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Integrated fertilization systems effects on yield, nodulation state and fatty acids composition of soybean (*Glycine max*)

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ABSTRACT

Combined application of organic and inorganic fertilizers can play an important role for increasing yield and quality of soybean (*Glycine max* L.). In order to study of effects of biofertilizers and nitrogen rates on yield, nodulation state and fatty acids composition of soybean, a factorial experiment was conducted based on randomized complete block design with three replications in 2012 and 2013. Factors were different rates of nitrogen fertilizer in four levels (without nitrogen and application 25, 50 and 75 kg urea/ ha) and seed inoculation with as biofertilizers in five levels (without inoculation, seed inoculation with *Pseudomonas putida* strain 41, *P. putida* strain 186, *Azotobacter chroococcum* strain 5 and *Bradyrhizobium japonicum*). The results showed that maximum of grain yield, plant height, number of filled pods and number of grains per plant were obtained from the highest level of nitrogen fertilizer (75 kg urea/ha) and *Rh*. inoculation. Furthermore, the highest rate of nitrogen usage (75 kg urea/ha) adversely inhibited nodulation of soybean. Number and dry weight of nodules/plant increased significantly with increasing nitrogen application rates up to 50 kg urea/ha. Seed inoculation with biofertilizers increased oil and protein contents. The maximum oil content was obtained by applying 50 kg urea/ha and seed inoculation with *Bradyrhizobium*. The saturated fatty acids (palmitic and stearic acids) declined in seed inoculation with *Bradyrhizobium* than the control, while it was vice versa in unsaturated fatty acids (linoleic, linoneic and oleic acids). Based on the results, it was concluded that application of suitable amounts of nitrogen fertilizer (i.e. between 50 and 75 kg urea/ha) as starter in seed inoculation with *Bradyrhizobium japonicum* can be recommended for profitable soybean production in the study area.

Key words: Linoleic acid, Linolenic acid, PGPR, Soybean, Yield

Legume crops are not only used as human diet but also used for improving soil fertility through biological nitrogen fixation. Among the legumes, soybean (*Glycine max* L.) is a very important recognized oil seed and protein crop in the world. As a legume, soybean can obtain a significant portion of its N requirement through symbiotic N_2 fixation when grown in association with effective and compatible *Rhizobium* strains (Walley 2005). On the other hand, *Bradyrhizobium japonicum* improves the growth and yield of this legume (Egamberdiyeva *et al*. 2004). According to Unkovich and Pate (2000), the amounts of N_2 fixed (kg/ha) by soybean have been up to 450 kg N/ha.

Although nitrogen is the key element in increasing of productivity, but large rates of fertilizer N loss to the environment could cause a serious environmental problem such as groundwater contamination (Caliskan *et al*. 2008). Chemical fertilizers combined with organic manures result in reduction of soil nitrate contents (Yang *et al*. 2005), increase of soil organic matter, improvement of soil properties, and increase of crop yield. Increasing and extending the role of biofertilizers such as *Rhizobium* can reduce the need for chemical fertilizers and decrease adverse environmental effects (Namvar *et al*. 2011).

Nitrogen fertilizer application and seed inoculation with biofertilizer can increase yield of legumes (Sogut 2006).

Soybean oil composition determines the oil quality. Luís *et al*. (2013) reported that inoculation with *Bradyrhizobium japonicum* enhances fatty acids content of soybean seeds. Dashti1 *et al*. (1997) suggested that application of plant growth-promoting rhizobacteria to soybean increases protein and yield. The present study was undertaken to know the effects of nitrogen rates and seed inoculation with biofertilizers on quality and quantity yield, nodulation state and fatty acids composition of soybean.

MATERIALS AND METHODS

Field experiment was conducted during 2012 and 2013 cropping season as factorial experiment based on randomized complete block design with three replications. Factors were different rates of nitrogen fertilizer in four levels (without nitrogen and application 25, 50 and 75 kg urea/ha) as N_0,

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N_1, N_2 and N_3, respectively and seed inoculation with biofertilizers in five levels (without inoculation, seed inoculation with *Pseudomonas putida* strain 41, *P. putida* strain 186, *Azotobacter chroococcum* strain 5 and *Bradyrhizobium japonicum*).

The area is located at latitude 36º 85/ N and longitude 54º 27/ E at an altitude of 13 m above the mean sea level. Climatically, the area is situated in the wet zone with moderate winter and hot summer. The soil was silty loam, with pH about 7.9 and EC about 2.3 ds/m.

