)	99.860

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

amplified by Polymerase Chain Reaction (PCR) using forward primer PolF[b] (5′ - TGC GAY CCS AAR GCB GAC TC - 3′) and reverse primer PolR[b] (5′ - ATS GCCATC ATY TCR CCG GA- 3′) as previously described (Poly *et al.,* 2001).

Morphological and Physiological Characterization

Growth of the isolated strains was optimized for pH in Tryptic Soya Broth (TSB) medium with a pH range from 4.0 to 10 (at increment of 1 pH unit). The strains were also tested for different concentrations of NaCl (0-7%) in TSB medium (pH 7.0). Growth experiments for pH optimization and NaCl tolerance were conducted at 28°C with vigorous shaking for 24 hours and optical density was monitored at 600 nm using a spectrophotometer (IMPLEN, Germany). Colonial morphology of bacterial strains was observed for shape, margins, surface, elevation, opacity, color and diameter (size) according to Tindall *et al.* (2007) after 24 hours growth at 28°C.

Pot Experiment for Evaluation of *Pseudomonas* Application on Wheat Growth

A pot experiment was conducted to evaluate the effect of *Pseudomonas* strains on wheat growth in comparison to un-inoculated control and fertilizer treatments at the soil and water testing laboratory Chakwal, Pakistan. Plastic pots (diameter 6 cm and depth 12 cm) were used for sowing that contained 300 g of non-sterilized soil. Non sterilized soil was used to check the strains efficacy in conditions nearest to the field conditions and to compare their effectiveness a negative un-inoculated soil was used as the control. The surface soil up to 30 cm used in this experiment was silt loam in texture (clay 18%, silt 55% and sand 27%) having pH of 7.3, EC_e= 1.5 dS m^{-1}, organic matter of 0.79%, Available phosphorus= 8.2 mg kg^{-1}, Exchangeable potassium= 110 mg kg^{-1}, and total nitrogen of 0.04%. All these soil characteristics were determined using the already developed methods previously described by Shahzad *et al.* (2014).

Bacterial inoculums were prepared by inoculating purified strains in TSB and incubated at 28°C for 48 hours. Surface disinfected seeds were inoculated by dipping into the bacterial culture for 5 min. Two seeds per pot were sown, but after germination, one plant was maintained. The experiment was conducted in completely randomized design having 10 treatments of bacterial and fertilizer application in comparison to un-inoculated control with 3 replications of each. To meet irrigation requirement, non sterilized distilled water was used. Different growth parameters including shoot length, shoot fresh and dry weight, root length, root fresh and dry weight were examined after 35 days of sowing.

RESULTS

Molecular Identification and Phylogenetic Analysis

Molecular identification based upon the 16S rRNA gene sequencing revealed that all of the four strains belonged to the genus *Pseudomonas*. The DNA sequence similarity percentage with closely related type strains ranged between 99.15 to 99.86%. On the basis of unambiguous sequence data, the strain NCCP-45 and 47 were closely related to *Pseudomonas beteli,* whereas NCCP-237 and 245 were related to *Pseudomonas lini* and *Pseudomonas plecoglossicidar,* respectively (Table 1). These identification results were also confirmed through the phylogenetic analysis. The phylogenetic tree constructed by Neighbor Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods (Figures 1 and 2) showed that the isolated strains belonged to genus *Pseudomonas*. However, there was little difference between NCCP-45 and 47 at species level as appeared from the divergence of the phylogenetic tree, and NCCP-237 was making its cluster with

