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Effects of seed inoculation by *Rhizobium* strains on chlorophyll content and protein percentage in common bean cultivars (*Phaseolus vulgaris* L.)

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Abstract

To study the effect of *Rhizobium* inoculation treatments (inoculation with strains number 133, 116 & their integrated application) and chemical nitrogen fertilizer application (based on soil analysis in form of Urea) on chlorophyll content and protein percentage of three common bean cultivars (viz. Bahman, Darkhshan and Sayyad) a field experiment was conducted at Agricultural Research Farm of College of Agriculture, University of Tehran (Karaj, Iran) during 2010 and 2011 growing seasons. The treatments arranged as factorial based on a randomized complete block design with three replications. The characteristics such as seed and leaf protein percentage, total chlorophyll content at flowering and podding stage were measured. The highest seed protein percentage of 28.02 was produced by Sayyad cv. when inoculated by rhizobium strain 116. The Darakhsan cv. treated with rhizobium strain 116 produced the highest leaf protein (27.67%) as compared to other fertilizing treatments. The highest total chlorophyll content (1.3 mg/gr) was measured in plants inoculated by Rhizobium strain 116. In response to an interaction effect, Drakhshan cultivar produced the highest total chlorophyll content (1.56 mg/gr) when treated by chemical nitrogen fertilizer. This could be explained by better access and easier nitrogen absorption in chemical nitrogen fertilizer treatment. The better vegetative growth in plants inoculated by rhizobium strains because of better access to nitrogen sources could stimulate other growth characteristics like total chlorophyll content in leaves. The results of this experiment suggest that application of rhizobium strains have the best effect on chlorophyll content and seed percentage.

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Introduction

Common bean (*Phaseolus vulgaris* L.) is the most edible legume representing 50% of grain legume for direct human consumption (McClean *et al.,* 2004). In some countries such as Mexico and Brazil, beans are the primary source of protein in human diets (Broughton *et al.,* 2003).

Nitrogen deficiency is considered as one the most important limiting factors in crop production. The mean nitrogen content in different crops varies between 1 to 6 percent. Nitrogen is the main element in bio-molecular structure of Nucleic acids, Adonezin Tri phosphate (ATP), Nicotinic acid, Nicotine amid Di Nucleotid (NAD), and proteins (Stacey *et al.,* 1992). The over application of nitrogen fertilizer in crop production have created adverse effects on soil, under-ground water and the environment.

The environmental friendly pro-biotic bacteria like rhizobium and their role in nitrogen fixation has come to the attention of the researchers in recent years. One of the most efficient symbiotic bacteria with legumes is Rhizobium leguminosarum (Giller, 2001). N2-fixing is important for crop plants as they increase N uptake and play a crucial role as Plant growth promoting rhizobacteria (PGPR) in biofertilization system (Zaidi and Mohammad, 2006). Thus, the application of such microbes as environment friendly biofertilizer may contribute to minimize the use of expensive chemical fertilizers. According to some researcher, nitrogen fixation is a genetically controlled quantitative characteristic and bean cultivars are very diversified in this trait (Anyango *et al.,* 1995).

There is a controversy in reports concerning the results by Rhizobium inoculation and over doses of chemical nitrogen applications (Graham, 1981). Despite of this, bean seed inoculation is still vastly practiced (Barron *et al.,* 2000). Tang *et al.* (2001) declared that seed inoculation along with minor amounts of chemical nitrogen and phosphorous fertilizer application at early growth stage (10 day after germination) could stimulate the root nodulation and increase biological nitrogen fixation. Nitrogen supply affects a wide range of physiological processes in higher plants (Cechin and Fumis, 2004). Application of a small amount of N at planting called as "starter N" reported to be beneficial to improve early growth and yield of soybean in most cases (Osborne and Riedell, 2006). Significant interactions have been reported between bean cultivars and different Rhizobium strains in regard to nodulation, biomass production and sap chemical components (Rodriguez-Navarrro, 2000). In a greenhouse and field experiment, inoculation of bean seed by domestic Iranian Rhizobium strains significantly increased total fixed nitrogen compared to control (Asadi Rahmani *et al.,* 2005). The chlorophyll content in leaf is a good indication of the nitrogen fixation (Kumawat *et al.,* 2000). The superiority of inoculated seeds in nitrogen fixation has been well documented (Yaman and Cinsoy, 1996).

