The Journal of Animal & Plant Sciences, 23(2): 2013, Page: 514-520
ISSN: 1018-7081

SCREENING OF MULTI-TRAITS RHIZOBACTERIA TO IMPROVE MAIZE GROWTH UNDER AXENIC CONDITIONS

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ABSTRACT

Soil microorganisms are helpful to improve the plant growth by different mechanisms like solubilization of inorganic phosphorus through production of organic acids or mineralization of organic P through phosphatase enzyme. Rhizobacteria are also capable to produce different growth regulators and enzymes. Now the challenge is isolation and screening of such multi-traits bacteria. To accomplish this task, different bacterial strains were isolated from the rhizosphere of maize. Along with their P solubilization/mineralization capabilities, bacteria were also tested for their auxin producing efficiency and ACC-deaminase activity to correlate these traits with P solubilization and ultimately screen the most efficient plant growth promoting rhizobacteria (PGPR). Out of seventy two bacterial isolates tested on Pikovskaya agar media, thirty isolates which showed clear zone (P-solubilizers) were initially selected. Out of these thirty bacterial isolates, fifteen showing higher phosphatase activity, ACC-deaminase activity and auxin production in liquid culture were selected for further evaluation for their growth promoting activities under axenic conditions. Inoculation with selected bacteria significantly increased shoot length, root length, shoot fresh and dry weight, root fresh and dry weights up to 39.7, 58.9, 99, 69.4, 97.7 and 87%, respectively over uninoculated control. Statistical analysis revealed that a positive correlation existed between the PGPR showing efficient plant growth and their *in vitro* traits of phosphatase activity, auxin production and ACC-deaminase activity. Study demonstrated that selection of multi-traits bacteria could be more effective tool to select PGPR than single trait to improve plant growth.

Key words: Screening, rhizobacteria, growth regulators, enzymes.

INTRODUCTION

Root associated bacteria of different genera and species that colonize the rhizosphere and improve plant growth when introduced onto seeds are often referred to as plant growth promoting rhizobacteria (PGPR). These can have an effect on plant growth both directly and indirectly through different mechanisms of action (Datta *et al*., 2011). A single bacterium can affect plant growth by one or more of these mechanisms, and also use different abilities for growth promotion at various stages during the life cycle of the plant (Glick *et al*., 1999).

Auxins are a class of phytohormones which are involved in the regulation of growth and development throughout the life cycle of plants. It is well documented that several soil microorganisms are actively involved in the synthesis of auxins in pure culture as well as in soil (Biswas *et al*., 2000 a, b). Simultaneous screening of rhizobacteria for growth promotion under gnotobiotic conditions and *in-vitro* production of auxin could be useful technique for selecting effective PGPR (Asghar *et al*., 2004).

Similarly, some PGPR with their ACC (1-aminocyclopropane-1-carboxylate) deaminase activity can improve nutrients acquisition by plant indirectly by increasing root development. Ethylene being an important hormone is concerned with the regulation of various physiological processes in plants (Arshad and Frankenberger, 2002; Owino *et al.*, 2006). Under nutritional stress such as P deficiency, the level of ethylene increases which is considered inhibitory to plant growth especially for root growth. Some phosphate solubilising bacteria can also produce ACC-deaminase as a plant growth promoting enzyme (Naik *et al.* 2008; Piromyou *et al*., 2011). Soil microorganisms containing ACC-deaminase activity are helpful in increasing root elongation through lowering ethylene levels in plant roots by converting ACC into NH_3 and α-ketobutyrate in plants (Penrose and Glick, 2003; Sharoona *et al*., 2007; Nadeem *et al*., 2006). It is likely that increased root growth could help in larger uptake of P for better plant growth, consequently, the bacteria having ACC-deaminase activity along with phosphatase enzymes or auxin production (i.e. multi-traits bacterial strains) capability could be more effective in increasing the P availability to plants and ultimately increase the yield. Therefore, in order to select most effective PGPR for plants, different strategies or approaches are being used by the researchers which include; promotion of root/shoot growth under axenic conditions; production of plant growth regulators or biologically active substances under *in vitro* conditions and/or evaluating ACC-deaminase activity of the root colonizing bacteria (rhizobacteria).