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

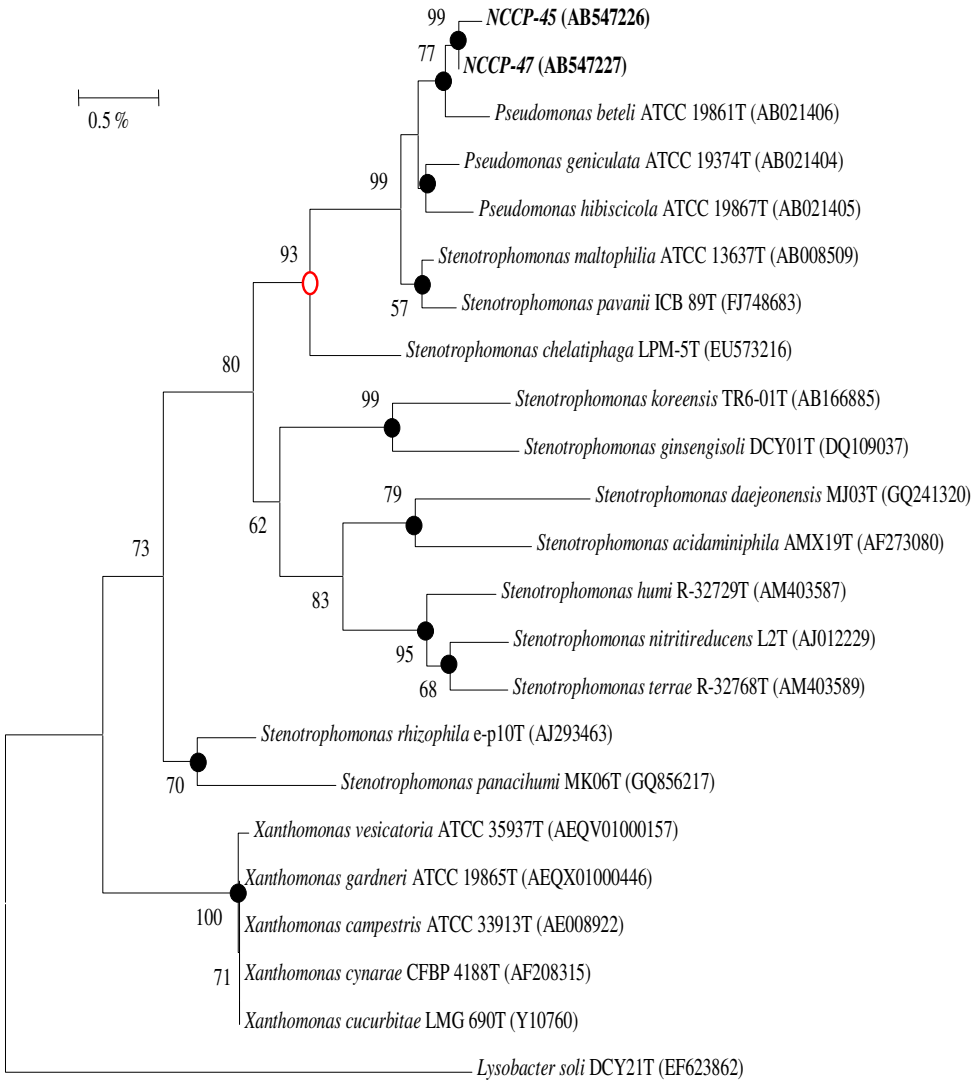

Figure 1. Phylogenetic tree showing the inter-relationship of strains NCCP-45 and NCCP-47 with closely related type species of *Pseudomonas* and other related genera inferred from sequences of 16S rRNA gene. The tree was generated using the neighbour-joining method contained in MEGA 5.2 software package and *Lysobacter soli* (EF623862) was used as an out group. Bootstrap values (more than 50%) are expressed as a percentage of 1,000 replications and are given at the branching point. Nodes indicated by empty circles were recovered by at least two algorithms, whereas nodes with solid circles were recovered by three algorithms (NJ, MLH and MP). The sequence of bar shows, 0.5% sequence divergence. The accession number of each type strain is shown in parenthesis.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

