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Research Article

Effect of Phosphorus and Nitrogen Concentrations on Root Colonization of Soybean (*GLYCINE MAX* L.) by *BRADYRHIZOBIUM JAPONICUM* and *PSEUDOMONAS PUTIDA*

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ABSTRACT:
The effect of N and P availability on the growth and colonisation of *Bradyrhizobium japonicum* and *Pseudomonas putida* on the rhizosphere of soybean (*Glycine max* L.) was studied. Three modified solution HNHP (high N -3000 µmol/L, high P -250 µmol/L), HNLP (high N-3000 µmol/l, low P -50 µmol/l), LNLP (low N-300 µmol/l, low P -250 µmol/l) were maintained in the gnotobiotic sand system. Decreasing of the N and P concentrations in growth medium inhibited the ability of *B. japonicum* strain NU1 cells to colonize soybean roots, CFU counts decreasing by 20 %, from $107.0x10^3$ (HNHP) to $86.5x10^3$ (LNLP) CFU/cm of root tip respectively. In competitive root tip colonization assay *P. putida* TSAU1 were better colonizers than *B. japonicum* NU1. Under Low N and P concentrations the root, shoot length, fresh and dry weight of soybean reduced by *B. japonicum*. Co-inoculation of soybean with *B. japonicum* NU1 and *P. putida* TSAU1 showed the highest stimulatory effect, by increasing significantly dry weight by 39% (HNHP) and 66% (LNLP) in comparison to inoculation with *B. japonicum* NU1 alone.

Key words: *nitrogen, phosphorus, colonization, soybean, Bradyrhizobium japonicum, Pseudomonas putida*

[I] NTRODUCTION

Soybean (*Glycine max* L.) is an important legume which contains high amount of protein and considered as an excellent rotation and intercropping crop by improving soil fertility [1]. The available essential nutrient elements such as N, P in soil to the soybean growth are one of the key components to determine their productivity [2,3]. According Araújo and Teixeira [4] the addition of P improves plant N status and promotes plant growth, whereas P deficiency affects negatively on those parameters. The inoculation of leguminous plants with rhizobium could improve N fixation, increase the nodule numbers, promote root growth, nutrient uptake (N,P,K) and yield of leguminous plants [5,6]. Understanding of biotic and abiotic factors such as temperature, soil pH, moisture, soil nutrients, indigenous microorganisms which affect the colonisation of

rhizobium in the rhizosphere is of primary importance for the effective use of rhizobial inoculants in legume production [7]. Bauske et al. [8] studied the effect of botanical aromatic compounds and seed surface pH on colonization of cotton plant by PGPR, whereas Chabot et al. [9] reported about the effect of phosphorus on root colonization by *Rhizobium leguminosarum biovar phaseoli* and on growth promotion of maize. Plant-growth-promoting bacteria isolated from the rhizosphere and phyllosphere were analysed for their colonization and growth-promoting effects on winter wheat and pea at different temperatures. The effect of different temperatures on the colonisation of *Cellulomonas* spp. in the rhizosphere of pea has been studied by Egamberdieva and Hoflich [10]. The present investigation is designed to investigate the effect of N and P availability on the plant growth and colonisation of *Bradyrhizobium japonicum* and *Pseudomonas putida* on the rhizosphere of soybean, using a gnotobiotic sand system.

[II] MATERIALS AND METHODS

2.1. Plant and bacterial strains

Soybean (*Glycine max* L.) cultivar Orzu used in this study was provided by the Tashkent State University of Agriculture, Uzbekistan. The root colonizing *Pseudomonas putida* strain TSAU1 and *Bradyrhizobium japonicum* strain NU1 were obtained from the culture collection of the Department of Microbiology and Biotechnology, National University of Uzbekistan. The *P. putida* strains were grown on King's B agar (KB) and *B. japonicum* strains on tryptone yeast extract agar (TY) [11] at 28°C.

