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Characterization of rhizobia nodulating faba bean plants isolated from soils of Jordan for plant growth promoting activities and N_2 fixation potential

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Abstract

Screening experiments were carried out to determine the presence and effectiveness of *Vicia faba* nodulating *Rhizobium* strains with plant growth promoting (PGP) traits from selected locations in Jordan. A total of 54 rhizobial isolates authenticated to nodulate faba bean plants were evaluated for indole acetic acid (IAA) production, phosphate solubilization and 1-aminocyclopropane-1- carboxylate (ACC) utilization. In vitro IAA production was observed in all isolates while phosphate solubilizing and ACC utilizing activities were demonstrated only in 15% of the isolates. Isolates varied significantly for IAA production as well as phosphate solubilization. The average IAA concentration recorded for isolates ranged from 75 to 25µgml^{-1}culture. Phosphate solubilization was more variable with solubilization index ranging from 2 to 4 on Pikovskaya's agar medium. On the basis of auxins biosynthesis, phosphate solubilization and ACC utilization a pot experiment was conducted for evaluating 2 isolates for the growth promotion and N_2 fixation of faba bean. Results showed that inoculation with rhizobial isolates having these PGP traits increased nodulation parameters (90.4% to 70.5% more nodules, 5-6 times larger nodule fresh weight/plant) than isolate lacking these traits. In addition these isolates had a positive impact on growth, N_2 fixation and shoot phosphorous (P) content. The two isolates with PGP activity selected in this study increased shoot dry weight by 40-55% and significantly elevated N_2 fixation potential and shoot P content compared to isolate without PGP activity. In conclusion the IAA production, phosphate solubization and ACC utilization showed by these isolates make them effective bio-inoculants to improving growth and N_2 fixation *in Vicia faba* plants**.**

Keywords: *Vicia faba, Rhizobium, N2 Fixation, Plant growth promoting factors.*

Introduction

The beneficial effect of *Rhizobium* species in terms of biological nitrogen- fixation (BNF) is well known and has been the subject of extensive previous studies (Mylona *et al.*, 1995; Garg and Geetanjali , 2007). Besides their central role in symbiotic BNF rhizobia are also known for their plant growth promoting (PGP) activities. For instance some rhizobia secrete growth hormones such as indole acetic acid (IAA) which has positive influence on plant growth and also plays an important role in the formation and development of root nodules (Lambrecht *et al*, 2000).

In addition rhizobia possess various activities of which phosphate solubilization and utilization of 1-aminocyclopropane-1-carboxylate (ACC) are highly valued. Phosphate (P) solubilization effectively releases P from inorganic and organic pools of total soil P and has the potential to improve plant yield and reduce fertilizer costs. In fact *Rhizobium* species with P solubilization potential are considered to have dual beneficial effects on plants from P mobilization and N_2-fixation (Peix *et al*., 2001). The ability to utilize ACC is widely spread among rhizobia (Nukui, *et al.*,

2004; Duan, *et al.*, 2009; Shahzad, *et al* 2010). This process, which is mediated by the enzyme ACC deaminase, involves cleavage of ACC (the plant ethylene precursor) to ammonia and α-ketobutyrate which is demonstrated to reduces ethylene production by plants. While ACC utilizing rhizobia are reported to possess better nodulation and N_2 fixation potentials compared to non utilizing species (Ma, *et al.*, 2003; Dey, *et al.*, 2004; Shahzad, *et al.*, 2008; Shaharoona *et al*., 2011) they are also considered of special importance to stressed plants. These bacteria are reported to limit the synthesis of stress induced ethylene which is believed to protect plants from the damage that may be caused by various biotic and abiotic stresses (Cheng *et al*. 2013; Zahir *et al*. 2009,; Wang *et al*., 2002).

The objectives of this work was to screen for IAA producing, phosphate solubilizing and ACC utilizing *Rhizobium* strains from nodules of faba bean (*Vicia faba* L.) plants grown in soils from different locations of Jordan in order to identify endogenous strains with high PGP activity. Apparently screening for effective rhizobia in terms of nitrogen fixation and enhancement of their quality in terms of various plant growth promoting activities is important both for agronomic purposes and for the maintenance of soil fertility.