The objective of this research was to evaluate the seed inoculation of different bean cultivars by different Rhizobium strains to identify the best inoculants in regard to protein percentage and chlorophyll content, for bean at field condition.

Materials and methods

Location and experimental design

This experiment was conducted in Research Farm of College of Agriculture, University of Tehran in Karaj, Iran during 2008 and 2009 growing seasons. The experimental site is located in 35: 56 northern latitude and 50: 58 eastern longitude with 1112.5 m height from the sea level. The treatments arranged as factorial based on a randomized complete block design with three replications. Two Rhizobium strains of 133, 116 & their integrated application (133+116), chemical nitrogen fertilizer application (based on soil analysis of 100kg N/ha from Urea source) and control (no inoculation or fertilizing treatment) were considered as the first variable and three common bean cultivars (viz. Bahman, Darakhshan & Sayyad) were the second variable in

this experiment. Sayyad and Darkhshan bean cultivars have a semi erect and erect growth habits with Columbia origin, respectively. The Rhizobium strains were provided by Soil and Water Research Institute of Iran.

Soil analysis

Soil analysis of the experimental site is presented in Table 1.

Seed inoculation

Seed inoculation was done according to Subba Rao, (1988) method. Inoculated seeds were dried in shade and instantly planted on May 9th and May 17th in 2008 and 2009, respectively.

Crop management

The experimental plot dimensions were 2.5 x 3 m consisting of 5 rows of cultivated bean 50cm apart. The seeds were planted 15cm apart on each row. A furrow irrigation system was employed during experimental period. All the plots were subjected to hand weeding 25 and 50 days after planting the seeds.

Chlorophyll determining

Leaf chlorophyll was measured at two growing stages. The first sampling was done from the third stem node during early flowering stage and the second sampling was done during 50% pod formation following the same processes. Chlorophyll measurement was done following Arnon, (1949) method.

Nitrogen content

Nitrogen content was measured by taking samples from the third node on the stem during early flowering stage and an Autoanalyzer Kjeltec 1030 apparatus was used to measure N percentage in plant samples. The protein content was calculated by total N x 6.25. The sampling for N content was done at physiological maturity when more than 50% of the pods were matured.

Statistical analyses

After the homogeneity test for collected data variance, statistical analysis was performed using Mstat-C software and the means were compared at $P<0.05\%$ probability level.

Results

Mean seed protein content was more in 2009 (26.9%) compared to 2008 (Table 5). Sayyad cultivar had the highest seed protein content of 27.4% across all fertilizing treatments while seed inoculation by Rhizobium strain 116 produced the highest protein percentage (27.2%) across all bean cultivars (Table 3). The significant interaction between fertilizing systems and bean cultivars caused that Sayyad cultivar produced the highest protein content of 27.9% at control treatment and when inoculated by Rhizobium strain 116 (28%), respectively (Table 4). Leaf protein content was significantly affected by bean cultivars, fertilizing systems as well as their interaction effect ($p<0.05$) (Table 2). Darakhshan cultivar showed the highest leaf protein content (26.5%) among the bean cultivars and seed inoculation by Rhizobium strain 116 produced the highest leaf protein content (25.4%) among the fertilizing systems, respectively. However, the highest leaf protein content (25.3%) was produced by Drakhshan cultivar when inoculated by Rhizobium strain 116 (Table 4). Leaf protein content was significantly higher in the second year of the experiment (2009) compared to the first year (2008) ($p<0.05$).

Table 1. Physical and chemical soil analysis of the experimental site (2008 and 2009).