2.2. Root tip colonization

Experiments under gnotobiotic conditions were carried out in test tubes (25 mm in diameter, 200 mm in length) as described by Simons et al. [12]. The tubes contained 60 g of a sterilized mixture of washed sand and vermiculite (1:1) soaked with 6 ml of diluted hoagland solution [13]. The modified high nitrogen（3000μmol/L) and high phosphorus (250μmol/L) hoagland solution [14]. were prepared as follow: stock content ×1000 (g/1000ml): KNO_3 101.10g; $Ca(NO_3)_2 \cdot 4H_2O$ 141.69; NH_4NO_3 32.02g; $MgCl_2$ 5.08; $CaCl_2$ 66.59g; $MgSO_4.7H_2O$ 123.235 g. $ZnSO_4.7H_2O$ 0.431 g; $CuSO_4.5H_2O$ 0.125 g; $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.1 g; $MnSO_4.H_2O$ 0.254 g; $(NH_4)_2SO_4$ 23.84; KH_2PO_4 34.022 g; Fe-EDTA(Na) 14.68 g; K_2SO_4 96 g, $NaB_4O_7.10H_2O$ 0.95 g. The modified high nitrogen（3000μmol/L）and low phosphorus (50μmol/L) hoagland solution [14] were prepared as follow: stock content ×1000 (g/1000ml): $KNO_3$101.10g; $Ca(NO_3)_2 \cdot 4H_2O$ 141.69; NH_4NO_3 32.02 g; $MgCl_2$ 5.08; $CaCl_2$ 66.59g; $MgSO_4.7H_2O$ 123.235 g. $ZnSO_4.7H_2O$ 0.431 g; $CuSO_4.5H_2O$ 0.125 g; $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.20 g; $MnSO_4.H_2O$ 0.254 g; $(NH_4)_2SO_4$ 23.84; KH_2PO_4 34.022 g; Fe-EDTA(Na) 14.68 g; K_2SO_4 113.27 g, $NaB_4O_7.10H_2O$ 0.95 g. The modified low nitrogen（300μmol/L）and low phosphorus (50μmol/L) hoagland solution [14] were prepared as follow: stock content ×1000 (g/1000ml): NH_4NO_3 12 g; $MgCl_2$ 5.08; $CaCl_2$ 133.18 g; $MgSO_4.7H_2O$ 123.24 g. $ZnSO_4.7H_2O$ 0.431 g; $CuSO_4.5H_2O$ 0.125 g; $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.20 g; K_2SO_4 52.28 g; $MnSO_4.H_2O$ 0.254 g; KH_2PO_4 34.022 g; Fe-EDTA(Na) 14.68 g; K_2SO_4 148 g, $NaB_4O_7.10H_2O$ 0.95 g.

The treatments were as follows: i) seeds inoculated with *B. japonicum* NU1 alone and ii) *B. japonicum* NU1 combined with *P. putida* TSAU1. Bacterial inoculants were grown and prepared and the seeds were inoculated as described by Jabborova [15]. Briefly, *B. japonicum* was grown overnight in TY broth and the *Pseudomonas* strains in KB broth. One ml of each culture was pelleted by centrifugation and the supernatant was discarded. Cell pellets were washed with 1 ml phosphate buffered saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and suspended in PBS. Cell suspensions were diluted to an optical density

of 0.1 at 620 nm, corresponding to a cell density of 10^8 cells/ml. For co-inoculation, the cell suspension with two strains was mixed in a ratio 1:1 and vortexes vigorously to yield a homogenous suspension. Germinated seeds were placed in the bacterial suspension with sterile forceps and shaken gently for a few seconds. After 10 min, inoculated seedlings were planted into sterile glass tubes, one seed per tube with ten replicates. The seedlings were grown in a growth cabinet with a 16-h light period at 22°C and an 8-h dark period at 16°C. After 14 days, the seedlings were removed from the sand and 1 cm of root tip was cut from the plantlets and transferred into a tube containing 1 ml of PBS. Bacterial cells were removed from the root tip by vortexing root tip in PBS. The homogenates were serially diluted and appropriate dilutions, 10^{-3} and 10^{-4}, were spread on two agar plates. KB agar supplemented with kanamycin (50 µg/ml) was used to select for intrinsically kanamycin resistant *Pseudomonas* strains. *Bradyrhizobium* counts were done on yeast mannitol (YEM) agar supplemented with Congo red, which distinguish *Bradyrhizobium* colonies from *Pseudomonas* colonies according to their differences in colour and shape. After incubation at 28°C, *Pseudomonas* colonies on KB agar were counted after 2 days and *Bradyrhizobium* colonies on YEM agar after 3 days. The number of bacteria was calculated as CFU per 1 cm of root tip.

2.3. Plant growth promotion in gnotobiotic systems

The effect of inoculation with *B. japonicum* strain NU1 alone and in combination with the *P. putida* strain TSAU1 on the growth of soybean seedlings was studied under gnotobiotic conditions with 10 replicate tubes as described above. The modified high nitrogen（3000µmol/L) and high phosphorus (250µmol/L), high nitrogen（3000µmol/L）and low phosphorus (50µmol/L), low nitrogen（300µmol/L）and low phosphorus (50µmol/L) hoagland solution were used as plant nutrition solution. At harvest, after14 days, the length of shoots and roots, the fresh weight and dry weight of whole plants were measured.