Phosphorus plays a significant role in plant growth and metabolism by supplying energy needed for metabolic processes (Lal, 2002). It has been reported that almost 80 to 90% soils from arid and semiarid regions of the world are deficient in available phosphorus (Memon *et al.*, 1992). Moreover, in soil, the main problem with P for plant uptake is its availability, which is in very minute quantity. The availability of P is affected by soil chemical properties as well as human management activities. Inogranic phosphorus can be mobilized to available form by the rhizobacteria (Laslo *et al.*, 2012). However, some of the total P in soils is present as organic matter forms as phospholipids, nucleotides and inositol phosphate (Turner *et al*., 2002). As plant cannot take up P as organic form directly, therefore, it must be first transformed into inorganic form after being mineralized. This process in soil is mediated by phosphatase enzyme; the main source of phosphatase enzyme in soil is microbial community (Ribeiro and Cardoso, 2012).

For isolation and screening of rhizobacteria to choose most effective PGPR, the combination of two or more strategies could be a superior technique as compared to using a single approach (Zahir *et al*., 2004; Ahmad *et al.*, 2008). Keeping in view these challenges, the present study was planned to isolate and screen the most effective multi-traits PGPR strains for maize crop and to evaluate growth promotion of inoculated plants under axenic conditions.

MATERIALS AND METHODS

Isolation of bacteria: Several rhizobacteria were isolated using glucose peptone agar media (GPAM). Dilution plate technique was employed under aseptic conditions for the isolation of rhizobacteria. Seventy two bacterial colonies showing prolific growth on glucose peptone agar media (GPAM) were isolated. The cultures were initially maintained by repeated streaking on fresh plates containing solid medium. The isolated strains were stored at 4^{o}C for subsequent use.

Phosphate solubilizing activity and phosphate solubilizing index (PSI): For the purpose of primary screening, the isolated rhizobacteria were screened for their phosphate-solubilizing ability on Pikovskaya (PVK) agar medium (Pikovskaya, 1948) containing tricalcium phosphate (TCP) as substrate. The qualitative P-solubilization potential was estimated by observing the large clear/halo zones on Pikovskaya agar media. Phosphate solubilizing index (PSI) of these isolates was measured using the following formula as proposed by Premono *et al.* (1996).

$$PSI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

Screening for phosphatase activity: For the mineralization of organic phosphorus, phosphate solubilizing bacteria produce phosphatase enzyme. For secondary screening, the isolates showing clear/halo zones on solid media were further evaluated for their phosphatase activity by following the modified protocol of Eivazi and Tabatabai (1977).

ACC-deaminase activity: Both qualitative and quantitative.1-amino cyclopropane 1-carboxylic acid (ACC) activity was measured. Qualitative ACC-metabolism assay was carried out by using the method proposed by Jacobson *et al.* (1994). While ACC-deaminase activity was quantified by measuring the amount of α-ketobutyrate formed when the enzyme ACC-deaminase cleaved ACC by the method as suggested by Penrose and Glick (2003).

Auxin production assay: Auxin production by the selected isolates as indole acetic acid (IAA) equivalents in the presence and absence of L-tryptophan was determined as described by Sarwar *et al*. (1992). Auxin compounds (IAA-equivalents) were determined by spectrophotometer, using Salkowski coloring reagent.

Preparation of growth medium: The inocula of the selected phosphatase producing bacterial isolates were prepared by growing them in 250 mL conical flasks containing general purpose media. The inoculated flasks were incubated at 28 ± 1 °C for 72 hours in the orbital shaking incubator at 100 rev min^{-1}. Uniform optical density at 535 nm was achieved by dilution to maintain uniform cell density (10^{8}-10^{9} colony forming units per mL). The suspension of the selected isolates was used for seed inoculation.