Year	Texture	Total mg/kg	P mg/kg	K mg/kg	pH
2008	loam	90	14.2	151	8
2009	loam	97	13.5	180	7.9

Total chlorophyll content in leaves in growth stages of flowering and pod formation was significantly higher in the first year (2008) of the experiment compared to the second year (2009) (Table 5). Total chlorophyll in Drakhshan cultivar significantly ($p<0.05$) exceeded (1.45 mg/gr) other bean

cultivars in both growing stages (early flowering and pod formation) (Table 3). The highest total chlorophyll content (1.3 mg/gr) was measured in plants inoculated by Rhizobium strain 116 (Table 3). In response to an interaction effect, Drakhshan cultivar produced the highest total chlorophyll content (1.56 mg/gr) when treated by chemical nitrogen fertilizer (Table 4). This could be explained by better access and easier nitrogen absorption in chemical nitrogen fertilizer treatment. In pod formation stage when more than 50% of pods were matured, the highest total chlorophyll content was obtained in plants inoculated by Rhizobium strain 133. This is a very important phenomenon because in this stage of plant growth the final yield is determined and the more chlorophyll content in the plants guarantees better seed formation and higher seed production.

In pod formation stage of the plant growth, Drakhshan cultivar inoculated by Rhizobium strain 113 produced the highest total chlorophyll content (0.89 mg/gr) (Table 4). Plants with higher total chlorophyll content performed better in leaf protein content which is a good index for better biological nitrogen absorption. Seed inoculation by Rhizobium strains performed superiority over control (no fertilizer treatment) and chemical nitrogen fertilizer treatment in percent protein in seed.

Table 2. Analysis of variance on Rhizobium strains effects on different traits of bean cultivars.

Sources of variance	df	Leaf Protein	Total Chlorophyll in podding stage	Total chlorophyll in flowering stage	Seed Protein
Year	1	2.364*	0.014*	0.018*	0.298
Replication*(year)	4	0.240	0.005	0.002	0.081
Variety (A)	2	71.889**	0.245**	1.638**	8.356**
Year*variety	2	0.229	0.001	0.002	0.063
Nitrogen (B)	4	5.761**	0.019**	0.106**	1.571**
Year*nitrogen	4	0.224	0.001	0.002	0.079
Variety * Nitogen	8	3.641**	0.025**	0.051**	1.785**
Year*variety* Nitrogen	8	0.178	0.001	0.0001	0.033
Error	56	0.343	0.003	0.004	0.363
Coefficient of variance (%)	-	2.37	7.53	5.33	2.24

Table 3. Mean comparisons of different traits of bean cultivars.

Treatments	Leaf protein (%)	Total chlorophyll in podding stage (mg/ gr wet leaf)	Total chlorophyll in flowering stage (mg/gr wet leaf)	Seed protein (%)
Variety				
Bahman variety	24.34 b	0.6526 b	1.112 b	26.52 b
Derakhshan variety	26.45 a	0.8057 a	1.454 a	26.54 b
Sayyad variety	23.43 c	0.6462 b	1.008 c	27.44 a

Nitrogen				
Control	24.35 bc	0.6604 c	1.100 d	26.91 ab
100% chemical nitrogen	25.25 a	0.7127 ab	1.238 b	26.93 ab
Strain no 133	24.69 b	0.7463 a	1.146 c	26.72 bc
Strain no 116	25.36 a	0.7076 b	1.296 a	27.20 a
133*116 strains	24.04 c	0.6803 bc	1.178 c	26.40 c

Table 4. The interaction effects of bean cultivars and fertilizing systems on different traits of bean.

