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Biomedicine and Nursing

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Impact of phosphorus and biofertilizer applications on leghemoglobin content, nitrate reductase, nitrite reductase enzyme activity in nodules of common bean (*Phaseolus Vulgaris* L.) under intercropping with maize(*Zea mays* L.)

M. Hussain Dar*[1], , N.Singh[1], I.Murtaza[2]

1 Department of Botany, Kurukshetra University, Kurukshetra, Haryana-136 119, India.
2 Department of Biochemistry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Corresponding author's e-mail: mhussaunidar@gmail.com, 7298986180

Abstract: Common bean, *Phaseolus vulgaris* L. c.v. Shalimar Rajmash was considered as a poor nitrogen fixing plant compared to other legumes. Even though there are several attempts to produce better varieties for improved N_2 fixation, no work has been done to evaluate the response of this crop to different biofertilizers under various levels of phosphorus. In order to study the effect of biofertilizers (*Rhizobium*, *Azotobacter, Arbuscular mycorrhizae*) under different levels of phosphorus (20 and 40 kg/ha) application on leghemoglobin content and enzyme activity (nitrate, nititre reductase) in nodules of common bean (*Phaseolus vulgaris* L.) in a sustainable production system, an experiment was conducted during *kharif* seasons of 2012 and 2013 at the Krishi Vigyan Kendra (KVK) of Shere-e-Kashmir University of Agricultural Sciences and Technology, Budgam, Jammu and Kashmir. The climate of the experimental site is temperate with mild summers and cold winters, showing wide variations in mean maximum and minimum temperatures. Temperature varies from 5˚C in winter to a maximum of 34˚C. The experiment was laid out in complete randomized block design (RBD). Different levels of DAP and various biofertilizers namely *Rhizobium* (*Rhizobium leguminosarum*), Azotobacter (*Azotobacter vinelandi*), VAM (*Glomus mosseae*) have been used during the research. *Rhizobium* with VAM @ 20 kg P/ha in the present research showed significant impact on all parameters of common bean.

[Hussain Dar, N.Singh, I.Murtaza. **Impact of phosphorus and biofertilizer applications on leghemoglobin content, nitrate reductase, nitrite reductase enzyme activity in nodules of common bean (*Phaseolus Vulgaris* L.) under intercropping with maize(*Zea mays* L.).** *Biomedicine and Nursing* 2022; 8(4):56-62]. ISSN 2379-8211 (print); ISSN 2379-8203 (online). http://www.nbmedicine.org. 07. doi:10.7537/marsbnj080422.07.

Key words: Common bean, phosphorus, biofertilizers, nitrate reductase, nitrite reductase.

INTRODUCTION

Phaseolus vulgaris L. c.v. Shalimar Rajmash (Common bean) has been considered as a poor nitrogen fixing crop compared to other legumes and only recently there have been attempts to select host plants of this species and for breeding better varieties of this crop for increased nitrogen fixation (Fernandes*et al.*1982). Even though there are several attempts to produce better varieties for improved N_2 fixation. The poor productivity of common bean is mainly due to imbalance application of nutrients and use of traditional varieties. Under such situations, use of *Rhizobium* and phosphate mobibilizing mirorganisms had shown advantage in enhancing common bean productivity (Jain *et al.*, 2012). To overcome the ecological problems resulting from the loss of plant nutrients and to increase crop yield, microrganisms that allow more efficient nutrient use or increase nutrient availability can provide sustainable solutions for present and future agricultural practices. It is well known that the biofertilizers contain a variety of beneficial microorganisms which accelerate and improve plant growth and protect plants from pests and diseases (Abou-Aly *et al.*, 2006). These are low-cost and ecofriendly inputs have tremendous potential for supplying nutrients which can reduce the dependence of chemical fertilizers. Also organic management resulted in significantly higher soil enzyme activities (Garcia-Ruiz *et al.*, 2008). But in commercial agriculture, the use of chemical fertilizers cannot be ruled out completely.