Screening trial for plant growth stimulation under axenic conditions: Based on *in vitro* characterization of PGPR strains, fifteen promising strains were selected for their plant growth promoting activities under axenic conditions. Surface-sterilized seeds were placed for germination in Petri dishes. Pre-germinated seeds were inoculated with fifteen selected isolates by dipping in respective broth for five minutes. In case of control, surface-sterilized pre-germinated seeds were treated with sterilized broth without inoculation. Inoculated seeds were transplanted to autoclaved glass jars filled with sand and 2% autoclaved FYM as organic amendment. Sterilized Hoagland solution (Hoagland and Arnon, 1950) was applied in the jars for providing nutrients to seedlings. Jars were placed in growth chamber at 28 ± 1 ^{o}C adjusted to 16 hours light and 8 hours dark period. Data were recorded after 20 days of sowing and the results were compared with least significant difference test (Steel *et al*. 1996).

RESULTS AND DISCUSSION

Qualitative P-solubilization potential estimated by observing the large clear/halo zones on agar media revealed that out of seventy two bacterial isolates tested, thirty isolates had P-solublizing ability (Data of thirty isolates is given in table-1). The isolates exhibited different sorts of phosphate solublizing index (PSI) ranging from 1.59 to 6.27. Four isolates (PS-01, PS-12, PS-41 and PS-51) showed PSI more than four. Eight isolates (PS-07, PS-17, PS-21, PS-30, PS-32, PS-52, PS-60 and PS-63) had PSI ranging from 3.10 to 3.91. This variation in utilization of substrate by these strains could be due to difference in their organic acids production, which is evident from our pH decrease experiment (data not shown) by the selected bacterial strains. Similar results have also been reported by Rashid *et al*. (2004). For the solubilization of organic phosphorus, phosphate solublizing bacteria secrete phosphatase enzyme, so an effort was made to assess the phosphatase activity of these thirty isolates. The results regarding phosphatase activity showed that all the isolates except, PS-23, exhibited phosphatase activity in liquid culture. Phosphatase activity obtained with fifteen isolates ranged from 11.18 to 42.87 µg PNP g^{-1} h^{-1}. It was evident from the *in vitro* tests that both solubilization of inorganic P and phosphatase activity (mineralization) abilities can coexist in same bacteria. Like our results Tao *et al.* (2008) reported the coexistence of both capabilities in single bacterium. Similarly, Ribeiro and Cardoso (2012) characterized several PGPR isolates for their phosphatase activities and found that majority of the isolates were phosphatases producers.

These thirty strains were also tested for ACC-deaminase activity. It was revealed that seventeen strains possessed ACC-deaminase activity on the basis of their ability to utilize ACC as a sole source of nitrogen. The data regarding quantitative ACC-deaminase activity showed that the selected isolates (17 strains) possessed ACC-deaminase activity with different degrees of efficacy (Table-1). Maximum ACC-deaminase activity was observed by eight isolates (PS-01, PS-12, PS-17, PS-31, PS-32, PS-40, PS-51 and PS-63) which ranged from 296 to 356 nmol α-ketobutyrate g^{-1} biomass h^{-1}. The results are in accordance with Naik *et al.* (2008) and Piromyou *et al*. (2011) they reported that rhizobacteria also produce ACC-deaminase as a plant growth promoting enzyme. Regarding auxin biosynthesis by these PGPR, out of thirty, seven isolates (PS-12, PS-17, PS-21, PS-32, PS-34, PS-51 and PS-71) resulted in maximum auxin (IAA) production in the absence of L-tryptophan which ranged from 1.72 to 3.18 ug/mL. Eleven isolates found negative for auxin production in the absence of L-TRP (Table-1). In the presence of L-tryptophan in the growth media, twelve isolates (PS-01, PS-12, PS-21, PS-30, PS-32, PS-41, PS-49, PS-51, PS-60, PS-63, PS-69 and PS-71) were found most efficient in producing auxin as IAA equivalents. The range of IAA production by these isolates was from 10.83 to 36.63 µg/mL. Two isolates (PS-50 and PS-55) could not to produce IAA either in the presence or absence of L-TRP. The isolates showed higher auxin production in the presence of precursor (L-tryptophan) as compared to without supplementation of L-TRP in the media. In line with this work Asghar *et al.* (2004) reported a several fold enhancement in auxin production by rhizobacteria with L-TRP than without L-TRP. Similar kinds of results were also reported by Ponmurugan and Gopi (2006) they found that phosphate solublizing bacteria (PSB) isolated from the rhizosphere of different field crops including maize, were capable of producing auxin under *in vitro* conditions.