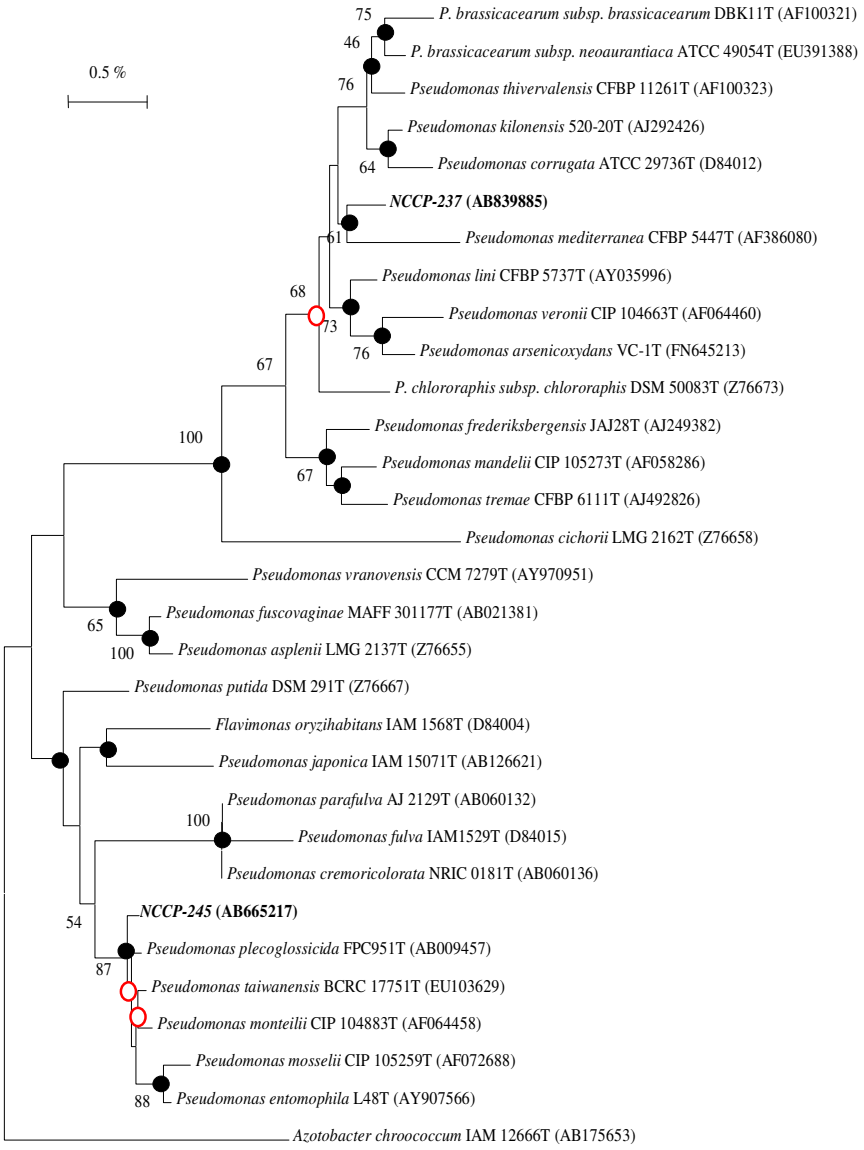

Figure 2. Phylogenetic tree showing the inter-relationship of strains NCCP-237 and NCCP-245 with the most closely related type species of *Pseudomonas* inferred from sequences of 16S rRNA gene. The tree was generated using the neighbour-joining method contained in MEGA 5.2 software package and *Azotobacter chroococcum* (AB175653) was used as an out group. Bootstrap values (more than 50%) are expressed as a percentage of 1,000 replications and are given at the branching point. Nodes indicated by empty circles were recovered by at least two algorithms, whereas nodes with solid circles were recovered by three algorithms (NJ, MLH and MP). The sequence of bar shows, 0.5% sequence divergence. The accession number of each type strain is shown in parenthesis.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

Pseudomonas mediterranea CFBP 5447T (AF386080).

Phenotypic Characterization

The four *Pseudomonas* strains purified through sub-culturing on the basis of colony morphology were the same in form, margins, and opacity (Table 2). However, NCCP-245 was different from the rest with smooth and dull surface. NCCP-45 had raised elevation while all others were convex in elevation. NCCP-47 was yellow as compared to the other strains which were off white to light yellow in color. The colony diameter ranged from 1-2 mm on TSA plates after 24 hours growth. The pH ranged from 5-10 and NaCl tolerance level lies between 0-6% (w/v) in TSB medium.

Phosphate Solubilization, IAA Production and Nitrogen Fixation

The quantitative estimation for solubilization of insoluble tri-calcium phosphate revealed that all the strains were able to solubilize insoluble mineral phosphate and their range was between 18.30 to 147.53 µg mL^{-1} (Table 3). The lowest quantity of insoluble mineral phosphate among isolated strains was solubilized by NCCP-47 and highest by NCCP-45. The phosphates solubilized by all *Pseudomonas* species were significantly different from one another. For quantitative analysis, the pH of the Pikovskaya's broth medium was adjusted to 7.0±0.05 before bacterial inoculation. The qualitative method revealed that only 2 strains (NCCP-245 and NCCP-45) were able to solubilize insoluble mineral phosphate by forming halo zones around their colonies on PVK medium and their Solubilization Efficiency (SE) range was 166.7 and 171.4%, respectively, while Solubilization Index (SI) was 2.7 for both of the strains. The qualitative indicators, *SI* "1" and *SE* "zero" indicated that bacterial strains

Table 2. Phenotypic characterization of *Pseudomonas* strains

Strain ID	Shape/Form	Margins	Surface	Elevation	Opacity	Chroma	Size (mm) Diameter	pH for growth (Optimum)	NaCl Tolerance (%)
NCCP-45	Circular	Entire	Smooth and Shiny	Raised*	Opaque	Off white	1.5-2.0	5-9 (7)	0-6
NCCP-47	Circular	Entire	Smooth and Shiny	Convex	Opaque	Yellow*	1.0-1.5	6-9 (7)	0-3
NCCP-237	Circular	Entire	Smooth and Shiny	Convex	Opaque	Light yellow	1.0-2.0	5-10 (7-8)	0-5
NCCP-245	Circular	Entire	Smooth and Dull*	Convex	Opaque	Light yellow	1.0-1.5	5-9 (7)	0-5

* Morphological characters that are differentiating their respective strains from NCCP-237 in addition to pH range for strain's growth.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

did not solubilize insoluble mineral phosphate on PVK agar medium.

The IAA production was measured spectophotometrically and in the absence of *L*-tryptophan. The lowest (7.61 µg mL^{-1}) and highest (11.49 µg mL^{-1}) IAA production was exhibited by NCCP-47 and NCCP-237, respectively. The addition of *L*-tryptophan further enhanced the IAA production of all the strains. In the presence of *L*-tryptophan, the lowest (11.76 µg mL^{-1}) and highest (20.73 µg mL^{-1}) IAA production was exhibited by NCCP-47 and NCCP-245, respectively. A considerable increase in IAA was observed due to addition of *L*-tryptophan (Table 3). The *nifH* gene analysis in comparison to 1 kb DNA ladder revealed that NCCP-45 and NCCP-245 were negative for nitrogen fixation (Table 3). However, NCCP-47 and NCCP-237 were positive for their nitrogen fixation ability showing that the amplified band size was almost near 400 bp.

Wheat Growth Promotion

The results of pot experiment on wheat shoot and root growth are presented in Tables 4 and 5. It was found that all the strains and fertilizer treatments, used either as independent or in combination, enhanced wheat growth positively in comparison to un-inoculated control, but NCCP-47 and NCCP-245 showed inconsistent and non-significant result as compared to un-inoculated control, especially for root growth. Overall, it was observed that co-inoculation in combination with half dose of recommended NPK fertilizer showed the maximum increase in wheat growth and development ranging from 58 to 140% for different shoot and root growth parameters. Interestingly, co-inoculation performed better in the treatment where only half dose of chemical fertilizers was applied. Furthermore, the growth of all recorded parameters for co-inoculation

Table 3. Plant growth promoting characterization of *Pseudomonas* strains.