Initiation of nodules development as well as efficiency of the symbiosis relationship between rhizobium and legumes is influenced by phosphorus P (Nyoki and Ndakidemi, 2014).In general, phosphorus is added to soil as inorganic phosphates, because the free inorganic P in soil solution plays a central role in P-cycling and plant nutrition (Pix *etal.*, 2001). However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is immobilized rapidly after application due to phosphate fixation by aluminum, calcium, iron, magnesium and

soil colloids (Rodriguez and Fraga, 1999) and becomes unavailable to plants (Singh and Kapoor, 1994). Therefore, P is often a limiting nutrient in agricultural soils. Microrganisms are also involved in a range of process that affect the transformation of soil P and thus an integral part of the soil P cycle (Chen *et al*., 2006). P -mobilisation ability of microrganisms considered to be one of the most important traits associated with plant P nutrition (Chen *et al*., 2006). It has been revealed from the studies on long-term fertilizer experiments that biofertilizers along with chemical fertilizers results in yield improvement and maintenance of soil fertility (Swarup, 1998). The objective of the present study was to find out the impact of different biofertilizers under various levels of phosphorus on enzyme activity in nodules of common bean.

MATERIALS AND METHODS

Experimental Site: The experiment was conducted at the KrishiVigyan Kendra (KVK) of Shere-e-Kashmir University of Agricultural Sciences and Technology, Budgam, Jammu and Kashmir. The climate of the experimental site is temperate with mild summers and cold winters, showing wide variations in mean maximum and minimum temperatures. Temperature varies from -5˚C in winter to a maximum of 34˚C. The soil at the experimental site was clay loam in texture. Nitrogen, phosphorus and potassium contents in soil of the experimental site were done by Modified Kjeldhal method (Jackson, 1973), Olsen's method (Jackson, 1967) and Flame photometer (Jackson,1967).

Treatments details and crop culture: For proper seed bed preparation, field was ploughed thoroughly twice with a tractor. The plot was properly leveled for even and efficient fertilizer and water distribution. The gross plot size was 16.5 square meters (m^2) and the net plot size was 9.6 square meters (m^2). The experiment was laid out in a complete randomized design (CRD)with each treatment replicated three times. The detailed treatments are presented in Table 2. Common bean variety "Shalimar Rajmash-1" and maize variety "C-15" were used for the present study. The seed were procured from KVK, Budgam, Jammu and Kashmir. The maize seeds were sown at row to row distance of 75 cm and plant to plant distance of 20 cm. The common bean seeds were sown in between the maize rows. Sowing was done in the last week of April, 2012 and 2013 and seeds were hand dibbled at a depth of about 2 cm in soil.

Biofertilizers and chemical fertilizers application: The seed were surface sterilized by sodium hypochlorite (0.1%) for 2 min., thoroughly rinsed with distilled water and soaked in distilled water for 6 h. before sowing in plots. Peat based *Rhizobium leguminosarum* inoculum, vesicular arbuscular mycorrhizae (*Glomus mosseae*) and *Azotobacter vinelandi* was procured from the Division of Microbiology, IARI (New Delhi) India. For *Rhizobium* and *Azotobacter* inoculation, the seeds were moistened in sugar solution (48%) before the application of inoculums to get a thin uniform coating of inoculum on the seeds, immediately before sowing the seeds in field. The seed were then shade dried after inoculation.The mycorrhizal inoculum was applied after seed sowing at the rate of 25 Kg/ha by planting holes method.

The fertilizers for maize (120 N, 30 P_2O_5 (kg/ha) and for common bean (30 N, 30 P_2O_5kg/ha) were applied according to plant population in the intercropping system. Phosphorus was applied as per the treatments. Half of the nitrogen and whole potassium were applied at sowing time as basal dose. The remaining nitrogen was top dressed when true leaves emerged after sowing (25 days.

Enzyme assays and leghaemoglobin content in root nodules

Leghaemoglobin content: It was determined in fresh uniform sized root nodules measuring about 0.5 cm in length. Nodules were carefully removed from the roots with sharp edged blade and washed with prechilled double distilled water. After washing, the nodules were blotted on filter paper, weighed and then finally crushed in prechilled sterilized pestle mortar containing 50.0 MmHCl, 5 Mm $MgCl_2$, 20 Mm KCl and 5m Mmercapto-ethanol. The slurry was centrifuged at 40˚C at 8,000 rpm for 15 min. The pellets were discarded and supernatant (SN) was made to known volume i.e. 4 ml/g fresh weight of nodules. In this supernatant, leghaemoglobin content was estimated as per the method of Hartree (1955). The 0.5 and 1 ml aliquots of clear extract were taken in test tubes. To each tube, 1.5 ml of 1 N NaOH was added and kept for half an hour at room temperature. After 30 minutes, 3 ml pyridine solution and 1.5 ml (10 % (W/V) sodium bisulphide added to each tube. Then distilled water was added to make the volume to 15 ml. The tubes were incubated for 30 minutes and the optical density recorded at 535 nm and 556 nm. Calibration curve was prepared by using a standard solution of haemin (100 µg/ml) by dissolving in 1N NaOH.

