

Inhibition of Nitrogenase Enzyme and Completely Suppression of Nodulation in Common Bean (*Phaseolus vulgaris* L.) at High Levels of Available Nitrogen

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Abstract: Among collected number of *Rhizobium leguminosarum* biovar *phaseoli* isolates from Egyptian soils, strains EBRI 2 and 26 nodulating common bean (*Phaseolus vulgaris* L.) effectively were examined for their ability to nodulate and fix nitrogen with common bean (*Phaseolus vulgaris* L.) at high levels of potassium nitrate or ammonium sulfate. The formation of nodules was markedly decreased with the increase of N dose with the examined strains (44% reduction rate with strain EBRI 2 and 68% reduction rate with EBRI 26) at 80 ppm N of potassium nitrate. The effect of nitrogen fertilizer was fluctuating on the nitrogenase enzyme, while the low dose of N (20 ppm) was promoting the N₂-fixation, the high dose of nitrogen (80 ppm) inhibited nitrogenase enzyme and suppressed nodulation. The same trend of results was observed at high levels of ammonium sulfate while the nodulation was completely diminished at 180 ppm. The nodulation was eliminated totally at 160 ppm N as potassium nitrate or 180 ppm N as ammonium sulfate with the two examined strains although the effective *Rhizobium* strains are present in the rhizosphere of plant, giving indication that high nitrogen availability is an important element controlling the success or failure of *Rhizobium* inoculation in the field.

Key words: Egyptian bean rhizobial isolates (EBRI) • High available of nitrogen • Nitrogenase enzyme

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the most important legume crops due to its high nutritional value as it can be used as green pods or dry seeds. Common bean should form symbiosis life as the other legumes with its symbiotic partner (*Rhizobium* strain) however, this kind of symbiosis relationship have many obstacles that responsible for the failure of nodulation. The failure of nodulation in common bean has been reported by many authors and this due to many factors such as salinity of soils [1-4], soil acidity [5,6] incompatibility between rhizobial strains and host plant [7-9], high temperature in the rhizosphere [10], susceptibility to bacteriophage [11,12], loosing of symbiotic plasmids [13], defect in nod genes based on genetic rearrangements between plasmid and genomic

DNA [14], low phosphorus [15], competition with native rhizobial strains [16,17] and high available nitrogen [18-20]. Consequently, we did a lot of efforts to establish a long term strategy to improve the nitrogen fixation with common bean through selection of effective nodulating common bean rhizobial strains and studying their genetic stability and diversity [9], selection of most competitive strains using the B- glucuronidase (*gus*) marker [17], selection of the strains more adapted to environmental stresses such as salinity and alkalinity [1], improve dual-strain inoculum of *Rhizobium* with some of diazotrophs that increased the common bean nodulation [20] and finally in this study we need to estimate the available nitrogen level which stimulate and increase the nodulation and nitrogen fixation with common bean, in order to grantee the success of inoculation under field conditions.

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MATERIALS AND METHODS

Rhizobium Strains and Growth Conditions: Rhizobial strains were isolated from the root nodules of common bean (*Phaseolus vulgaris* L.) after surface sterilization as described by Somasegaran and Hoben [22]. Strains EBRI 2 and 26 were identified as *R. etli* bv. *phaseoli* as done by Shamseldin and Werner [23], based on the full sequences of 16S rDNA that deposited in the Genebank under accession numbers (EY 221174 and AY 221177) and the hybridization with *nifH* gene [23]. Rhizobial strains were grown in liquid or solid 20E medium [24] and were kept in 50% glycerol at -70°C for further studies.

