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Differential Responses of Oxidative Stress Indices in different Cultivars of *Phaseolus vulgaris* L. subjected to Rhizobacterial Inoculation

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Abstract: The present study aims to unravel the mechanism of differential response of oxidative stress indices in response to rhizobacterial inoculation in *Phaseolus vulgaris* L. Oxidative burst triggered at the early events of the symbiotic interaction especially during nodulation stage which is necessary for the efficient development of healthy root nodules. To test our hypothesis, common bean seeds were inoculated with *Rhizobium* spp or co-inoculated with rhizobacteria and compared with uninoculated control plants. Afterward, at late flowering stage, the common bean nodules were collected, and the levels of ascorbic acid (AsA), hydrogen peroxide and lipid peroxidation activities were evaluated. There was constant increase in oxidative stress indices such as ascorbic acid, hydrogen peroxide (H_2O_2), and lipid peroxidation which might attributes to generation of free radicals during nodulation stage. However, ascorbic acid content in nodules was much higher than that in the root. Our results also provide new insights into the mechanisms involved in the interaction between bacteria as a symbiont and reinforces the generation of reactive oxygen species. We concluded that the additive effects of *Rhizobium leguminasarum along* with *Bacillus megaterium* induces greater oxidative stress relative to other treatments in roots and nodules of common bean.

Keywords: *Phaseolus vulgaris* L., oxidative stress, ascorbic acid, lipid peroxidation, H_2O_2.

INTRODUCTION

The plant–microbe interactions determines a beneficial effects and are considered as primary determinants of nitrogen fixing potential and soil fertility (Santos *et al.*, 2018). Rhizobacteria also known as root-colonizing bacteria are known to influence plant growth by various physiological and biochemical mechanisms. Moreover, these bacteria exhibited multiple plant growth promoting properties, such as nitrogen fixation, enhanced salinity tolerance, drought stress and solubilize phosphates and other essential nutrients (Rodrigues *et al.*, 2013; Steiner *et al.*, 2020).

Gopalakrishnan *et al.* (2015) described rhizobia as the unique rhizobacteria that can fix atmospheric nitrogen in symbiotic association with legumes. Besides nitrogen fixation, rhizobia performs several plant growth promoting functions like production of phytohormones, lipochitooligosaccharides (LCOs), siderophores, hydrogen cyanide (HCN) ACC deaminase. exopolysaccharides (EPS), phosphate solubilizing enzymes and bio-control activity against phytopathogens which could improve plant growth and yield (Aeron *et al.*, 2017). Akinrinlola *et al.*, (2018) isolated 12 strains of Bacilli among which *Bacillus megaterium*, *B. safensis*, *B. simplex*, and *Paenibacillus graminis* were found to be efficacious in growth of soybean. Wang *et al*., (2018) reported that root nodules of leguminous plants are very rich in nutrients which attracts various types of rhizobacteria to colonize plants opportunistically.

However, oxidative burst in the plant tissue is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant metabolism. Hasanuzzaman *et al.*, (2020) studied the oxidative indices during and after the establishment of symbiosis in cowpea with *Bradyrhizobium* sp. They found that plants inoculated with *Bradyrhizobium* sp. showed higher levels of lipid peroxidation and H_2O_2 levels as compared to controls at the flowering and at the beginning of senescence respectively. Puppo *et al.* (2011) concluded that hydrogen peroxide (H_2O_2) and nitric oxide (NO) appear to play an important signalling role in the establishment and the functioning of legume-rhizobium symbiosis. Sainz *et al*., (2015) observed that decrease in reactive oxygen species (ROS) level prevents root hair curling and infection threads formation. Park *et al*., (2017) determined the oxidative burst triggered at the early events of the symbiotic relationship between *Bacillus aryabhattai* SRB02 in common bean. They found that reduced H_2O_2 levels showed low nodule number, a reduction in the proportion of red nodules (%) and an increase in the lipid peroxidation. Torres-Jerez *et al*., (2017) reported that *M. truncatula* have higher rates of

N_2 fixation and detected an overexpress GME (GDP mannosepyrophosphorylase) leading to elevated ascorbic acid content. Depending on the legume species, this ascorbic acid concentration was 15–40% of that observed in the leaves and slightly greater than that in the roots (Matamoros *et al*., 2006). Given this context, this study proposed to test the hypothesis that successful inoculation with *Rhizobium* spp. and rhizobacteria would lead to generation of free radicals especially during nodulation stage which might attributes to higher nitrogen fixing potential of common bean.