Strain ID	P Solubilization (µg mL^{-1}) quantitative analysis		P Solubilization (µg mL^{-1}) quantitative analysis		*nif*H gene Detection	Indole Acetic Acid production (IAA= µg mL^{-1}±SD)		
			S.I	S.E	*nif*H gene	Without *L*-tryptophan	With *L*-tryptophan	% Increase[c]
NCCP-45	147.53±5.33 (a)	4.83±0.02 (c)	2.7	171.4	-	9.23±1.39 (ab)	14.35±1.27 (b)	55.45%
NCCP-47	18.30±0.38 (d)	5.23±0.02 (b)	1.0	0.0	+	7.61±1.35 (b)	11.76±1.13 (b)	54.59%
NCCP-237	103.58±3.30 (b)	4.42±0.03 (d)	1.0	0.0	+	11.49±1.77 (a)	17.81±1.82 (a)	55.02%
NCCP-245	43.80±0.38 (c)	5.78±0.05 (a)	2.7	166.7	-	11.02±2.08 (a)	20.73±1.95 (a)	88.01%

[a] All the means are average of three replications and means with different letters are significantly different from one another compared on the basis of *LSD* at 5% level of significance, [b] pH reduction of Pikovskaya broth medium which was adjusted to seven before bacterial inoculation, [c] percent increase in IAA production due to addition of *L*-tryptophan.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

Table 4. Effect of seed inoculation with *Pseudomonas* strains on shoot growth of wheat.

Treatments	Shoot length (cm)[a]		Fresh shoot weight (g)[a]		Dry shoot weight (g)[a]	
	Mean±SD	% Increase[c]	Mean±SD	% Increase	Mean±SD	% Increase
Un-inoculated Control	18.33 ± 0.80 (g)	0.00	0.68 ± 0.07 (f)	0.00	0.14 ± 0.01 (h)	0.00
Strain NCCP-45	21.77 ± 0.87 (e)	18.73	0.93 ± 0.08 (de)	37.30	0.18 ± 0.01 (ef)	34.15
Strain NCCP-47	19.43 ± 0.85 (fg)	6.00	0.87 ± 0.07 (e)	28.43	0.16 ± 0.01 (g)	17.07
Strain NCCP-237	23.77 ± 1.00 (d)	29.64	1.05 ± 0.07 (cd)	54.90	0.20 ± 0.02 (de)	48.78
Strain NCCP-245	20.33 ± 0.96 (ef)	10.91	0.83 ± 0.11 (ef)	22.06	0.16 ± 0.02 (fg)	19.51
Co-inoculation[b]	27.13 ± 1.11 (b)	48.00	1.35 ± 0.09 (b)	98.04	0.25 ± 0.01 (b)	85.37
Co-inoculation+ Half dose (N,P,K)	29.10 ± 1.05 (a)	58.73	1.51 ± 0.11 (a)	122.06	0.29 ± 0.01 (a)	112.20
NCCP-237 + Half dose (N,P,K)	25.90 ±1.20 (bc)	41.27	1.16 ± 0.10 (c)	71.08	0.23 ± 0.01 (c)	68.29
Half dose of (N,P,K)	25.00 ± 1.05 (cd)	36.36	1.08 ± 0.11 (cd)	58.82	0.22 ± 0.02 (cd)	58.54
Full dose of (N,P,K)	27.23 ± 1.25 (b)	48.55	1.44 ± 0.12 (ab)	111.76	0.27± 0.02 (ab)	100.00

[a] Mean ± standard deviation from three replicates. Means within a column followed by similar letters given in parenthesis show no significant difference (P= 0.05) by least significant difference test. [b] Co-inoculation means consortium of all four strains. [c] % Increase within a column shows difference of production for applied treatments in comparison to control.

Table 5. Effect of seed inoculation with *Pseudomonas* strains on root growth of wheat**.**

Treatments	Root length (cm)[a]		Fresh root weight (g)[a]		Dry root weight (g)[a]	
	Mean±SD	% Increase[c]	Mean±SD	% Increase	Mean±SD	% Increase
Un-inoculated Control	5.73 ± 0.57 (g)	0.00	1.03 ± 0.05 (g)	0.00	0.11 ± 0.01 (f)	0.00
Strain NCCP-45	6.87 ± 0.42 (ef)	19.77	1.38 ± 0.10 (ef)	34.42	0.14 ± 0.01 (de)	27.76
Strain NCCP-47	6.17 ± 0.32 (fg)	7.56	1.11 ± 0.08 (g)	7.79	0.12 ± 0.01 (ef)	11.04
Strain NCCP-237	7.43 ± 0.40 (de)	29.65	1.43 ± 0.15 (ef)	39.61	0.15 ± 0.01 (d)	45.11
Strain NCCP-245	6.43 ± 0.55 (fg)	12.21	1.23 ± 0.07 (fg)	19.81	0.12 ± 0.01 (ef)	17.03
Co-inoculation[ω]	9.10 ± 0.46 (b)	58.72	2.01 ± 0.14 (bc)	95.45	0.20 ± 0.02 (b)	93.06
Co-inoculation+ Half dose (NPK)	10.37 ± 0.45 (a)	80.81	2.42 ± 0.15 (a)	135.39	0.25 ± 0.02 (a)	140.06
NCCP-237+ Half dose (NPK)	8.50 ± 0.46 (bc)	48.26	1.85 ± 0.20 (cd)	80.19	0.19 ± 0.02 (bc)	77.60
Half dose of (NPK)	8.07 ± 0.55 (cd)	40.70	1.62 ± 0.17 (de)	57.79	0.17 ± 0.02 (c)	65.30
Full dose of (NPK)	9.20 ± 0.45 (b)	60.47	2.22 ± 0.21 (ab)	115.91	0.23 ± 0.02 (a)	118.61