Nitrate reductase activity (NRA): Nitrate reductase activity was assayed by the method of Jaworski (1971) with the slight modifications suggested by Muthuchelian *et al.* (1993). Fresh nodules (0.5g) were incubated in vials containing 5 ml of incubation medium prepared by mixing 0.1 N KNO_3 (1 ml), 0.1 M phosphate buffer of pH 7.5 (3.75 ml), 0.1per cent of Triton X-100 (0.01 ml) and 1per cent propanol (0.25 ml). Incubation was carried out in dark for 1 h at room temperature (28±2˚C) with occasional shakings. Aliquots of 0.2 ml from the incubation mixture were analysed for nitrite after 60 min. To 0.2 ml of incubation medium, 1.8 ml of distilled water, 1 ml of 3

% sulphanilamide in 3N HCl and 1 ml of 0.02 % N- (1-naphthyl) ethylene-diamine dihydrochloride were added in quick succession. This was incubated for 15 min in darkness for colour development and absorbance was read at 540 nm with a suitable blank in a spectrophotometer.

Nitrite reductase activity (NiRA): Nitrite reductase activity (NiRA) was assayed by the method of Wray and Fido (1990) by using dithionite-reduced methyl viologen as an artificial electron donor. About 0.5 g fresh nodules were ground in a prechilled mortar with pistle containing 2ml of distilled water. Then the extract was filtered through a filter paper to get 1ml of assay. To 10µl assay 25 µl of each of potassium phosphate buffer (pH7.5), potassium nitrite (2.5 Mm KNO_2) and sodium dithionite (20 Mm sodium dithionite (prepared freshly in 290 Mm sodium bicarbonate) was added. Then 25 µl methyl viologen (3 Mm methyl viologen) was added for blue colour development. The material was incubated for 25 min for disappearance of colour and finally 0 .7ml of distilled water, 0.6 ml of sulphanilamide (1%, w/v, in 3 N HCl) and 0.6 ml of N-(1-naphthyl) ethylene-diamine dihydrochloride (0.1%, w/v) was added and then incubated again for 15 min. The absorbance was noted at 540 nm with a suitable blank in a spectrophotometer.

STATISTICAL ANALYSIS

The data collected was analyzed statistically by online Statistical Analysis (OPSTAT, CCS Haryana Agricultural University, Hisar). The experiment was conducted in randomized block design with three replications and thirteen treatments. The significance of data obtained was judged from the critical difference at 5% level of significance.

RESULTS

The results revealed that application of biofertilizers under different levels showed significant effect on leghaemoglobin content in nodules of common bean [Table-3].Treatment receiving dual inoculation of *Rhizobium* + VAM + 20 Kg P/ha showed maximum leghemoglobin content (27.51 mg/g FW) followed by treatment receiving dual inoculation of Azotobacter + VAM + 20 Kg P/ha (25.45 mg/g FW) and treatment receiving triple inoculation of *Rhizobium* + VAM + *Azotobacter* (24.34 mg/g FW) as compared to other treatments and control. Treatments receiving dual inoculation of biofertilizers (T_7, T_8) also showed significant difference as compared to single inoculation treatments (T_2, T_3 and T_4) as well as control. Significantly lowest leghaemoglobin content (920.15 mg/g FW) was recorded in control plants (T_1) .

The data recorded on enzymatic activities in nodules of common bean *viz.*, nitrate reductase activity (µmole NO_2/h/g FW) and nitrite reductase activity (µmole NO_2/h/g FW) at flowering stage is presented in Table 3. All nodules showed NRA, but with wide and statistically significant differences [Table-3]. Among all the treatments, nodules of treatment T_9 observed significantly highest nitrate reductase activity (9.22 µmole NO_2/h/g FW) followed by T_{10} and T_{13} (9.14 and 9.07 µmole NO_2/h/g FW) as compared to other treatments and control. Treatments receiving dual inoculation of Rhizobium + VAM and Azotobacter + VAM) also showed significant nitrate reductase enzyme activity as compared to single inoculation treatments and control. The lowest nitrate reductase activity (3.40 µmole NO_2/h/g FW) was recorded in control plants treatment (T_1).