Screening trial for plant growth stimulation under axenic conditions: On the basis of P-solubilization/mineralization, ACC-deaminase activity and auxin production, fifteen best isolates were selected for further evaluation regarding their plant growth promoting activities in the jar trial under axenic conditions. Inoculation of maize seeds with selected bacterial isolates showed significant increase in shoot length except with two isolates (PS-21 and PS-60) compared with uninoculated control (Table-2). Out of fifteen isolates tested, six isolates (PS-01, PS-12, PS-32, PS-41, PS-51 and PS-71) increased shoot length up to 39.7% compared to uninoculated control. Regarding shoot fresh weight of seedlings, maximum increase (up to 99% over uninoculated control) was recorded in response to inoculation with four isolates (PS-01, PS-12, PS-41 and PS-51). The most efficient among these isolates was PS-51 while three isolates (PS-40, PS-52 and PS-71) gave non-significant results but still caused up to 5.8% increase in shoot fresh weight. In case of shoot dry weight inoculation increased it up to 69.4% over uninoculated control. Inoculation with two isolates (PS-31 and PS-71) resulted in minimum increase in shoot dry weight which was up to 5.5% higher than uninoculated control.

Data (Table-2) showed that inoculation with all bacterial isolates increased the root length significantly compared to uninoculated control except three isolates (PS-40, PS-52 and PS-60). Increase in the root length, up to 58.9% was observed in response to inoculation with PS-32, the same strain was most efficient regarding its ACC-deaminase activity. The lowest increase in root length was observed in case of inoculation with PS-52 and PS-60 that was up to 1% more than uninoculated control. Regarding root fresh weight, all the isolates significantly contributed in increasing root fresh weight compared to the uninoculated control except with three isolates (PS-21, PS-60 and PS-71). Results revealed that

PS-41 was most efficient to increase the root fresh weight (up to 97.7%) over uninoculated control.

The inoculation with selected bacterial isolates also improved the root dry weight significantly which ranged from 24 to 87% over uninoculated control. Maximum increase in root dry weight was observed by inoculation with PS-12, which was up to 88.2% more than uninoculated control. Minimum increase in root dry weight as a result of inoculation was 6.6% compared to uninoculated control.

Table-1: *In vitro* characterization of rhizobacteria for different traits.

average of three repeats ± S.E)