Analysis of variance was performed using the Excel program package version 11 for Windows 2007 (Microsoft Corporation), Student's *t*-test and least significant differences (LSD) were applied to compare means at $P<0.05$.

[III] RESULTS

3.1. Root tip colonization

The association and colonization of *Rhizobium* on surface of roots involve direct competition with other rhizosphere bacteria. In our experiments the N and P availability in growth medium affect on the plant growth and colonization of *B. japonicum* NU1 alone and in combination with *Pseudomonas putida* TSAU1 in the rhizosphere of soybean. Decreasing of the N and P concentrations in growth medium inhibited the ability of *B. japonicum* strain NU1 cells to colonize soybean roots, CFU counts decreasing by 20 %, from $107.0x10^3$ (HNHP) to $86.5x10^3$ (LNLP) CFU/cm of root tip. Our competitive root tip colonization test showed that *P. putida* TSAU1 were better colonizers than *B. japonicum* NU1 **[Figure 1**]. At high N and high P condition the colony counts were $35x10^3$ CFU/cm for *P. putida* TSAU1 and 19.4 $x10^3$ for *B. japonicum* NU1.

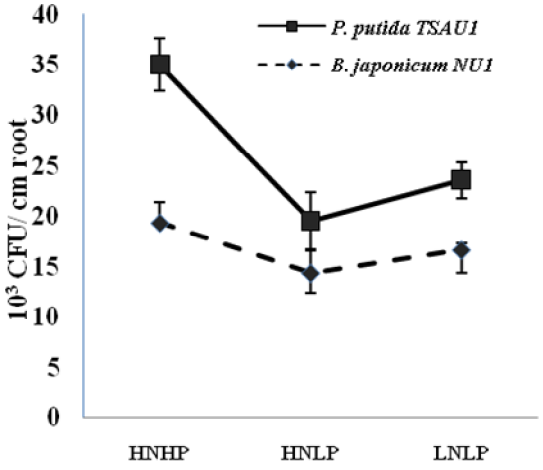

Fig:1. Competitive soybean root tip colonization ability of *Bradyrhizobium japonicum* NU1 strain in competition with *Pseudomonas putida* TSAU1 (plants

grown in gnotobiotic sand system with various N and P content; HNHP- high N (3000 µmol/l) and high P (250 µmol/l); high N (3000 µmol/l) and low P (50 µmol/l); with low N (300 µmol/l) and low P (250 µmol/l)).

3.2. Plant growth promotion

Under Low N and P concentrations the root, shoot length, fresh and dry weight of soybean also reduced up to 18, 8, 26, 29 % by *B. japonicum* respectively presumably due to the reduced availability of nutrients required for the growth **[Table 1].** Potential root colonization of plant root by *Pseudomonas* is important factor in the ability of bacteria promote growth and development. However lower concentration of N and P (LN -300 µmol/l, LP -250 µmol/l) reduced the root, shoot length and dry weight of soybean inoculated with *B. japonicum* NU1 combined with *P. putida* TSAU1 **[Table 1].** However, co-inoculation of soybean with *B. japonicum* NU1 and *P. putida* TSAU1 showed the highest stimulatory effect, by increasing significantly root length and dry weight by 39% in comparison to inoculation with *B. japonicum* NU1 alone under high N (3000 µmol/l) and high P (250 µmol/l) condition **[Table 1].**

Treatments	Root length[a]	Shoot length[a]	Dry weight[b]
high N (3000 µmol/l) and high P (250 µmol/l)			
B. japonicum NU1	12.7 ± 1.15	17.2 ± 0.5	0.11 ± 0.01
B.japonicum NU1+*P. putida* TSAU1	17.3 ± 0.6*	18.0 ± 0.8	0.15 ± 0.08
high N (3000 µmol/l) and low P (50 µmol/l)			
B. japonicum NU1	10.3 ± 1.5	17.0 ± 2.64	0.13 ± 0.01
B.japonicum NU1+*P. putida* TSAU1	14.3 ± 1.15	18.7 ± 0.57	0.12 ± 0.03
with low N (300 µmol/l) and low P (250 µmol/l)			
B. japonicum NU1	12.5 ± 1.29	16.0 ± 2.00	0.08 ± 0.01
B.japonicum NU1+*P. putida* TSAU1	15.5 ± 1.00*	21.7 ± 2.08*	0.13± 0.04*