In each level, nitrogen fertilizer was divided into two equal parts; the first part of the N was broadcasted by hand and at 6–8 leaf, second parts used before flowering stage. In each plot there were 5 rows 4 m long. Plots and blocks were separated by 1 m unplanted distances. Seed placement was done by hand in individual hills at inter-row and intra-row spacing of 60 × 4.8 cm. Soybean seed (var. DPX) was planted in 10 July 2012 and 21 July 2013. Seeds were inoculated with *Rhizobium japanicum* and plant growth promoting rhizobacteria at the rate of approximately $1{\times}10^8$ colony forming units (CFU)/ml just before planting that was obtained from the Soil and Fertilizer Research Institute, Tehran, Iran.

For inoculation, seeds were coated with gum Arabic as an adhesive and rolled into the suspension of bacteria until uniformly coated. Two seeds were sown per hill and two week after emergence and at 4–5 leaf stage thinned to one plant per hill. The field was immediately irrigated after planting. In each experimental plot, two beside rows and 0.5 m from beginning and ending of planting lines were removed as margin and measurements were done on three rows in the middle lines.

To study the nodules of root, five pots were sown in each plot. Each pot consisted of three plants. The pots in each plot were removed at harvest, and the soybean plants were uprooted carefully. Roots were washed using slow running water to remove soil particles and organic debris. After washing, the number of nodules per root system was counted and their weight was recorded after drying in an oven at 60ºC (Namvar *et al*. 2011). The plants were harvested at maturity and yield components such as plant height, filled and unfilled pods/plant and number of grains/plant was recorded on 8 randomly selected plants in each plot.

Determination oil and protein contents: Seeds oil was extracted based on Folch *et al*. (1957) protocol by using of rotary evaporators. Extracted fatty acids were transformed to their methyl esters (FAME) using the Metcalf *et al*. (1966) method, and were determined using a gas chromatography (Unicam 4600) equipped with a FID detector. Nitrogen concentration of seeds was determined by Kjeldaul analysis. The protein amount was calculated by multiplying the nitrogen concentration by 6.25.

Grain yield: Three central rows each 1 m long were harvested in each plot. The total grain weight for sampled material was recorded and converted into grain yield (g/m^2). Analysis of variance technique was used to test the significance and LSD at 5% probability level was used to compare the treatment's means. The main and interaction effects of the treatments were determined by analysis of variance by statistical software *SAS*.

RESULTS AND DISCUSSION

Precipitation and temperature were generally similar in both growing seasons. The effects of nitrogen rates and biofertilizer application were significant for number of grains/plant, number of nodules and weight of nodules/plant, grain yield and yield attributes, oil and protein content and fatty acids compound of soybean.

Number of grains per plant, number of nodules and weight of nodules per plant

Number of grains/plant, number and weight of nodules/plant showed significant response to nitrogen rates and biofertilizer inoculation. The highest number of grains/plant (17.39), number (18.24 nodules/plant) and weight (24.03/plant) of nodules/plant recorded in inoculation by rhizobium and application of 50 kg urea/ha that was statistically on a par with 75 kg urea/ha application. The lowest values of these traits was observed in control (Table 2).

The highest rate of nitrogen application (75 kg urea/ha) reduced the number and weight of nodules/plant by 57 and 66%, respectively, in comparison with application of 50 kg urea/ha, and 33 and 44% compared to no application of nitrogen, respectively (Table 1). A negative exponential relationship was observed between N fertilizer rate and N_2 fixation when N was applied in the top 0–20 cm of soil or on the soil surface (Salvagiotti *et al*. 2008). Biological N fixation begins around 2-5 weeks after planting and therefore, N uptake from biological N fixation is negligible in early growth stages. Thus, application of small amount of N at planting called as "starter N" is beneficial to improve early growth and yield of legumes in most cases (Caliskan *et al*. 2008).

Moreover, inoculated plants showed more number and weight of nodules/plant than non-inoculated plants. *Rhizobium* inoculation increased the number and weight of nodules/plant by 46 and 51.57%, respectively, compared to non-inoculated plants or control (Table 1). Plants inoculated with rhizobium increased about 46%, 24.1%, 17.2%, and 10.34% higher number of nodules per plant and 51.7%, 27%, 14.43% and 10.23% higher weight of nodules/plant compared to no inoculated, inoculated by *Pseudomonas* 41, *Pseudomonas* 186 and *Azotobacter*, respectively (Table 1).