Bacterial Isolates	Phosphate Solubilizing Index PSI	Phosphatase Activity (µ g PNP $g^{-1}h^{-1}$)	ACC-deaminase activity (nmol α-ketobutyrate g^{-1}biomass h^{-1})	Auxin Production as IAA equivalents (µg/mL)	
				Without L-Tryptophan	With L-Tryptophan
PS-01	6.27 ± 0.18	35.18 ± 1.5	335 ± 9	1.15 ± 0.2	21.11 ± 1.1
PS-03	2.11 ± 0.06	9.85 ± 0.7	ND	ND	4.16 ± 0.4
PS-07	3.67 ± 0.64	11.33 ± 0.7	279 ± 6	0.93 ± 0.3	9.70 ± 1.0
PS-12	4.87 ± 0.97	42.87 ± 1.5	351 ± 4	1.94 ± 0.3	24.80 ± 4.1
PS-17	3.17 ± 0.30	19.54 ± 0.63	317 ± 10	1.81 ± 0.4	8.11 ± 1.4
PS-21	3.45 ± 0.40	11.49 ± 1	259 ± 10	1.96 ± 0.3	12.64 ± 1.0
PS-23	1.59 ± 0.03	ND	ND	ND	2.22 ± 0.4
PS-24	2.29 ± 0.11	8.12 ± 0.8	ND	0.62 ± 0.2	3.62 ± 0.2
PS-26	2.46 ± 0.14	8.81 ± 0.6	ND	ND	3.47 ± 0.3
PS-27	1.70 ± 0.09	3.26 ± 0.6	ND	1.18 ± 0.3	2.15 ± 0.4
PS-30	3.25 ± 0.15	11.57 ± 0.6	260 ± 11	0.77 ± 0.1	16.02 ± 0.6
PS-31	2.75 ± 0.31	12.95 ± 1.02	337 ± 8	1.20 ± 0.3	6.65 ± 0.6
PS-32	3.91 ± 0.33	29.44 ± 1.6	356 ± 13	1.72 ± 0.3	25.13 ± 3.7
PS-34	2.17 ± 0.05	7.85 ± 0.7	ND	1.75 ± 0.6	3.40 ± 0.5
PS-36	2.57 ± 0.27	9.70 ± 1.1	ND	ND	9.53 ± 0.5
PS-40	2.71 ± 0.16	11.37 ± 1.3	296 ± 4	0.66 ± 0.3	7.11 ± 0.5
PS-41	4.39 ± 0.35	21.08 ± 1.2	240 ± 4	1.32 ± 0.2	30.56 ± 2.0
PS-47	2.83 ± 0.28	8.65 ± 0.6	ND	ND	3.74 ± 0.5
PS-49	2.55 ± 0.25	9.53 ± 0.6	ND	ND	11.32 ± 0.4
PS-50	2.74 ± 0.10	8.86 ± 0.5	ND	ND	ND
PS-51	4.34 ± 0.48	25.43 ± 1.7	316 ± 7	3.18 ± 0.6	36.63 ± 2.0
PS-52	3.54 ± 0.25	12.42 ± 1.0	190 ± 7	1.53 ± 0.3	6.64 ± 0.6
PS-55	2.47 ± 0.12	8.55 ± 0.8	210 ± 8	ND	ND
PS-56	2.25 ± 0.07	6.99 ± 0.6	ND	0.65 ± 0.2	8.14 ± 0.6
PS-60	3.10 ± 0.27	11.18 ± 0.8	230 ± 6	0.74 ± 0.3	10.83 ± 0.5
PS-63	3.63 ± 0.52	15.80 ± 0.7	336 ± 11	0.62 ± 0.2	14.13 ± 0.6
PS-66	2.64 ± 0.07	8.31 ± 0.6	ND	ND	7.59 ± 0.2
PS-67	2.26 ± 0.01	4.51 ± 1.3	ND	ND	6.25 ± 0.3
PS-69	2.33 ± 0.05	7.92 ± 0.6	220 ± 5	ND	11.04 ± 0.5
PS-71	2.85 ± 0.23	19.86 ± 1.6	236 ± 10	2.70 ± 0.6	17.92 ± 0.6

ND= Not detectable

Data from axenic trial revealed that most of the PGPR isolates showed growth promoting activity but with variable efficacy. All the selected isolates significantly increased the shoot and root length, fresh and dry weights as compared to uninoculated control. A significant and a positive correlation was found between ACC-deaminase activity (r = 0.53), phosphatase activity (r = 0.47) and auxin production (r = 0.69) with shoot biomass. Similarly, root length was also positively correlated with ACC-deaminase activity (r = 0. 50), phosphatase activity (r = 0.70) and auxin production (r = 0.64) in the jar trial. The inoculation concurrently improved plant growth. This growth increase may be attributed to auxin production (Piromyou *et al*., 2011), ACC-deaminase (Naik *et al*., 2008; Piromyou *et al*., 2011), production of organic acids (Fankem *et al*., 2008) or phosphatase (Ribeiro and Cardoso, 2012) to solubilize/mineralize P, thereby increasing phosphate nutrition of inoculated plants. Similarly, Kapri and Tewari (2010) also got increased shoot /root length, fresh

and dry weight of shoot/root of chickpea due to inoculation with phosphate solubilizing and phosphatase producing *Trichoderma* sp. Results suggested that seed treatment with these phosphate-solubilizing bacteria enhanced seedling length as previously reported by Sharma *et al*. (2007). Similarly, while investigating the effect of PSB inoculation on growth promotion of cowpea, Linu *et al.* (2009) found that *Burkholderia* sp. gave better results which have been previously evaluated by Pandey *et al*. (2005) to have phosphate solubilizing capability, produced auxin, ACC-deaminase and also nitrogen fixing ability. Likewise Chabot *et al*. (1996) also reported an increase in dry matter of lettuce and maize due to inoculation with phosphate solubilizing *Rhizobium leguminosarum* biovar *phaseoli* capable of solublizing both organic and inorganic phosphorus sources when tested *in vitro*. Study demonstrates that multi-traits bacteria could be more effective PGPR than single trait bacteria to improve plant growth.