The more variability was observed among different treatments for nitrite reductase enzyme activity in nodules of common bean. The results further revealed that significantly maximum nitrite reductase activity (80.87 µmole NO_2/h/g FW) was recorded in T_9 (*Rhizobium* + VAM + 20 kg P/ha) followed by T_{10} and T_{13} (79.22 and 79.1 µmole NO_2/h/g FW, respectively). The lowest nitrite reductase activity (29.58 µmole NO_2/h/g FW) was recorded in control plants (T_1).

DISCUSSION

Quality and crop yield mainly depends on the interplay of various biochemical functions of the plant in addition to the impact of growing environment. The cause and effect relationship is difficult to understand mainly because of complexity in understanding the interplay of several processes and functions. It has been observed that the common bean crop response to P is dependent on P available in the soil (Mallarino and Rueben, 2005). It was observed in the present study that the treatments differ significantly with respect to leghaemoglobin contents in nodules, nitrate and nitrites reductase activity. The results revealed that the maximum leghaemoglobin content, nitrate and nitrite reductase activity were reported with the treatment combination of *Rhizobium* + VAM + 20 Kg P/ha. The increase in leghaemoglobin content of the root nodules might be due to the improved availability of phosphorus to the root nodules due to combined application of inorganic phosphorus fertilizer and nitrogen and phosphorus biofertilizers. Also higher leghemoglobin content in *Rhizobium* + VAM @ 20 kg P/ha was mainly due to better root and nodules development (Sidhu *et al*., 2002). Similar to our results, there was observed a positive effect of phosphorus application on nodule leghaemoglobin content in *Lablab purpureus* and *Cassia tora* (Naeem and Khan, 2005). In conformity with our study, there was noted beneficial effect of inorganic phosphorus fertilizer as well as that of nitrogen and phosphorus biofertilizers on leghaemoglobin content in chickpea by Dutta and Prohit (2009).

Generally, NRA (Nitrate reductase activity) is higher in the nodules than in other plant parts (Ashraf

and Iram, 2005). The highest NRA was obtained from the nodules at the initiation of flowering and declined thereafter. Nitrate is reduced to NO_2 by NR. The NO_3 accumulation and assimilation in cell depends on NR activity.Nitrate reductase activity in nodules is related to the leghaemoglobin content of nodules (Caba*et al*., 1990) where root nodules are able to reduce NO_3 accumulation rapidly (Giannakis *et al.,* 1988). Gairola *et al.,* (2009) found that an increase in NRA decreases the accumulation of nitrate. The presence of phosphorus in the nutrient solution has earlier been reported to induce greater nitrate assimilation in corn (Magalhaes *et al*., 1998). The improvement in nitrate reductase and nitrite reductase activity in this study could be as a result of adequate availability of nitrogen and phosphorus at the site of their metabolism, owing to the application of phosphorus and nitrogen and phosphorus biofertilizers. A combination of control NR catalytic activity, NR protein degradation and NR expression provide a rigid control of the NO_2 concentration.This enzyme is up-regulated in the presence of nitrate, light, high concentration of CO_2 and photosynthetic production (sucrose).Nitrite is known to interfere with the overall process of nitrogen fixation (Streeter, 1986). A byproduct of NRA, NO_2, hinders the function of leghaemoglobin as well as nitrogenase (Becana and Sprent, 1987). This can be observed due to accumulation of toxic levels of NO_2 from the nitrate reductase reaction (Caba*et al*., 1990). It was strongly suggested that NO_3 accumulation affects the N_2 fixation process through formation of NO_2 and binding of leghaemogloebin (Lb) to form nitrosyl-Lb, which is unable to bind O_2 (Arrese-Igor *et al*., 1998). It oxidizes leghemoglobin (Riguard and Puppo, 1997) and inhibits invitro activity of nitrogenease (Trinchant and Riguard, 1980). The enzyme nitrate reductase catalyzes the reduction of nitrate to nitrite which is the first step in assimilation of nitrate by the plants. Rahman *et al*., 2010 reported that dual inoculation of biofertilizers and inorganic phosphorus showed the highest nitrate and nitrite reductase activity. Significantly higher nitrite reductase activity due to the inoculation of chick pea with biofertilizers was also reported by Eusufzai*et al*., 1999. Maximum activities of nitrate reductase and nitrite reductase in the leaves of chick pea might be the reason for the enhanced yield and quality of chickpea reported by Moin*et al*., 2014. Integrated application of P with different biofertilizers is highly recommended in common bean for enhancing nodulation or N_2 fixation and nitrate and nitrite reductase enzyme activity in nodules of common bean.