Application of rhizobium showed the highest number of grains/plant (17.3% increase over control) and in *Pseudomonas* 41, *Pesedomonas* 186 and *Azotobcter* (14.22%, 8.55%, 4.2% increase over control) plants, respectively. Application of 75 kg urea/ha in inoculated plants by rhizobium increased the number of grains/plant by 45.9% compared to control. Inoculation with *Rhizobium* had the greatest effect on number of grains/plant in 50 and 75 kg urea/ha rather than other fertilizer levels that may be due to more effectiveness of *Rh*. inoculation in these levels compared to other levels of nitrogen usage.

Table 1 Effects of biofertilizer and nitrogen rates on grain filling rate, oil and protein contents of soybean (mean of two years or combined analysis of the two years (2012-2013)

Treatment	PH (cm)	NGP	GY (g/m²)	100-GW (g)	NUFP	NFP	NN/plant	WN/plant
Nitrogen rates (kg urea/ha)								
N_0=0	143d	76.9dd	414.6d	21.6d	8.7a	51.8d	6.3b	8.4b
N_1=25	150.9c	97c	500.2c	22.2c	4.5b	61.1c	10.9a	14.2a
N_2=50	155.3b	115.4b	563.6b	22.6b	2.6c	70b	9.7a	13.7a
N_3=75	160.9a	134.7a	691a	23.8a	.99d	79.6a	4.2c	4.7c
LSD (p<0.05)	2.52	8.02	37.7	0.302	1.1	6.2	1.2	1.8
Biofertilizers								
S_0= no inoculation as control	146.6e	91.8e	342.7d	19.7d	5.41a	58e	7.8d	9.2d
S_1=*Pesedomonad putida* 41	149.1d	95.3d	498.2c	22b	4.73b	60.4d	11.0c	13.9c
S_2= *Pesedomonad putida* 186	150.4c	101.6c	520.6bc	21.3c	4.03c	63.3c	12.0b	16.3b
S_3=*Azotobacter chrochoccoum*	151.7b	106.4b	540.2b	21.9b	3.34d	65.6b	13.03b	17.1b
S_4=*Bradyrhizobium japanicum*	152.6a	111.1a	688.7a	22.9a	2.85e	68.2a	14.5a	19.05a
LSD (p<0.05)	0.6	2.03	31.7	0.31	0.262	1.03	0.988	1.4
Nitrogen (N)	**	**	*	**	*	**	**	*
Biofertilizer(B)	*	**	*	*	**	*	*	*
N*B	ns	**	ns	ns	ns	ns	*	**
CV (%)	11	9.48	12.5	8.7	13.2	14.1	9.46	8.32
		PC (%)	*OC (%)*	*PA (%)*	*SA (%)*	*OA (%)*	*LILA (%)*	*LINLA (%)*
Nitrogen rates (kg/ha urea)								
N_0=0		37.2 c	21c	12.06a	4.53a	21d	50c	6.66b
N_1=25		40b	23.4b	11.73a	3.66a	22.2c	51.26b	7.53a
N_2=50		42.5a	24.3a	11.13ab	4.93a	23.1b	52.86a	8.2a
N_3=75		43.6a	24a	10.46b	4.53a	24a	53.32a	8.17 a
LSD (P=0.05)		1.13	0.598	0.942	2.2	0.512	1.2	0.845
Biofertilizers								
B0 = no inoculation		40d	21d	12a	4.58a	22.5d	50.5b	6.91b
B1=*Pesedomonad* 41		41.2c	22.58c	11.66ab	4.41 b	23.6c	52.41ab	7.58ab
B2=*Pesedomonad* 186		42.3b	23.2bc	11.41ab	4.28c	23.8c	52ab	7.75ab
B3=*Azotobacter*		44a	23.64ab	10.75b	4.16 d	24.2b	52.91ab	7.91a
B4=*Bradyrhizobium*		44.3a	24a	10.91b	4 e	24.8a	53.75a	8.08a
LSD (P=0.05)		0.688	0.44	1.053	0.927	1.87	2.43	0.945
Nitrogen (N)		*	*	**	*	**	**	**
Biofertilizer (B)		**	*	ns	ns	ns	ns	**
N × B		**	**	ns	ns	ns	**	ns

ns, * and ** show no significant and significant differences at 0.05, 0.01 probability level, respectively; pH: Plant height; NGP: Number of grains/Plant; GY: Grain yield; 100-GW: 100 Grains weight; NUFP: Number of unfilled pods; NFP: Number of filled pods; NN: Number of nodules; WN: Weight of nodules; OC: oil content, PC: protein content; PA: palmitic acid; SA: stearic acid; OA: oleic acid; LILA: linoleic acid, LINLA: linolenic acid