MATERIAL AND METHODS

This was carried out in the Molecular Biology Laboratory Division of Biochemistry Sher-e-Kashmir University of Agricultural Sciences & Technology. Two Local common bean BR-104 & BR-50 obtained from Regional Agricultural Research Sub Station, Bhaderwah (Jammu division) and two common bean (VL-63 and VL-125) obtained from IIPR Kanpur and VPKAS ICAR, Almora. Two *Rhizobium* spp. namely *Rhizobium leguminasarum* (NAIMCC-B-00862) (T1) and *Rhizobium phaseoli* (NAIMCC-B-00431) (Plate 13) were obtained and were used as commercial bioinoculant or biofertiliers as they are known for their nitrogen fixation potential. Two rhizobacteria *Bacillus megaterium* & *Bacillus aryabhattai were* successfully isolated from root nodules of *Phaseolus vulgaris* L. The cultures were grown on Yeast Agar Mannitol Agar and maintained at 28°C obtained from ICAR-National Bureau of Agriculturally important microorganisms (NBAIM), Kushmaur, Mau Nath Bhanjan, Uttar Pradesh (India). The overall study was conducted according to the table shown in Table 1.

Table 1.

Selected common bean from germplasm screening	1. BR-104 2. VL-125 3. VL-63 4. BR-50	
Treatment	1. *Rhizobium leguminasarum* (NAIMCC-B-00862)	**T1**
	2. *Rhizobium phaseoli* (NAIMCC-B-00431)	**T2**
	3. *Bacillus megaterium strain* (ROA047)	**T3**
	4. *Bacillus aryabhattai strain* (HFBP06)	**T4**
	5. *Rhizobium leguminasarum + Bacillus megaterium strain* (ROA047)	**T5**
	6. Nitrogen	**T6**
	7. Control	

Ascorbic acid was determined according to the modified method of Mukherjee and Choudhuri (1983). The estimation is based on the reduction of dinitrophenylhydrazine (in acidic medium) by ascorbic acid to phenyl hydrazone which results in the formation of dark pinkish colored complex. Fresh plant tissue (root or nodule), 0.2 g was homogenized with 2 ml 6% trichloroacetic acid. Homogenate was centrifuged at 7000 g at 4°C for 10 minutes. The supernatant were used for estimation of total ascorbic acid.

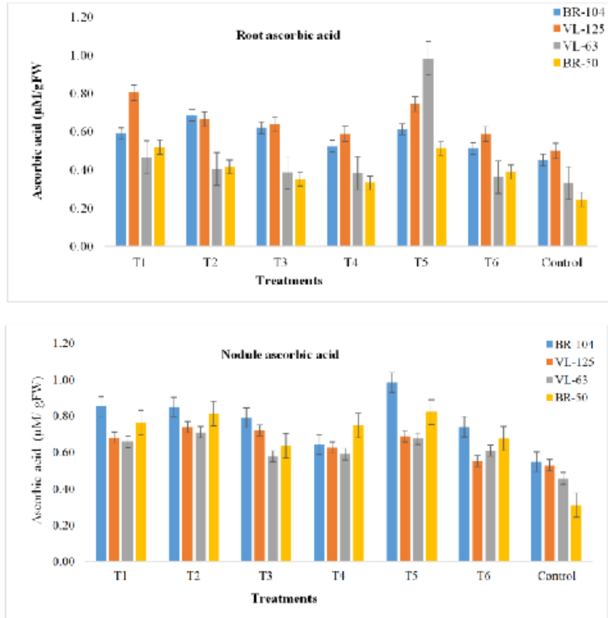
Hydrogen peroxide levels were measured calorimetrically as described according to the modified method of Velikova *et al*., (2000). Fresh nodule and nodule (approximately 0.2 g) was homogenized at 0 to 4°C in 1.0 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm (Eppendorf) for 15 minutes at 4°C. The reaction mixture consists of 0.2 mL of supernatant, 1 mL of 10 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 1.0 mL of 1.0 M potassium iodide (KI, freshly prepared).