Table 1. The length, fresh and dry weight of root and shoot of soybean in gnotobiotic sand culture system with different N and P content

[a] Shoot and root length cm; [b] Fresh and dry weight, gram/plant; ± SD. Plants were grown for 14 days in plant growth chamber, values represent means for six plants (N = 6), *significantly different at $P<0.05$

[IV] DISCUSSION

The colonization of leguminous root hairs by rhizobial cells is fundamental for the establishment of the legume - *Rhizobium* symbiosis in order to continue improving plant growth and development [15,16]. The colonization of root hairs by rhizobial cells, are especially sensitive to biotic and abiotic factors such as soil nutrient content, pH, salinity, temperature [17]. The changes of N and P concentration in the rhizosphere will directly affect the mobility of several nutrients and the activity of diverse groups of symbiotic and free-living microorganisms [19]. Marschner et al. [2] also observed that nitrogen deficiency decreased both plant growth and root colonization by *P. fluorescens* 2-79RLI at the root tip. Medeiros et al. [20] observed where the addition of increased doses of ammonium and nitrate inhibited the population of *G. diazotrophicus*. The higher number of bacterial cells in the rhizosphere of soybean grown in high N and high P condition may be due to increased leakage of solutes into the rhizosphere as a result of impaired exudate retention in the rhizosphere [2]. An increase in the number of *Pseudomonas* bacteria indicated that strain was able to grow faster within the plant rhizosphere competing use of the root exudates as a possible source of carbon. There have been several studies investigating colonization of leguminous plant root by *Pseudomonas* strains [21,15,22]. According Lugtenberg et al. [16] the

growth rate of bacterial strains in the rhizosphere will depend on the ability to take up components essential for cell growth and/or maintenance. It is known that pseudomonads are motile by one or several polar flagella and thus allowing bacteria to reach and utilize a large number of carbon sources and nitrogen compounds as the roots grow [23]. The root length was decreased up to 11% and dry weight up to 32% compared to control plants. This could be due to the fact that nitrogen and phosphorus both being essential constituent of plant tissue, influence the plant growth and development [24]. Singh et al. [25] observed that the plant height, and dry matter accumulation of black gram increased significantly with higher nitrogen and phosphorus concentrations.

The positive effect of combined bacterial treatments on plant growth has already been described for other leguminous crops. Co-inoculation with *Pseudomonas* spp. and *Rhizobium* spp. enhanced nodulation and plant biomass and grain yield in various leguminous species including chickpea [26], pea [27], and goats rue [28].

[V] CONCLUSION

We observed that plant growth stimulation by co-inoculation of soybean was higher in low N and P condition compared to the single-inoculation. Whether this stimulation is caused by some compounds produced by the *Pseudomonas* strains or alternatively induced by quantitatively or qualitatively improved plant exudates remain subjects for other studies.

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REFERENCES

1. Aslam M, Mahmood IA, Ahmad S, Peoples MB, Schwenke GD, Herridge DF (2003) Contribution of chickpea nitrogen fixation to increased wheat production and soil organic fertility in rain-fed cropping. Biol Fertil Soils 38:9-64.
2. Marschner P, Gerendás J, Sattelmacher B (1999) Effect of N concentration and N source on root colonization by *Pseudomonas fluorescens* 2-79RLI. Plant Soil 215(2): 135-141.
3. Sanginga N, Thottappilly G, Dashiell K (2000) Effectiveness of rhizobia modulating recent promiscuous soybean selections in the most Savanna of Nigeria. Soil Biol Biochem 32: 127-133.
4. Araújo AP, Teixeira MG (2000) Ontogenetic variations on absorption and utilization of phosphorus in common bean cultivars under biological nitrogen fixation. Plant Soil 225:1–10.
5. Okereke GU, Onochie CC (1996) Screening of native and foreign *Bradyrhizobium japonicum* strains for N2-fixation in soybean. World J Microb Biotech 12:639-641Bauske EM, Backman PA, Harper KM, Brannen PM, Kabana RR, Kloepper JW (1997) Effect of botanical aromatic compounds and seed surface pH on growth and colonization of cotton plant-growth promoting rhizobacteria. Bioc Sci Technol **7**:415-421.
6. Cheng F, Cao G, Wang X, Zhao J, Yan X, Liao H. (2009) Isolation and application of effective nitrogen fixation rhizobial strains on low-phosphorus acid soils in South China. Chin Sci Bull 54(3): 412-420.
7. Schroth MN, Becker JO **(**1990) Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion, In: Hornby D. (eds.), Biological control of soil-borne plant pathogens. CAB International, Wallingford, United Kingdom, 389–414.
8. Bauske EM, Backman PA, Harper KM, Brannen PM, Kabana RR, Kloepper JW (1997)

Effect of botanical aromatic compounds and seed surface pH on growth and colonization of cotton plant-growth promoting rhizobacteria. Bioc Sci Technol **7**:415-421.