Table 1: Chemical properties of soil at the experimental site

S. No	Chemical properties	Value obtained	Method employed
1.	Available nitrogen (kg/ha)	210.2	Modified Kjeldhal method (Jackson, 1973)
2.	Available phosphorus (kg/ha	16.4	Olsen's method (Jackson, 1967)
3.	Available potassium (kg/ha)	270.5	Flame photometer (Jackson, 1967)
4.	Soil pH (1:2.5 soil: water)	7.90	pH meter (Piper, 1966)

Table 2:Treatment details of the experiment

S.No	Treatment combinations used	Treatment code
1	Maize + common bean (control).	T_1
2	Maize+ common bean treated with *Rhizobium* .	T_2
3	Maize + common bean both treated with *Azotobacter*.	T_3
4	Maize + common bean both treated with Arbuscular mycorrhizae	T_4
5	Maize + common bean both supplied with 20 kg phosphorus (P)/ha	T_5
6	Maize + common bean both supplied with40 kg P/ha	T_6
7	Maize + common bean treated with *Rhizobium* + Arbuscular mycorrhizae	T_7
8	Maize + common bean treated with *Azotobacter* + Arbuscular mycorrhizae	T_8
9	Maize + common bean treated with *Rhizobium* + Arbuscular mycorrhizae + 20kg P/ha	T_9
10	Maize + common bean treated with *Azotobacter* + Arbuscular mycorrhizae + 20 kg P/ha	T_{10}
11	Maize + common treated with *Rhizobium* + Arbuscular mycorrhizae + 40 kg P/ha	T_{11}
12	Maize + common bean treated with *Azotobacter* + Arbuscular mycorrhizae + 40 kg P/ha	T_{12}
13	Maize + common bean treated with *Rhizobium* + *Azotobacter* + Arbuscular mycorrhizae	T_{13}

Table 3:- Impact of phosphorus and biofertilizers on leghaemoglobin content, nitrate reductase activity (NRA) and nitrite reductase activity (NiRA) in nodules of common bean under intercropping of common bean + maize.

Treatments	Leghemoglobin content (mg/g fresh weight of nodules	Nitrate reductase activity (µmoleNO_2 /h/g FW)	Nitrite reductase activity (µmole NO_2/h/g FW)
T_1 (Control)	20.15±0.01	3.40 ±0.09	29.58±0.10
T_2 (*Rhizobium*)	22.16±0.02	4.87 ±0.06	39.77±0.06
T_3 (*Azotobacter*)	21.26±0.03	4.51±0.09	39.54±0.12
T_4 (VAM)	21.49±0.02	4.68±0.08	39.61±0.04
T_5 (20 kg P)	21.67±0.01	4.71±0.06	39.68±0.06
T_6 (40 kg P)	21.47±0.02	4.53±0.08	39.43±0.05
T_7 (Rhz.+ VAM)	23.37±0.02	5.96±1.01	49.63±0.12
T_8 (Az.+VAM)	23.06±0.01	5.67±0.09	49.37±0.09
T_9 (Rhz.+ VAM+20kg P)	27.51±0.04	9.22±0.19	80.87±1.37
T_{10} (Az.+ VAM+20 kg P)	25.45±0.02	9.14±0.16	79.22±1.34
T_{11} (Rhz.+VAM+40 kg P)	23.68±0.01	8.77±1.12	75.60±0.04
T_{12} (Az.+VAM+40 kg P)	23.81±0.03	8.90±1.07	75.73±0.05
T_{13} (Rhz.+Az.+VAM)	24.34±0.05	9.07±1.08	79.12±0.81
C.D.@ 5%	0.040	0.124	1.479

Rhz. = *Rhizobium*, Az. = *Azotobacter*, VAM = *Vesicular arbuscular mycorrhizae*, P = Phosphorus, C.D. = Critical Difference

CONCLUSION

Use of P as fertilizer is common practise among farming community through the world but application of P to legumes is limited especially in the developing countries. The results of present investigation, revealed that integrated application of mineral P fertilizer, Rhizobium, VAM, Azotobacter in combination significantly increased the various biochemical parameters of common bean. Application of P fertilizer (@20 kg P/ha) along with Rhizobium, VAM gave significant increase of various biochemical parameters in common bean under intercropping system with maize in temperate regions of Kashmir, Srinagar,India. Integrated application of P with

Rhizobium, Azotobacter and VAM is highly recommended in common bean + maize system for improving various biochemical parameters of common bean, enhancing N_2 fixation and also for sustainable agricultral purposes and healthy food production is recommended.

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