[a] Mean±standard deviation from three replicates. Means within a column followed by similar letters given in parenthesis show no significant difference (P= 0.05) by least significant difference test. [b] Co-inoculation means consortium of all four strains. [c] % Increase within a column shows difference of production for applied treatments in comparison to control.

treatment was very much closer to the full dose of NPK treatment and difference between these two treatments was non-significant. However, significantly higher dry root weight (0.23 g) was achieved in full dose of NPK treatment than the co-inoculation (0.20 g). The strain NCCP-237 with triple PGPR traits, in combination with half dose of recommended NPK fertilizer showed better results than the sole application of strains and un-inoculated control.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

DISCUSSION

Rhizobacteria were isolated from the rhizospheric soil and roots of mash bean (*Vigna mungo* L.) grown at the research farm of Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan. The isolated strains were subjected to molecular identification and polyphasic characterization. The 16S rRNA gene sequence depicted that all of the four strains belonged to the *Pseudomonas* sp. Two isolates were closely related to *Pseudomonas beteli* (ATCC 19861^T) and *Pseudomonas lini* (CFBP 5737^T) and *Pseudomonas plecoglossicida* (FPC 951^T). The strain NCCP-237 made its cluster with *Pseudomonas mediterranea* (AF386080) in all of the phylogenetic trees. Phylogenetic analysis also confirmed the similarity of two strains with *Pseudomonas beteli* (ATCC 19861T). These two strains also proved to be different species based upon morphology, physiological and PGPR characterization. These results were in accordance with Hayat *et al.* (2013) who reported that RH-3 and RH-5 were closely related to *Bacillus subtilis* subsp. *Inaquosorum* (BGSC 3A28T), but both of the isolated strains were different in PGPR and morphological traits. Currently, the 16S rRNA gene sequence analysis is the primary key to analyze the microbial phylogeny (Dewhirst *et al.,* 2005). The 16S rRNA gene sequencing is considered less authenticated at the species level and has poor discriminatory power for some genera (Mignard and Flandrois, 2006). The genus *Bacillus* is a good example of this as Ahmed *et al.* (2007) also reported that the type strains of *B. boroniphilus* and *B. jeotgli* share > 99.5% sequence similarity with regard to their 16S rRNA genes, but at the DNA level, these exhibited only 36.4% relatedness in reciprocal hybridization reactions, which proved that the two strains were different from each other and belonged to two separate species.

Molecularly identified *Pseudomonas* species were tested for PGPR characterization including P solubilization, IAA production, and *nifH* gene detection. P solubilization was observed only in two strains when subjected to qualitative analysis on PVK agar medium. However, the experiment showed that all the strains were capable of solubilizing tri-calcium phosphate quantitatively and pH of the broth culture medium decreased significantly during incubation. The pH reduction indicates the production of acids like gluconic acid, citric and propionic acid etc. and acidification caused by metabolic processes play important role in P solubilization (Chen *et al.,* 2006). These results are in agreement with earlier studies of Rodríguez and Fraga (1999) who reported that *Bacilli, Rhizobia* and *Pseudomonas* were efficient phosphate solubilizing bacteria but they could vary in qualitative and quantitative analysis for P solubilization. Abd El-Azeem *et al.* (2007) also reported the solubilization of insoluble mineral phosphate in the range of 1.53 to 360 µg mL^{-1} as well as the pH reduction from initial value of 7.1 to values varying between 4.16 and 6.45. A negative correlation between P solubilized and pH of broth culture was observed and this has been supported by the findings of Keneni *et al.* (2010).

It has been estimated that 80% of bacteria isolated from the rhizosphere can produce plant growth regulator IAA (Patten and Glick, 1996). In our study, the IAA production was measured colorometrically and it was observed that in the absence of *L*-tryptophan, the lowest and highest IAA production was exhibited by NCCP-47 and NCCP-237 closely related to *Pseudomonas beteli* and *Pseudomonas lini*, respectively. The addition of *L*-tryptophan further enhanced the IAA producing capacity of all the strains ranging from 54% to 88% (Table 3). All the isolated *Pseudomonas* sp. were the low IAA producer as their production was below 40.0 µg mL^{-1} (Anjum *et al.,* 2011), but all the strains showed considerable IAA production which may be beneficial for plants growth. Our results are

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

also in agreement with Xie *et al*. (1996) who reported the IAA production in *Pseudomonas* isolates.

The isolated *Pseudomonas* strains were analyzed through PCR for the presence or absence of *nifH* gene for the nitrogen fixing ability. The *nifH* gene analysis revealed that 2 strains were positive and 2 were negative for their *nifH* gene presence. De Meyer *et al.* (2011) used this analysis to check the nitrogen fixing ability and phylogenetic relationship of *Pseudomonas* and several other bacterial genera. This gene involves two identical subunits of dinitrogenase reductase that are mainly responsible for nitrogen fixation and to develop its phylogeny (Dobert *et al.,* 1994; Chen *et al.,* 2003).