Lipid peroxidation is oxidative degradation of lipid-fatty acids by reactive oxygen species and hence it is considered as one of the measure of oxidative stress in the cells. Lipid peroxidation (LPO) was referred to malondialdehyde (MDA) contents estimated as thiobarbituric acid (TBA) (Heath and Packer 1968). Lipid peroxidation in root and nodules of common bean was assayed using the thiobarbituric acid (TBARS) method according to Singh *et al*., (2007) in terms of malondialdehyde (MDA). The absorbance of the supernatant was recorded at 532 nm and the TBARS content was calculated according to its extinction coefficient of 155 mM^{-1} cm^{-1}.

RESULT

The average AsA content of the four cultivars increased significantly after inoculation, but no major differences in AsA content were detected among the four cultivar at the late flowering stage of common bean as shown in Fig. 1. In case of cultivar BR 104, highest significant increase in root and nodule ascorbic acid content was recorded when inoculated with T5 as compared to uninoculated control. In the cultivar VL 63, content of nodule ascorbic acid was recorded highest with increasing percentage of 54.35% when inoculated with T2 while least increase of was observed with treatment T3 relative to control. Similarly, in cultivar BR 50, root ascorbic acid among all the treatment was significantly increased by with treatment T1 followed by T5 and T2 as compared to control. However nodule AsA was in the increasing order of T5, T2 and T4, T3 respectively over control.

The root sample of cultivar BR-104 showed MDA content to highest increase in T5 followed T2 relative to control plant. Similarly, in nodules of VL 125, highest MDA contents in nodules was increased by almost 100% (T1) while lowest MDA in nodules increased by 27.6% in plants supplemented with nitrogen as compared to uninoculated control.

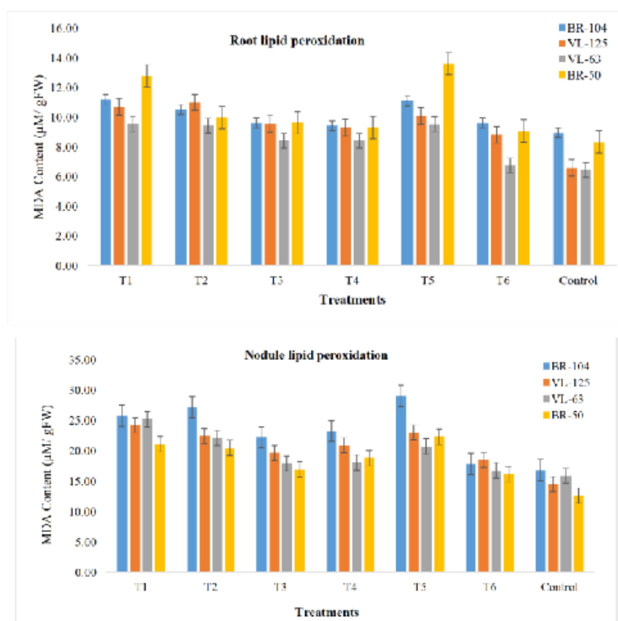


Rhizobium leguminasarum (NAIMCC-B-00862) (T1), *Rhizobium phaseoli* (NAIMCC-B-00431) (T2), *Bacillus megaterium* strain (ROA047) (T3), *Bacillus aryabhattai* strain (HFBP06) (T4), *Rhizobium leguminasarum* NAIMCC-B-00862) + *Bacillus megaterium* strain (ROA047) (T5), Nitrogen (T6).

*Mean values having different alphabets are significantly different from each other at $p<0.005$

Fig. 1. Effects of rhizobacterial treatments on ascorbic acid (µM/g FW) content in root and nodule of different cultivars of *Phaseolus vulgaris* L.

Likewise, in the cultivar VL-63 roots highest MDA contents was found to highest when inoculated with T1 while lowest significant increase was recorded in plants treated with nitrogen supplement as compared to control. In the cultivar BR-50, roots MDA content significantly increased by 63% treated with T5 followed by T1 with 53% increase as compared to control. And in nodules, highest MDA content was recorded highest with T5 while lowest nodule MDA contents increase by around 34% as compared to uninoculated control. Hydrogen peroxide content was reported highest in roots of BR 104 while nodule highest H_2O_2 content was found in VL 125 when treated with T5 as shown in Fig. 2.