Plant growth and some yield attributes

Application of high N rates (75 kg urea/ha) increased number of filled pods/plant, grain yield, plant height and 100-grains weight in comparison with control. Application of high N rates (75 kg urea/ha) resulted 9.24% and 11.1% increase in 100-grains weight and plant height compared with control, respectively. Plants inoculated with bio fertilizer showed higher 100-grains weight and plant height compared to control plants. Application of 75 kg urea/ha increased about 11.1%, 6.2%, and 4% higher plant height compared to application of 0, 25, 50 kg urea/ha, respectively. Plants inoculated with rhizobium increased about 13.97%, 3.9%, 6.9% and 4.3% higher 100-grains weight compared to application of no inoculated, inoculated by *Pseudomonas* strain 41, *Pseudomonas* strain 186 and *Azotobacter,* respectively (Table 1). These results are in line with the findings of Achakzai and Bangulzai (2006); Amany (2007) and Caliskan *et al*. (2008) who reported that 100-grains weight and plant height was increased with application of nitrogen fertilizer.

The number of unfilled pods/plant was 8.7 in control and decreased to 0.99 in application of 75 kg urea/ha. It was vise versa in the number of filled pods/plant. Increasing the number of unfilled pods/plant may be due to the less assimilation in plant for filling of whole pods in high levels of nitrogen application. *Biofertilizer* inoculation increased

Table 2 Mean comparison effects of biofertilizer and nitrogen rates on some studied traits in soybean (mean of two years or combined analysis of the two years (2012-2013)

Treatment compound	NGP	NN per plant	WN per plant	OC(%)	PC(%)	LINLA(%)
N_0S_0	9.4k	3.37h	3.8j	18.111	33.7J	46 c
N_0S_1	12.2hi	5.04fg	6.7hi	19.18j	34.3i	47.6cd
N_0S_2	12i	5.04fg	5.7hij	18.88k	35.7h	53.3 ab
N_0S_3	11. 3 j	4.22gh	4.75ji	19.45i	39.0def	52.3 abc
N_0S_4	14.5de	6.31ef	8.42fg	19.85gh	39def	53.6ab
N_1S_0	12i	5.04fg	6.73ghi	19.46i	37.9g	49.6 bcd
N_1S_1	13.5g	7.8de	9.24ef	20.19ef	39.49cde	53 ab
N_1S_2	13.8fg	7.83d	10.98de	19.77h	39.7cd	53 ab
N_1S_3	12.8h	8.72d	11.4d	20.34de	39.4cde	51.33 abc
N_1S_4	16b	10.9c	14.25c	21.11ab	40.1bc	53 ab
N_2S_0	14.67 d	6.2f	7.11gh	20.31def	38.4fg	52 abc
N_2S_1	15cd	13.b61b	16.93b	20.91bc	39.7cd	53.11 ab
N_2S_2	13.9efg1	13.6bb	17.68b	20.83c	39.5cde	53 ab
N_2S_3	15.95b	13.71b	17.11 b	20.05fg	39.2de	55a
N_2S_4	17.39a	18.24a	24.03 a	21.29a	40.5b	54.6a
N_3S_0	14.38def	5.97f	6.85 gh	19.85gh	38.7efg	52.6 ab
N_3S_1	14.7d	13.12b	16.32b	20.55d	40.2bc	51.3 abc
N_3S_2	13.64g	13.17b	17.04b	20.52d	40.2bc	53.3 ab
N_3S_3	15.64bc	13.22b	16.5b	20.48d	38.9def	53 ab
N_3S_4	17.05a	17.58a	23.16a	21.29a	41.7a	53.66 ab
LSD ($P<0.05$)	0.657	1.51	2.01	0.265	.849	4.7
Significance	**	*	**	**	**	**

Means with similar letters in each column are not significantly different, NGP: Number of grains/plant; NN: Number of nodules; WN: weight of nodules; OC: oil content, PC: protein content; LINLA: linolenic acid.

significantly the number of filled pods and decreased unfilled pods compared to control in each plant (Table 1).

The highest grain yield (670 g/m^2) was obtained in application of 75 kg urea/ha and 697.5 g/m^2 in seed inoculation by rhizobium which was statistically significant to other treatments. Comparing with the uninoculated treatments, the treatment of biofertilizer (rhizobium, *Azospirillum strain 41*, *Azospirillum strain 186* and *Azotobacter*) increased nearly by 35.7%, 24%, 21.2% and 27% grain yield, respectively. Similar findings were also reported by Malik *et al*. (2006).