In physiological study it was observed that the isolated strains could survive from pH 5 to 9, except NCCP-237 which also showed growth at pH 10. The maximum NaCl tolerance (6% w/v) was observed for NCCP-45. This showed that all of the strains were able to survive in alkaline condition, but at low level of salinity with respect to tolerance and optimization. Our results are in agreement with Kwon *et al.* (2003) and Nishimori *et al.* (2000) who reported similar type of results for the growth and survival of different *Pseudomonas* species under similar these physiological conditions.

The pot experiment clearly showed that NCCP-45 and NCCP-237 significantly increased the wheat growth compared to un-inoculated control, probably due to their efficient P solubilizing capacity in addition to other PGPR traits including IAA production and *nifH* gene. Both of these strains are good candidates to use as bio-inoculants in future. It was also observed that co-inoculation of all strains positively affected the wheat growth even without NPK, and their performance was very much closer to full dose of NPK fertilizer application. This was most probably due to more changes occurring in the soil system as a result of multi-traits/effects of strains like P solubilization, nitrogen fixation, and IAA production making soil nutrients more readily available to plants. Co-inoculation of all four strains in combination with half dose of recommended NPK fertilizer produced even better results than full NPK fertilizer treatment due to phytohormone synthesis and more uptake of nutrients. This proved that the isolated *Pseudomonas* strains are good source of enhancing fertilizer use efficiency and thus crop growth/ production. The above mechanism was also reported in earlier studies (Abbas-Zadeh *et al.,* 2010; Zabihi *et al.,* 2011). Duarah *et al.* (2011) also stated that the application of PGPR+50% NPK produced better crop growth than 100% NPK alone.

In the present study, it was concluded that all of the isolated *Pseudomonas* strains were slightly different from one another in their morphology. Molecular identification on the basis of 16S rRNA gene sequencing and phylogenetic analysis also revealed that all the isolated strains belonged to genus *Pseudomonas*. All of the strains were positive for IAA production either in the presence or absence of *L*-tryptophan and tri-calcium phosphate solubilization by reducing the pH of broth culture medium. However, *nifH* gene was present only in two strains including NCCP-45 and NCCP-237. Based upon the plant growth promotion, NCCP-237 may prove as an effective bio-inoculant since it was positive for all three PGPR traits. In comparison to this, NCCP-45 was even more efficient for P-solubilization and also positive for IAA production but *nifH* gene was missing making it less effective than NCCP-237 in plant growth promotion. It was concluded that all of the remaining three strains, except NCCP-237, should be applied in the form of consortium for inoculation on crops, but maximum profitability can be achieved through the integrated use of *Pseudomonas* co-inoculation and 50% of the recommended rate of mineral fertilizers. In addition to physiological examination of the strains, plant growth promotion also indicated that all of the isolated *Pseudomonas* strains could survive in alkaline soils. Severe salinity problem may adversely affect the

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

efficiency of bacteria, as all of these strains have very low limit of NaCl tolerance.

ACKNOWLEDGEMENTS

This work was partially supported by financial assistance from PSDP funded Project "Research for Agricultural Development Program (RADP)" under a sub-project (Grant No. CS-55/RADP/PARC) entitled "Establishment of Microbial Bio-Resource Laboratories: National Culture Collection of Pakistan (NCCP)" from Pakistan Agricultural Research Council (PARC); and partly by Pakistan Science Foundation under PSF project No. PSF/Res/P-PMAS-AAUR-Agr-374.