Growth of Common Bean (*Phaseolus vulgaris* L.) under High Levels of Available Nitrogen and Nodulation Parameters: Seeds of the Egyptian common bean cultivar Giza 6 that obtained from the germplasm collection of the Agriculture Research Center, El-Giza, Egypt were checked by nicked eye to select seeds with the same size and free from infection of disease. Then were surface sterilized by soaking them in 70% ethanol for three minutes followed by four minutes in 3% sodium hypochlorite and rinsed several times with sterile water and left to imbibe for one hour in sterile water and then were germinated on 1% agar plates at 30°C. After germination, seedlings of five days old were transplanted in sterilized Leonard jars with vermiculite: perlite (1:1 v:v) as substrate and N-free solution [24] under aseptic conditions. Different concentrations of N as potassium nitrate or ammonium sulfate were sterilized by filtration and added to the nutrient solution after autoclaving. Then seedlings were inoculated by adding 1 ml of rhizobial cultures with about 10^7 cells ml^{-1} for each Leonard jar and jars were covered with aluminum foil paper for two days. Conditions of controlled growth chamber were mentioned in details by Shamseldin and Werner [17]. Plants were harvested after 25 days from inoculation at the beginning of flowering stage. Acetylene reduction assays (ARA) were performed as described by Bender and Rolfe [25]. Nodulation parameters as nodule number, nodule fresh weight and acetylene reduction records were statistically analyzed using the least significant difference test according to Sendecor and Cochran [26].

RESULTS AND DISCUSSION

Under the intensification of agriculture policies especially in developing countries such as Egypt, to meet the increased demand of food, farmers use excessive

amounts of nitrogen fertilizers in their farming systems to increase the production of crops and this reflect notoriously on nodulation and nitrogen fixation. In this study, we are trying to know the level of available nitrogen which stimulates the nodulation and nitrogen fixation with common bean as important legume crop for human nutrition in the poor countries. Therefore, strains EBRI 2 and 26 that nodulated common bean and identified as *R. etli* bv. *phaseoli* [23] were used to select the best strain for giving high nitrogen fixation rate at high nitrogen level in the soil. Obtained results show that nodulation was markedly reduced with the raising of nitrogen fertilizer dose. This negative effect was observed with both examined strains (Table 1). Nodule records were 93 and 120 nodules plant^{-1} with strains EBRI 2 and EBRI 26 without nitrogen application, while these numbers decreased significantly to 52 and 38 nodules plant^{-1} at 80 ppm potassium nitrate. These results are in the same line with those noticed by Danso *et al.* [27] who reported the reduction in nodulation and inhibition of N_2 fixation at 80 ppm N with soybean. The same results were obtained for nodule biomass with 512 mg without nitrogen addition compared to 173 mg plant^{-1} at 80 ppm N for strain EBRI 2 and 488 mg nodule biomass reduced to 125 mg for strain EBRI 26. At the application of 160 ppm N, there are no nodules observed at all indicating that the nodulation was suppressed completely. Both rhizobial inoculation and nitrogen fertilization supported higher shoot biomass production compared with the un-inoculated and unfertilized plants. The application of nitrogen fertilizer dose of 160 ppm N was the most efficient in this respect. The effectiveness values gave indication that the inoculation with EBRI 2 plus 20 or 40 ppm N gave the best values (68.1%) compared with the full N dose (100%) with a good values also for nodule numbers, nodule and shoot weights and acetylene reduction assay. Generally, parameters of acetylene reduction decreased gradually depending on the dose of nitrogen fertilized used. Strikingly, the low dose of nitrogen (20 ppm) promoted the nitrogen fixation ($20.4 \text{ nmol C}_2\text{H}_4 \text{ h}^{-1} \text{ mg nodules}$) compared with the inoculation without nitrogen fertilization ($17.9 \text{ nmol C}_2\text{H}_4 \text{ h}^{-1} \text{ mg nodules}$) by both strains and these results are in agreement with those obtained by Eaglesham [28].

Results in Table 2 show the reduction in nodule numbers under high available nitrogen as ammonium sulfate, the reduction rate was about (83% reduction) at 160 ppm N with the two examined strains. The same trend of results was observed while the nodulation was completely diminished at 180 ppm N. The higher inhibitory

Table 1: Nodulation and nitrogen fixation parameters of cultivar Giza 6 under application of potassium nitrate, 25 days after inoculation