Quality parameters

Maximum of protein content (41.7 %) was recorded at 75 kg urea/ha application when seed inoculed with *Rhizobium*, protein content was 19% higher than control treatment and minimum (33.71%) was recorded under control. It seems that applying integrated fertilizers provides more available nitrogen for the plant, possibly by preventing the dissipation of nitrogen due to the presence of bio fertilizer, and, therefore, the protein content of the integrated fertilizer levels, which consisted of biofertilizer and chemical fertilizer, was higher compared to the other levels. Luís *et al*. (2013) reported that inoculation with *Bradyrhizobium japonicum* enhances protein content of soybean seeds. Soybean takes part in a nitrogen-fixing symbiosis with several species of *Bradyrhizobium* genus, including *Bradyrhizobium japonicum*, which improves the growth, yield, nitrogen and protein content of this legume (Malik *et al.* 2006). Rudresh *et al*. (2005) and Sogut (2006) reported inoculation of seeds with *Rhizobium* increases nodulation, nitrogen uptake and could be possible reason for increase of protein content and yield parameters of legume crops. In fact, the protein content of soybean seeds increases as the access to nitrogen increases. Basu *et al*. (2008) have also reported that the highest protein content for integrated treatments was found with chemical and biofertilizer.

Oil content in seeds was progressively increased with increasing levels of N up to 50 kg urea/ha; however, no significant difference was between 50 and 75 kg urea/ha application (Table 1). Application of 75 kg urea/ha and seed inoculation by rhizobium showed the highest oil content (14.5% increase over control) and in *Pseudomonas* 41, *Pesedomonas* 186 and *Azotobcter* (about 11.5% increase over control) plants, respectively. The lowest oil content was recorded in control (Table 2). It was vice versa of protein content which increased with increasing levels of N. Roche *et al.* (2006) reported that in oil crops, an increase in oil concentration is generally associated with a decrease in protein concentration as a result of a dilution effect. The synthesis of grain starch and oil mostly relies on current photosynthesis. Luís *et al*. (2013) reported that inoculation with *Bradyrhizobium japonicum* enhances the organic and fatty acids content of soybean seeds. Dashti1 *et al*. (1997) suggested that application of plant growth-promoting rhizobacteria to soybean increases protein and dry matter yield.

Soybean oil composition determines the oil quality. Soybean oil is composed of saturated and unsaturated fatty acids. Linoleic acid (C18:2) was the most abundant fatty acid, ranging between 50% and 53.32%, followed by oleic acid (C18:1) and linolenic acid (C18:3), with contents of (21-24% and 6.66-8.17%) in various levels of nitrogen fertilizer. The amount of palmitic acid (C16:0) and stearic acid (C18:1) were 10.46-12.06% and 3.66-4.93%, respectively (Table 1). These ranges were similar to those reported by Yin *et al*. (2005) in soybean. Application of 75 kg urea/ha increased about 6.22%, 3.86% and 0.8% higher linoleic acid content compared to application of 0 and 25 and 50 kg urea/ha, respectively (Table 2). But no significant difference was between 50 and 75 kg urea/ha application (Table 1).

Subedi and Ma (2009) found that lack of assimilate supply could result in a dramatic decline in grain weight and its composition such as starch and oil. Inoculation with rhizobium induced a 6% increase of linoleic acid content compared to control (Table 2). The saturated fatty acids (palmetic and stearic acids) declined in seed inoculation with *Bradyrhizobium* than the control, while it was vice versa in unsaturated fatty acids (linoleic, linoneic and oleic acids). Inoculation with rhizobium decreased content of palmitic acid (about 9.1% low control) and stearic acid (about 11.4% low control) compared to control (Table 1). Luís *et al*. (2013) reported that inoculation with *Bradyrhizobium japonicum* enhances unsaturated fatty acids content of soybean seeds. Similar results were obtained in seed inoculation by *Pseudomonas* 41, *Pesedomonas* 186 and *Azotobcter*. Dashti1 *et al*. (1997) suggested that application of plant growth-promoting rhizobacteria to soybean increases oil content.

It was concluded that application of suitable amounts of nitrogen fertilizer (i.e. between 50 and 75 kg/ha urea) as starter in seed inoculation with *Bradyrhizobium japonicum* can be recommended for profitable soybean production in the study area.

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