REFERENCES

1. Abbas-Zadeh, P., Saleh-Rastin, N., Asadi-Rahmani, H., Khavazi, K., Soltani, A., Shoary-Nejati, A. R. and Miransari, M. 2010. Plant Growth Promoting Activities of *Fluorescent pseudomonads*, Isolated from the Iranian Soils. *Acta Physiol. Plant*., **32,** 281-288.
2. Abd El-Azeem, S. A. M., Mehana, T. A. and Shabayek, A. A. 2007. Some Plant Growth Promoting Traits of Rhizobacteria Isolated from Suez Canal Region, Egypt. *African Crop Sci. Conf. Proceed.,* **8:** 1517-1525.
3. Abdoli, M., Saeidi, M., Honarmand, S. J. and Azhand, M. 2013. The Effect of Foliar Application of Indole-3-Acetic Acid (IAA) and Roles of Ear Photosynthesis on Grain Yield Production of Two Wheat Cultivars (*Triticum aestivum* L.) under Post Anthesis Water Deficit. *Intl. Res. J. Appl. Basic. Sci.,* **4(6):** 1506-1513.
4. Ahmed, I., Yokota, A. and Fujiwara, T. 2007. A Novel Highly Boron Tolerant Bacterium, *Bacillus boroniphilus* sp. nov., Isolated from Soil, that Requires Boron for Its Growth. *Extremophile.,* **11**: 217–224.
5. Anjum, M.A., Zahir, Z. A., Arshad, M. and Ashraf, M. 2011. Isolation and Screening of Rhizobia for Auxin Biosynthesis and Growth Promotion of Mung Bean (*Vigna radiata* L.) Seedlings under Axenic Conditions. *Soil Environ*., **30**: 18-26.
6. Bowen, G. D. and Rovira, A. D. 1999. The Rhizosphere and Its Management To Improve Plant Growth, In: "*Advances in Agronomy*", (Ed.): Donald, L. S.. Academic Press, London. PP. 1-102.
7. Chen, W. M., Moulin, L., Bontemps, C., Vandamme, P., Bena, G. and Boivin-Masson, C. 2003. Legume Symbiotic Nitrogen Fixation by Beta-proteobacteria is Widespread in Nature. *J. Bacteriol.,* **185**: 7266-7272.
8. Chen, Y. P., Rekha, P. D., Arun, A. B., Shen, F. T., Lai, W. A. and Young, C. C. 2006. Phosphate Solubilizing Bacteria from Subtropical Soil and Their Tricalcium Phosphate Solubilizing Abilities. *Appl. Soil. Ecol.,* **34**: 33-41.
9. de Lorenzo, V. 2000. *Pseudomonas* Entering the Post-genomic Era. *Environ. Microbiol.,* **2**: 349-354.
10. De Meyer, S. E., Van Hoorde, K., Vekeman, B., Braeckman, T. and Willems, A. 2011. Genetic Diversity of Rhizobia Associated with Indigenous Legumes in Different Regions of Flanders (Belgium). *Soil. Biol. Biochem.,* **43**: 2384-2396.
11. Dewhirst, F. E., Shen, Z., Scimeca, M. S., Stokes, L. N., Boumenna, T., Chen, T., Paster, B. J. and Fox, J. G. 2005. Discordant 16S and 23S rRNA Gene Phylogenies for the Genus *Helicobacter*: Implications for Phylogenetic Inference and Systematics. *J. Bacteriol.,* **187**: 6106-6118.
12. Dey, R., Pal, K. K., Bhatt, D. M. and Chauhan, S. M. 2004: Growth Aromotion and Yield Enhancement of Peanut (*Arachis hypogaea* L.) by Application of Plant Growth-promoting Rhizobacteria. *Microbiol. Res.,* **159**: 371-394.
13. Dobert, R. C., Breil, B. T. and Triplett, E. W. 1994. DNA Sequence of the Common Nodulation Genes of *Bradyrhizobium elkanii* and Their Phylogenetic Relationship to Those of Other Nodulating Bacteria. *Mol. Plant-Microbe Interac.: MPMI.,* **7**: 564-572.
14. Duarah, I., Deka, M., Saikia, N. and Deka Boruah, H. P. 2011. Phosphate Solubilizers Enhance NPK Fertilizer Use Efficiency in Rice and Legume Cultivation. *3 Biotech*., **1**: 227–238.
15. Gaby, J. C. and Buckley, D. H. 2014. A Comprehensive Aligned *nifH* Gene Database: A Multipurpose Tool for Studies

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

of Nitrogen-fixing Bacteria. *Database.*, **2014:** Article ID: bau001; doi:10.1093/database/bau001.