Materials and Methods

Soil Sampling and isolation of Rhizobia

Soil samples were collected from 7 locations in Jordan during the period from June 2012 to June 2013 (Fig.1). At each sampling site the upper 5 cm of the soil surface was discarded and 2-3 kg of the soil was collected. For the isolation of rhizobia, clean and disinfected plastic pots were filled with individual soil samples and 2 to 3 surface sterilized seeds of locally cultivated faba bean (*Vicia faba* L.) were grown in each pot. All pots were kept in the greenhouse at 20 to 25° C, 60-70% relative humidity and normal sunlight (12-13 hr) and watered whenever needed. After three to four weeks, healthy looking plants were uprooted and root nodules were collected. Rhizobia were isolated from nodules and authenticated by the procedure of Vincent (1970). Pure cultures of nodule forming rhizobia were maintained on yeast extract mannitol (YEM) agar slants at 4 ˚C and each isolate was given a JV prefix and a number for identification (Table1).

Table (1). . Rhizobial strains isolated from root nodule of *Vicia faba* L. plants grown in soils from different locations in Jordan

Location	Isolates
Amman	JV1-JV12
Irbid	JV13-JV16
Jerash	JV17
Al-Salt	JV18-JV28
Madaba	JV29-JV38
Zarqa'a	JV39-JV41
Jordan Valley	JV42-JV54

Screening of Rhizobial Isolates for Plant Growth Promoting Activities

ACC Utilization

Rhizobial isolates were screened for ACC utilization using the procedure of Penrose and Glick (2003) with modifications. Individual rhizobial isolates were cultured in 5ml tryptone yeast (TY) broth for 48 h at 25°C -28°C. Cultured cells were harvested by centrifugation at 5000 rpm for 10 min. The cell pellets were re-suspended in 2ml Burk's nitrogen-free medium (Wilson and Knight, 1952) and 200 µl washed cells (O.D = 0.8 nm) of each isolate were transferred aseptically to 2.5 ml Burk's broth medium supplemented with 3 mM ACC as the sole source of nitrogen. Cells were grown on a rotary shaker (200 rpm) at 28º C for 48 h and the resulting culture was used for inoculation onto solid Burk's nitrogen free agar medium containing ACC (3mM). Plates were incubated for 72 h at 30 °C and colony forming isolates on this medium were considered as ACC utilizers.

Phosphate Solubilization

Rhizobial isolates were screened and assessed for phosphate solubilization on Pikovskaya's (PVK) agar medium (Pikovskaya, 1948). PVK agar plates were inoculated with 10 µl of 24 h culture of individual isolates. Plates were incubated for 10 days at 28° C and the formation of clear halo zone by the cultured isolate indicated the ability of the isolate to solubilize Ca-phosphate. Phosphate solubilizing was evaluated according to Mehta and Nautiyal (2001) using the equation:

Phosphate solubilization index = (Total diameter (halo+colony zone))/(Diameter of colony).

Indol acetic acid (IAA) production

IAA production by each isolate was determined calorimetrically using Van Urk Salkowski reagent (Ehmann, 1977). Individual isolates were grown in TY medium supplemented with 0.1% L-tryptophan and incubated at 28°C for 72 h. Cultures were centrifuged at 10000 rpm for 10 min. and 1ml of the resulting supernatant was mixed with 1ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$ solution) and kept at room temperature in the dark for 30 min. IAA concentration in the mixture was determined from a standard curve after measuring its absorbance at 530 nm.

Symbiotic Effectiveness of Rhizobial Isolates

Plant Growth and Rhizobial Inoculation

Surface sterilized seeds were planted in small pots containing 1 kg of sterile soil and were then reduced after germination into single seedling per pot. Young (3-4 days old) uniform seedlings in each pot were inoculated with 1 ml (10^{10} cfu) of fresh culture of the *Rhizobium* isolate under investigation and control (un-inoculated seedlings) were planted in parallel. Seedlings in pots were allowed to grow in the greenhouse at 20-25^{o}C under normal sunlight for three weeks. Throughout this period, plants were watered with sterile water whenever needed. All treatments were arranged in a randomized block design with 5 replicates per treatment. Three weeks after inoculation, the following parameters were estimated for each treatment; shoot fresh weight, shoot dry weight, nodule numbers per plant, nodule fresh weight, nodule leghaemoglobin (LHb) content (determined by the method of Wilson and Reisenauer, 1962) .