Treatments	No. of nodules plant ⁻¹	FW of nodules mg plant ⁻¹	FW of shoots g plant ⁻¹	Effectiveness %	nM C ₂ H ₄ h ⁻¹ mg ⁻¹ nodules
Control	0.0e	0.0d	5.1cb	-	0.0c
Full dose N (160 ppm)	0.0e	0.0d	14.4a	100	0.0c
Inoculated by EBRI2	93b	512a	8.3b	57.96	17.9a
Inoculated by EBRI 2+20 ppm N	69cb	375b	9.8b	68.2	20.4a
Inoculated by EBRI 2+40 ppm N	71cb	355b	9.8b	68.1	12.9ba
Inoculated by EBRI 2+80 ppm N	52d	173c	14.9a	103.4	10.7b
Inoculated by EBRI 2+160 ppm N	0.0e	0.0d	15.7a	109	0.0c
Inoculated by EBRI 26	120a	488a	6.8cb	47.2	17.7a
Inoculated by EBRI 26+20 ppm N	77b	286b	7.5b	52.1	21.0a
Inoculated by EBRI 26+40 ppm N	82b	254cb	8.6b	59.7	12.2b
Inoculated by EBRI 26+80 ppm N	38d	125c	10.0b	69.4	8.6b
Inoculated by EBRI 26+160 ppm N	0.0e	0.0d	16.9a	117.4	0.0c
L.S.D 0.05	16.4	96.4	2.75	-	5.29

FW: fresh weight, % Effectiveness = fresh weight of inoculated plants or inoculated plus fertilized plants ÷ fresh weight of full fertilized plants.

Table 2: Nodulation and nitrogen fixation parameters of cultivar Giza 6 with the application of nitrogen fertilizer ammonium sulfate, 25 days after inoculation

Treatments	No. of nodules plant ⁻¹	FW of nodules mg plant ⁻¹	FW of shoots g plant ⁻¹	Effectiveness %	nM C ₂ H ₄ h ⁻¹ mg ⁻¹ nodules
Control	0.0f	0.0e	4.9dc	-	0.0d
Full dose N (160 ppm)	0.0f	0.0e	13.2b	100	0.0d
Inoculated by EBRI 2	88b	495a	9c	68.2	18.2a
Inoculated by EBRI 2+20 ppm N	71b	420b	9.3cb	70	21.5a
Inoculated by EBRI 2+40 ppm N	62b	400b	8.9c	67	15.3ba
Inoculated by EBRI 2+80 ppm N	44d	295cb	11.9b	90	11.5b
Inoculated by EBRI 2+160 ppm N	15e	125d	14.2ab	107	1.5c
Inoculated by EBRI 2+180 ppm N	0.0f	0.0e	15.2a	115	0.0d
Inoculated by EBRI 26	132a	512a	7.52c	64.5	19.0a
Inoculated by EBRI 26+20 ppm N	90b	460a	8.4c	63	22.3a
Inoculated by EBRI 26+40 ppm N	75b	365b	9.1c	69	13.7ba
Inoculated by EBRI 26+80 ppm N	45d	320cb	10.6b	80	9.2b
Inoculated by EBRI 26+160 ppm N	22d	180d	17.3a	131	1.3c
Inoculated by EBRI 26+180 ppm N	0.0f	0.0e	16.7a	126	0.0d
L.S.D 0.05	12	83.5	2.95	-	4.85

FW: fresh weight, % Effectiveness = fresh weight of inoculated plants or inoculated plus fertilized plants ÷ fresh weight of full fertilized plants

effect of nitrate than ammonium was reported by Straub *et al.* [20] who mentioned that this attributed to a direct competition between nitrate reductase and nitrogenase enzyme for reducing power or this higher negative effect of nitrate is due to the formation of nitrite as intermediate of nitrate reductase and the later inhibits the function of nitrogenase or leghemoglobin [29]. Acetylene reduction assay was decreased with the increase of available nitrogen but the low dose of N 20 ppm increased the nitrogenase enzyme. Inoculation with strains EBRI 2 and EBRI 26 plus 80 ppm N were the best treatments as they gave effectiveness of 103% and 70% of the full fertilized plants under application of potassium nitrate while they gave 90% and 80% effectiveness with ammonium sulfate application.

Ultimately, we conclude that the addition of ammonium sulfate has lesser negative effect on nodulation and nitrogen fixation than potassium nitrate and the application of 20 ppm N or 40 ppm N in addition to rhizobial inoculation has not much negative effects on nodulation and nitrogen fixation with common bean under the addition of potassium nitrate or ammonium sulfate, giving indication that the inoculation can be success at low levels of N application (20 ppm and 40 ppm N) but it can be suppressed totally at 160 ppm or higher levels of N application. Our study also revealed that the completely success of inoculation with *Rhizobium* strains can be done in newly reclaimed soils that characterized by low available nitrogen or in the arable soils that suffer from the deficiency of soluble nitrogen.

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