	Treatments	leaf protein (%)	Total chlorophyll in podding stage (mg/gr wet leaf)	Total chlorophyll in flowering stage (mg/gr wet leaf)	Seed protein (%)
Bahman variety	Control	24.35 ef	0.6368 fg	0.9910 f	26.07 e
	100% chemical nitrogen	25.11 d	0.6300 fg	1.126 e	26.70 de
	Strain no 133	24.14 fg	0.6512 efg	1.059 ef	26.24 e
	Strain no 116	24.89 de	0.7378 cd	1.314 d	27.58 abc
	133*116 strains	23.22 hi	0.6070 g	1.072 ef	26.00 e
Derakhshan variety	Control	25.91 c	0.8228 ab	1.306 d	26.79 de
	100% chemical nitrogen	26.71 b	0.8192 b	1.560 a	27.08 cd
	Strain no 133	25.32 cd	0.8860 a	1.530 ab	26.67 de
	Strain no 116	27.67 a	0.7163 de	1.462 bc	25.99 e
	133*116 strains	26.62 b	0.7842 bc	1.414 c	26.15 e
Sayyad variety	Control	22.80 ij	0.5215 h	1.003 f	27.87 ab
	100% chemical nitrogen	23.92 fgh	0.6890 def	1.028 f	27.02 cd
	Strain no 133	24.61 def	0.7018 def	0.8483 g	27.25 bcd
	Strain no 116	23.51 gh	0.6687 defg	1.111 e	28.02 a
	133*116 strains	22.28 j	0.6498 efg	1.048 ef	27.05 cd
ns ،*and **: non-significant, significant diferences at 1% and at 5% probability levels					

Table 5. Mean comparisons of different traits of bean cultivars in two years of the experiment.

Years	leaf protein (%)	Total chlorophyll in podding stage (mg/gr wet leaf	Total chlorophyll in flowering stage (mg/gr wet leaf	Seed protein (%)
2010	2.053 b	0.398 a	0.807 a	26.774 b
2011	2.075 a	.0386 b	0.791 b	26.889 a

Discussion

Rhizobium bacteria synthesize phytohormones like Auxin as secondary metabolites in inoculated plants. These results corresponds to the results reported by Gamini Senevirante and Ekanayake, (2000) working with different strains of *Bradyrhizobium japonicum* and soybean.

Giller (2001) indicated that Rhizobium bacteria increase nitrogen absorption from soil and stimulate plant biomass production. Moreover, growth

promoting substances (phytohormones) are produced by these organisms.

Nitrogen is the most important element in protein synthesis and its increase in optimum conditions increases the amount of protein. Shehata and Khawas (2003) showed that application of biological fertilizer on sunflower increased seed protein. Significant difference in nitrogen fixation ability among different Rhizobium bacteria species and its effect in shoot protein content in soybean were reported by Vederia *et al*. (2001). Hafeez *et al*. (2000) also reported significant differences in shoot protein content among lentil cultivars when inoculated by different *R. leguminosarum* bv. *Viciae* strains.

In this study, Derakhshan cultivar produced the highest chlorophyll content in both stages, among all the evaluated bean cultivars. Similar to our study, it has been reported that Rhizobial inoculation may influence the physiological growth conditions of leguminous plants (Lanier *et al.,* 2005) by increasing leaf photosynthesis (Zhou *et al.,* 2006) and chlorophyll contents in the leaves (Tajini *et al.,* 2008). These results support the findings of present experiment. Higher chlorophyll content in plants in response to Rhizobium inoculation have been confirmed by other researchers (Matos and Schroder 1989; Yaman and Cinsoy, 1996). The better vegetative growth in plants inoculated by rhizobium strains because of better access to nitrogen sources could stimulate other growth characteristics like total chlorophyll content in leaves. A similar result was reported by Zarei *et al.* (2011). Nitrogen supply affects a wide range of physiological processes in higher plants.

Conclusions

Seed inoculation by Rhizobium strains performed superiority over control (no fertilizer treatment) and chemical nitrogen fertilizer treatment in regard to percent protein and chlorophyll content in seed. The results of this experiment suggest that under the environmental conditions of this experiment, application of rhizobium strains have the capability to replace the chemical nitrogen fertilizer in bean production.

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