Percent N_2 fixation

250 mg of powdered dry shoot from inoculated and un-inoculated plants were digested using 10 ml of concentrated sulfuric acid (98%) and 3 ml prechloric acid. The Nitrogen concentration of each digest was determined by distillation in H_3BO_3 (4%) and titration with 0.01 M H_2SO_4 (AOAC, 1990). Shoot nitrogen contents were then used to estimate percent nitrogen fixed according to Rennie (1984) using the equation:

Percent N_2 fixed = 100*(Total nitrogen content of inoculated plant – total nitrogen of control)/ total nitrogen of inoculated plant)

Shoot Phosphorous Content

For digest preparation, 40 ml HCl (6 M) and few drops HNO_3 (69.5 %) were added to 5 g finely ground dry shoot material and boiled. After cooling the volume of the digest was made to 250 ml with distilled water. To 1ml of filtered digest 4 ml sulfuric acid – ammonium molybdate solution and 1ml Stannous solution were added and the volume was made to 100ml with distilled water. The absorbance of the resulting solution was read at 650 nm (AOAC, 1990) and phosphorous content was determined from a standard curve.

Statistical Analyses

All data were analyzed statistically using Graph Pad Prism 5 program using the analysis of variance "ANOVA" and T-test at *P* value < 0.05.

Results

Fifty four indigenous rhizobia, authenticated to nodulate fab beans, were isolated from root nodules of *Vicia faba* L. plants grown in soils collected from seven locations in Jordan. All 54 isolates were capable of IAA production (Table 2) although, variations were observed among isolates. Based on the level of IAA production, isolates were divided into 3 groups; group 1; high IAA producers (>50µgml^{-1}), group 2; moderate IAA producers (25-50µgml^{-1}), group 3; low IAA producers (< 25µgml^{-1}). The results in Fig. 1 showed that 18.5% of the isolates belonged to group 1 while the remaining 53.7% and 27.8% of the isolates were moderate and low IAA producers, respectively.

Table (2). IAA production by *Vicia faba* nodulating rhizobia isolated from the soils of Jordan

Character	Isolates	Percentage of total isolates
IAA production (Group 1) >50µgml^{-1}	JV6, JV7, JV9, JV10, JV23, JV28, JV30,JV37, JV47 and JV53	18.5%
(Group 2) 25-50µgml^{-1}	JV1, JV3, JV4, JV5, JV8, JV12, JV13, JV14, JV15, JV16, JV17, JV19, JV21, JV22, JV26, JV31, JV33, JV34, JV36, JV39, JV40, JV41, JV42, JV44, JV46, JV49, JV50, JV52 and JV54	53.7%
(Group 3) (< 25µgml^{-1}	JV2, JV11, JV18, JV20, JV24, JV25, JV27, JV29, JV32, JV35,JV38,JV43, JV45,JV48 and JV51	27.8%
Phosphate solubilization	JV2, JV3, JV18, JV24 ,JV25, JV 30, JV31, JV32 and JV52	16.7%
ACC utilization	JV3, JV9, JV11, JV19,JV31, JV32, JV41, JV43 and JV48	14.8%

Fig.1 Map of Jordan showing soil collection sites

Of the 54 rhizobial isolates tested for phosphate solubilization, only 9 isolates; JV2, JV3, JV18, JV24 ,JV25, JV 30, JV31, JV32 and JV52 were identified as phosphate solubilizers with a solubization index ranging from 2 to 4 on Pikovskaya's agar medium. The remaining isolates either had very low phosphate solubilization potential or could not solubilize phosphate suggesting that phosphate solubilization is not a widely spread character among isolates.

Based on their ability to grow on Burk's minimal medium plates with ACC as the sole source of nitrogen the results presented in table (2) showed that only 8 isolates; JV3, JV9, JV11, JV19, JV32, JV41, JV43 and JV48 were able to grow on ACC supplemented plates after 3 days of incubation indicating that only 15% of the isolates were capable of ACC utilization.