Table-2: Effect of selected bacterial isolates on growth parameters of maize seedlings under axenic conditions (Average of three repeats)

	Shoot parameters			Root parameters		
Bacterial isolate	**Shoot length (cm)**	**Shoot fresh weight (g/plant)**	**Shoot dry weight (g/plant)**	**Root length (cm)**	**Root fresh weight (g/plant)**	**Root dry weight (g/plant)**
Un-inoculated Control	38.27 ^{f}	1.33 ^{e}	0.127 ^{g}	16.20 ^{e}	0.733 ^{h}	0.136 ^{f}
PS-01	51.97 ^{a}	2.34 ^{b}	0.207 ^{ab}	23.83 ^{ab}	1.220 ^{b}	0.240 ^{a}
PS-07	47.43 ^{c}	1.85 ^{d}	0.160 $^{d-g}$	20.13 ^{d}	0.917 ^{ef}	0.168 $^{b-f}$
PS-12	53.47 ^{a}	2.06 ^{c}	0.216 ^{a}	25.60 ^{a}	1.450 ^{a}	0.255 ^{a}
PS-17	48.53 ^{bc}	1.84 ^{d}	0.172 ^{cd}	22.03 $^{b-d}$	0.863 ^{fg}	0.178 $^{b-f}$
PS-21	39.70 ^{ef}	1.83 ^{d}	0.160 $^{d-g}$	21.17 ^{cd}	0.733 ^{h}	0.202 $^{a-e}$
PS-30	47.17 ^{c}	1.88 ^{cd}	0.167 $^{c-e}$	21.73 $^{b-d}$	0.987 $^{d-f}$	0.206 $^{a-c}$
PS-31	47.23 ^{c}	1.94 ^{cd}	0.134 $^{e-g}$	22.63 ^{bc}	0.967 $^{d-f}$	0.144 $^{d-f}$
PS-32	51.23 ^{ab}	1.98 ^{cd}	0.197 $^{a-c}$	25.73 ^{a}	1.163 ^{bc}	0.244 ^{a}
PS-40	41.97 ^{de}	1.40 ^{e}	0.170 $^{c-e}$	17.33 ^{e}	0.967 $^{d-f}$	0.168 $^{b-f}$
PS-41	51.13 ^{ab}	2.03 ^{cd}	0.175 $^{b-d}$	23.57 ^{ab}	1.177 ^{bc}	0.213 ^{ab}
PS-51	50.83 ^{ab}	2.64 ^{a}	0.183 $^{a-d}$	23.03 ^{bc}	1.080 ^{cd}	0.205 $^{a-d}$
PS-52	42.63 ^{de}	1.35 ^{e}	0.166 $^{c-f}$	16.23 ^{e}	0.903 ^{ef}	0.145 $^{c-f}$
PS-60	38.43 ^{f}	1.84 ^{d}	0.166 $^{c-e}$	16.37 ^{e}	0.743 ^{gh}	0.143 ^{ef}
PS-63	43.37 ^{d}	1.88 ^{cd}	0.161 $^{c-g}$	21.00 ^{cd}	1.020 ^{de}	0.203 $^{a-e}$
PS-71	48.50 ^{bc}	1.34 ^{e}	0.129 ^{fg}	22.83 ^{bc}	0.743 ^{gh}	0.200 $^{a-e}$
LSD Value	***3.2852***	***0.2074***	***0.0368***	***2.3444***	***0.1245***	***0.0616***

*Means sharing the same letter (s) do not differ significantly at $P < 0.05$ according to Least Significant Difference Test

Conclusion: The study revealed that screening of PGPR with P solubilization/mineralization, auxin production and ACC deaminase activity traits could be highly effective for improving growth of maize crop. It is concluded from the study that phosphate solublizing bacteria enhance the growth through simultaneous exudation of organic acids and/or through releasing phosphatases, ACC-deaminase and auxin production. It can be emphasized that during screening and selection strategies, the selection of bacteria multi-traits PGPR could be more effective approach.

Acknowledgement: This research work (part of Ph.D) was conducted in the Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad and funds were provided by Higher Education Commission (HEC) Islamabad. Their contribution is highly acknowledged.

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