9. Chabot R, Beauchamp CJ, Kloepper JW, Antoun H (1998) Effect of phosphorus on root colonization and growth promotion of maize by bioluminescent mutants of phosphate-solubilizing *Rhizobium leguminosarum* biovar *phaseoli*. Soil Biol Bioch 30: 1615-1618.
10. Egamberdiyeva D, Hoflich G (2002) Root colonization and growth promotion of winter wheat and pea by *Cellulomonas* spp. at different temperatures. J Plant Growth Regul 38:219-224.
11. Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Microb 84:188-198.
12. Simons M, van der Bij AJ, Brand I, de Weger LA, Wijffelman CA, Lugtenberg BJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting Pseudomonas bacteria. Mol Plant Microbe Interact 9:600–607.
13. Vincent J (1970) A Manual for the Practical Study of Root-nodule Bacteria. Blackwell, Oxford.
14. Lynch J (1990) The rhizosphere. Wiley, London, UK, 458.
15. Jabborova D (2012) Improving of the growth, development and symbiotic performance of soybean using pseudomonas strains. Bulletin of Uzbek Academy of Sciences 2:64-67.
16. Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomanas*. Ann Rev Phytopat 39: 461-490.
17. Rekha PD, Lai WA, Arun AB, Young CC (2007) Effect of free and encapsulated *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104on plant growth under gnotobiotic condition. Biores Tech 98:447-451.
18. Räsänen LA, Saijets S, Jokinen K, Lindström K (2003) Evaluation of the roles of two compatible solutes, glycine betaine and trehalose, for the Acacia senegal–Sinorhizobium symbiosis exposed to drought stress. Plant Soil 260:237-251.
19. Siqueira JO, Moreira FMS (1997) Microbial populations and activities in highly weathered acidic soils: highlights of the Brazilian research in Plant-Soil interactions at low pH: Sustainable Agriculture and Forestry Production. In: Moniz, A.C. et al. (Eds) Brazilian Soil Science Society, Campinas, Brazil. pp 139-156.
20. Medeiros A, Polidoro J, Reis V (2006) Nitrogen source effect on *Gluconacetobacter diazotrophicus* colonization of sugarcane (*Saccharum* Spp.). Plant Soil 279(1-2):141-152.
21. Miller CD, KimYC, Anderson AJ (2001) Competitiveness in root colonization by *Pseudomonas putida* requires the *rpoS* gene. Can J Microb 47: 41-48.
22. Jabborova D., Qodirova D, Egamberdieva D (2014) Plant growth stimulation and root colonisation of soybean (Glycine max L.) by *Bradyrhizobium japonicum* and *Pseudomonas putida* as affected by phosphorus and nitrogen source. In: XIVth International Congress of Bacteriology, Applied Microbiology, Mycology and Virology, Proceeding book, p-139, Montréal, Canada.
23. Morales A, Garland JL, Lim DV (1996) Survival of potentially pathogenic human-associated bacteria in the rhizosphere of hydroponically grown wheat. FEMS Microb Ecol 20:155–162.
24. Kumar R, Chandra R (2008) Influence of PGPR and PSB on *Rhizobium leguminosarum* bv. Viciae competition and symbiotic performance in lentil. World J Agr Sci 4(3):297-301.
25. Singh RP, Gupta SC, Yadav AS (2008) Effect of levels and sources of phosphorus and PSB on growth and yield of blackgram (*Vigna mungo* L. Hepper.). Legume Research 31(2):139-141.

26.Goel AK, Sindhu SS, Dadarwal KR (2000) Stimulation of nodulation and plant growth of chickpea (*Cicer arietinum*) by *Pseudomonas* spp. antagonistic to fungal pathogens. Biol Fert Soil 36:391-396

27.Dileep Kumar BS, Berggren I, Mårtensson AM (2001) Potential for improving pea production by co-inoculation with fluorescent *Pseudomonas* and *Rhizobium.* Plant Soil 229:25–34.

28. Egamberdieva D, Berg G, Lindstrom K, Rasanen L (2010) Root colonising *Pseudomonas* spp. improve growth and symbiosis performance of fodder galega (*Galega orientalis* LAM) grown in potting soil. Eur J Soil Biol 46 (3-4):269-272 .