Two ACC utilizing phosphate solubilizing and IAA producing isolates (JV3, JV32) and one ACC non-utilizing phosphate non solubilizing isolate (JV36) were finally selected and tested for their nitrogen fixation potential. The results presented in Fig. 2 showed that the three selected *Rhizobium* isolates colonized faba bean roots and induced nodulation, but isolates JV3 and JV32 were better nodulators producing 90.4% and 70.5% more nodules, respectively, than isolate JV36. In addition strong positive correlation between nodule number and nodule fresh weight was noted (Fig.2). Average nodule fresh weight/plant was 5-6 times greater in plants inoculated with isolates JV3 and JV32 compared to those inoculated with isolate JV36. However, no significant difference in mean LHb content/g nodule tissue was observed among the three isolates.

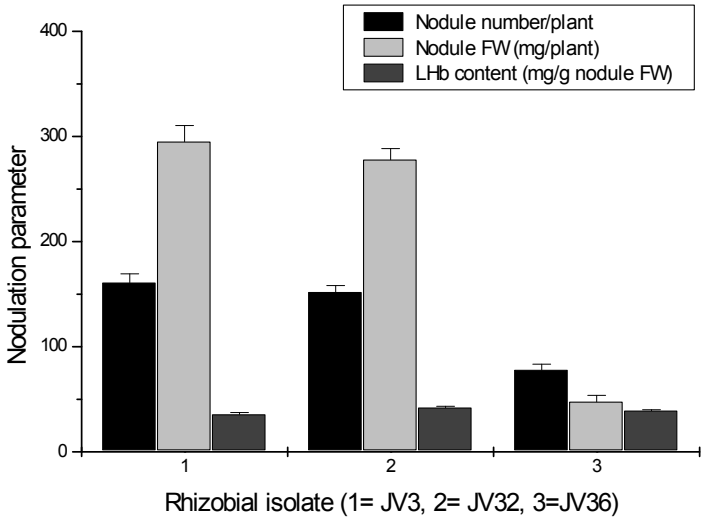

Fig.2 Nodulation parameters of JV3 and JV32 rhizobial isolates showing PGP activity (IAA production, phosphate solubilization and ACC utilization) as compared to isolate JV36 lacking these traits.
(Results are the mean of 5 replicates± SE)

The highest increase in shoot dry weight, as the main indicator of nitrogen fixing ability and growth promotion, was detected in plants inoculated with isolate JV3 followed by plants inoculated with isolate JV32. These isolates resulted in 40% and 55 % increase in dry weight, respectively over control lacking PGP traits (Fig.3).

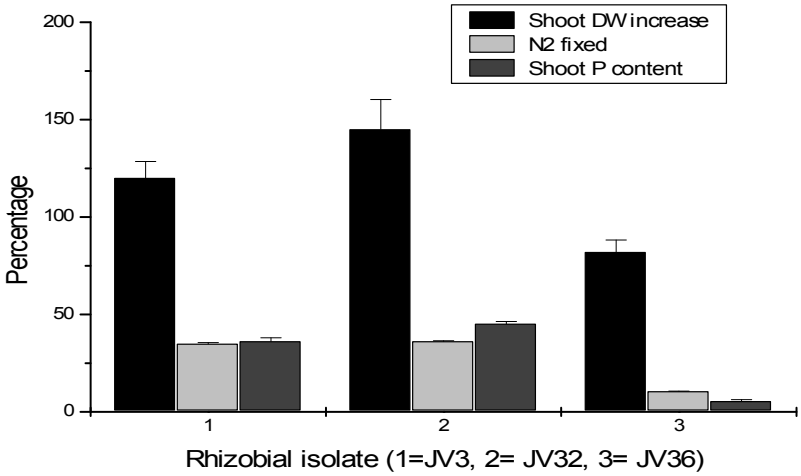

Fig. 3 Effect of inoculating *Vicia faba* plants with JV3 and JV32 rhizobial isolates showing PGP activity on shoot DW, percent N_2 fixed and shoot P content as compared to plants inoculated with isolate JV36 lacking PGP activity.
(Results are the mean of 5 replicates± SE)

Shoot P and N content of plants inoculated with isolates JV3 and JV32, which exhibited phosphate solubilizing activity *in vitro*, were closely similar but significantly higher than those found in plants inoculated with isolate JV36 (Fig. 3). In these plants shoot P and N were 2-3 times greater than those detected in plants inoculated with isolate JV36. While P content of shoots inoculated with isolate JV36 was not elevated it resulted in low (8%) increase in shoot N content compared to un-inoculated control.