16. Glick, B. R. 1995. The Enhancement of Plant Growth by Free-living Bacteria. *Can. J. Microbiol.,* **41**: 109-117.
17. Hayat, R., Ali, S., Amara, U., Khalid, R. and Ahmed, I. 2010. Soil Beneficial Bacteria and Their Role in Plant Growth Promotion: A Review. *Ann. Microbiol.,* **60**: 579-598.
18. Hayat, R., Khalid, R., Ehsan, M., Ahmed, I., Yokota, A. and Ali, S. 2013. Molecular Characterization of Soil Bacteria for Improving Crop Yield in Pakistan. *Pak. J. Bot.,* **45**: 1045-1055.
19. Herman, M. A. B., Nault, B. A. and Smart, C. D. 2008. Effects of Plant Growth-promoting Rhizobacteria on Bell Pepper Production and Green Peach Aphid Infestations in New York. *Crop Protec.,* **27**: 996-1002.
20. Keneni. A., Assefa, F. and Prabu, P. C. 2010. Isolation of Phosphate Solubilizing Bacteria from the Rhizosphere of Faba Bean of Ethiopia and Their Abilities on Solubilizing Insoluble Phosphates. *J. Agr. Sci. Tech., **12**:* 79-89.
21. Kwon, S. W., Kim, J. S., Park, I. C., Yoon, S. H., Park, D. H., Lim C. K. and Go, S. J. 2003. *Pseudomonas koreensis* sp. nov., *Pseudomonas umsongensis* sp. nov. and *Pseudomonas jinjuensis* sp. nov., Novel Species from farm Soils in Korea. *Int. J. Syst. Evol. Microbiol.,* **53**: 21-27.
22. Mignard, S. and Flandrois, J. P. 2006. 16S rRNA Sequencing in Routine Bacterial Identification: A 30-month Experiment. *J. Microbiol. Method.,* **67**: 574-581.
23. Mózner, Z., Tabi, A. and Csutora, M. 2012. Modifying the Yield Factor Based on More Efficient Use of Fertilizer: The Environmental Impacts of Intensive and Extensive Agricultural Practices. *Eco. Ind.*, **16**: 58-66.
24. Nishimori, E., Kita-Tsukamoto, K. and Wakabayashi, H. 2000. *Pseudomonas plecoglossicida* sp. nov., the Causative Agent of Bacterial Haemorrhagic Ascites of Ayu, *Plecoglossus altivelis*. *Int. J. Syst. Evol. Microbiol.,* **50:** 83-89.
25. Patten, C. L. and Glick, B. R. 1996. Bacterial Biosynthesis of Indole-3-acetic Acid. *Can. J. Microbiol.,* **42**: 207-220.
26. Poly, F., Ranjard, L., Nazaret, S., Gourbière, F. and Monrozier, L. J. 2001. Comparison of *nifH* Gene Pools in Soils and Soil Microenvironments with Contrasting Properties. *Appl. Environ. Microbiol.,* **67**: 2255-2262.
27. Rodríguez, H. and Fraga, R. 1999. Phosphate Solubilizing Bacteria and Their Role in Plant Growth Promotion. *Biotechnol. Adv.,* **17**: 319-339.
28. Roohi, A., Ahmed, I., Iqbal, M. and Jamil, M. 2012. Preliminary Isolation and Characterization of Halotolerant and Halophilic Bacteria from Salt Mines of Karak, Pakistan. *Pak. J. Bot*., **44**: 365-370.
29. Shahzad, S. M., Khalid, A., Arif, M. S., Riaz, M., Ashraf, M., Iqbal, Z. and Yasmeen, T. 2014. Co-inoculation Integrated with P-enriched Compost Improved Nodulation and Growth of Chickpea (*Cicer arietinum* L.) under Irrigated and Rainfed Farming Systems. *Biol. Fertil. Soil.*, **50**:1–12.
30. Tindall, J. B., Sikorski, J., Simbert, A. R. and Krieg, R. N. 2007. Phenotypic Characterization and the Principles of Comparative Systematics, In: "Methods for General and Molecular Microbiology", (Eds.): Reddy, C. A., Beveridge, T. J., Breznak, J. A., Marzluf, G. A., Schmidt, T. M. S., Snyder, L. R.. American Society for Microbiology, Washington, DC, PP. 330-393.
31. Torsvik, V., Goksoyr, J. and Daae, F. L. 1990. High Diversity in DNA of Soil Bacteria. *Appl. Environ. Microbiol.,* **56**: 782-787.
32. Wu, S. C., Cao, Z. H., Li, Z. G., Cheung, K. C. and Wong, M. H. 2005. Effects of Biofertilizer Containing N-fixer, P and K Solubilizers and AM Fungi on Maize Growth: a Greenhouse Trial. *Geoderma,* **125**: 155-166.
33. Xie, H., Pasternak, J. J. and Glick, B. R. 1996. Isolation and Characterization of Mutants of the Plant Growth-promoting Rhizobacterium *Pseudomonas putida* GR12-2 that Overproduce Indole Acetic Acid. *Curr. Microbiol*., **32:** 67-71
34. Zabihi, H. R., Savaghebi, G.R., Khavazi, K., Ganjali, A. and Miransari, M. 2011. *Pseudomonas* Bacteria and Phosphorous Fertilization, Affecting Wheat (*Triticum aestivum* L.) Yield and P Uptake under Greenhouse and Field Conditions. *Acta Physiol Plant*., **33**: 145-152.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]

شناسایی مولکولی و تعیین ویژگی های گونه های سودوموناس حل کننده فسفات جدا سازی شده از ریشه گاه ماش (*Vigna Mungo* L.) برای افزایش رشد گندم

م. احسان، ی.احمد، ر. حیات، م. اقبال، ن. بی‌بی، و ن. خالد

چکیده

مایه تلقیح های زیستی نقش مستعدی در افزایش رشد گیاهان دارند. در پژوهش حاضر، توان سویه های سودو موناس به عنوان مایه تلقیح های زیستی بر مبنای افزایش رشد و ویژگی های فیزیولوژیکی گیاه گندم ارزیابی شد. نتایج توالی ژنی 16S rRNA و تجزیه تبارزایی(فیلو ژنتیک) آشکار ساخت که ۴ مورد از سویه های جدا سازی شده متعلق به جنس سودوموناس بودند. این سویه ها قادر به حل کردن فسفر و تولید ایندول استیک اسید بودند ولی بر مبنای حضور یا غیبت ژن *nif*H از طریق تکثیر از واکنش زنجیره ای پلیمراز، فقط دو سویه ازنظر توان تثبیت نیتروژن مثبت بودند. در ادامه، آزمایش گلدانی نشان داد که کاربرد همزمان سویه های سودوموناس به عنوان مایه تلقیح -همراه (co-inoculant) و ۵۰٪ کود های معدنی مصرفی بیشترین اثر (از ۵۸٪ تا ۱۴۰٪) را روی پارامترهای مختلف رشد شاخسار و ریشه گندم داشت. سویه NCCP-45 و NCCP-237 به ترتیب با *Pseudomonas beteli* و *Pseudomonas lini* رابطه نزدیک داشتند.به این قرار، برای افزایش بهره وری تولید گیاه می توان این سویه های جدا شده را به عنوان مایه یک کودزیستی به کار برد.

[Downloaded from jast.modares.ac.ir on 2024-04-17]

[DOR: 20.1001.1.16807073.2016.18.3.2.4]