Discussion

The use of *Rhizobium* with PGP activities is becoming a popular approach for improving growth and N_2 fixation in legumes. In the present study 54 *Vicia faba* nodulating rhizobia from the soils of Jordan were screened in vitro for PGP activities including auxin production, phosphate solubilization and ACC utilization. All isolates produced auxin (IAA) but with different degree of efficacy. About 15% of isolates, however, exhibited phosphate solubilization and ACC utilization. Two isolates having maximum auxin production P-solubilization and ACC utilization were selected and further evaluated for improving growth, nodulation and N_2 fixation in faba bean plants in a pot experiment. Results revealed that inoculation with these isolates increased faba bean growth, nodulation and N_2 fixation with greater degrees of efficacy than isolates lacking these features. Auxin production by rhizobia is often considered to improve growth and N_2 fixation in many legumes including, beans, lentil, chickpea and pea (Yanni, 1992; Huang and Erickson, 2007; Anjum *et al.*, 2011; Zafar-ul-Hye *et al*., 2013). The positive influence of co-inoculation of rhizobia and auxin producing rhizobacteria on growth and nodule formation in legumes (Yadegari *et al*., 2008; Stajković *et al*., 2011; Hungria *et al*., 2013) emphasizes the role of this hormone in nodule development. The value of employing P solubilizing and ACC utilizing rhizobia in improving nodulation and N_2 fixation is well documented. Previous work demonstrated that nodulation and N_2-fixation are P-dependent (Barea *et al*., 2005). Improved phosphorous nutrition by P solubilizing rhizobia was reported to influences overall plant growth, root development and nitrogenase activity which is positively correlated with increased nodulation and N_2-fixation (Kenasa *et al*., 2014, Singh *et al*., 2014). The results of this work extends support for these findings and suggest that both P solubilizing and ACC utilizing rhizobia are more superior in promoting faba bean growth and nodulation than isolates lacking these traits. The ability of some rhizobia to utilize ACC was reported to improve nodulation and plant growth in various legumes (Zafar-ul-Hye *et al*., 2013). Presence of ACC utilizing rhizobia on the roots of legumes is thought to suppress endogenous synthesis of ethylene during rhizobial infection and thus may facilitate nodulation and improve growth and yield. Stimulation of nodulation by Ag+ (which inhibits ethylene action) and by amino ethoxyvinyl glycine (AVG) (a chemical inhibitor of endogenous ethylene biosynthesis) strongly supports this premise (Schmidt *et al.*, 1999). Apparently the findings of this study agree with previously reported results demonstrating the positive influence of rhizobia with PGP activities on nodulation parameters and plant growth and suggests that the use of rhizobial inoculant that have some other qualities in addition to nitrogen fixing capacity such as production of plant growth promoting hormones, solubilization of phosphates and utilization of ACC could be very useful strategy for improving legume crops.

Conclusion

Fifty four rhizobial isolates nodulating faba bean plants isolated from selected locations in Jordan were studied for PGP activity and N_2 fixation potential. All isolates were able to produce IAA, 8 isolates were found positive for phosphate solubilization and 7 were positive for ACC utilization. Rhizobial isolates showing the three PGP activities were tested for their N_2 fixation potential in faba bean plants and compared with isolates lacking these activities. Results suggested that rhizobia with PGP activity are more superior to those without such activities in promoting faba bean growth and in stimulating the level of N_2 fixed and plant P accumulation. Thus, the potential capacity of the present rhizobial isolate in IAA production, phosphate solubilization and ACC utilization can be employed as biological fertilizer and would have a significant role in achieving sustainable agriculture.

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