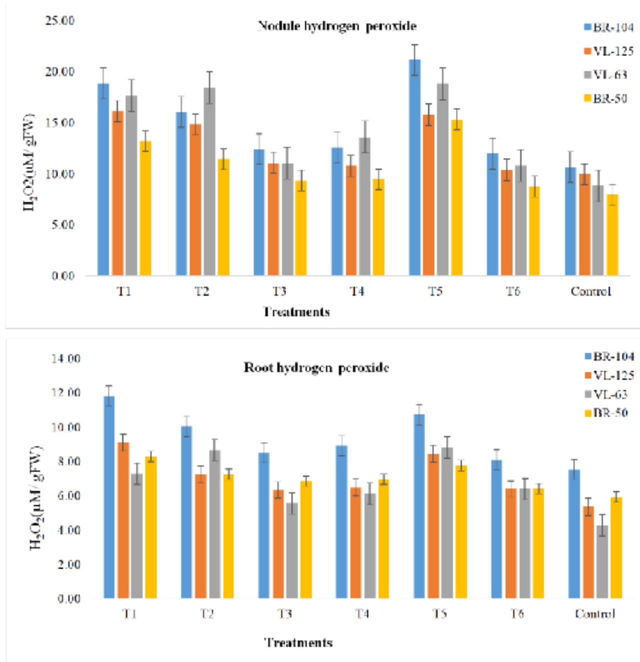


Rhizobium leguminasarum (NAIMCC-B-00862) (T1), *Rhizobium phaseoli* (NAIMCC-B-00431) (T2), *Bacillus megaterium* strain (ROA047) (T3), *Bacillus aryabhattai* strain (HFBP06) (T4), *Rhizobium leguminasarum* NAIMCC-B-00862) + *Bacillus megaterium* strain (ROA047) (T5), Nitrogen (T6).

*Mean values having different alphabets are significantly different from each other at $p<0.005$

Fig. 2. Effects of rhizobacterial treatments on malondialdehyde (MDA) (µM/ gFW) content in root and nodule of different cultivars of *Phaseolus vulgaris* L.

The hydrogen peroxide (H_2O_2) production was quantified in roots of BR-104 with highest increase of 57% when inoculated with T1 while lowest significant increase of 7% was recorded when plants were supplemented with nitrogen as compared to control. However, the production of H_2O_2 in nodule was much higher compared to root with highest increase of in plants inoculated with T5 while lowest concentration was increased by 16.4% when inoculated with T3 relative to control. The VL-63 cultivar inoculated with T5 observe highest increase in the order of T2, T1 and T4 as compared to uninoculated control in roots. With the cultivar BR-50, plants inoculated with T1 recorded highest increase in root H_2O_2 production while lowest increase was 8.26% in nitrogen supplemented plant as compared to uninoculated control. Significant H_2O_2 concentration in nodule was found to increase by 9.78% in plants treated with nitrogen supplement as compared to control as shown in Fig. 3.



Rhizobium leguminasarum (NAIMCC-B-00862) (T1), *Rhizobium phaseoli* (NAIMCC-B-00431) (T2), *Bacillus megaterium* strain (ROA047) (T3), *Bacillus aryabhattai* strain (HFBP06) (T4), *Rhizobium leguminasarum* NAIMCC-B-00862) + *Bacillus megaterium* strain (ROA047) (T5), Nitrogen (T6).

Mean values having different alphabets are significantly different from each other at $p<0.005$

Fig. 3. Effects of rhizobacterial treatments on hydrogen peroxide (µM/ gFW) content in root and nodule of different cultivars of *Phaseolus vulgaris* L.

DISCUSSION

In present investigation, the concentration of nodule's ascorbic acid was higher as compared to root's ascorbic acid which is in line with the study reported by Matamoros *et al.*, (2006) describing presence of high amount of Ascorbic acid (AsA) at the apex of the nodules. Similar observations were reported in root nodules of the legume pulse *P. mungo* contained high amount of AsA, much higher than the amount of AsA present in the young roots Ghosh *et al.*, (2006). Earlier, it was thought that the legume root nodules were unable to synthesize AsA and this capacity was lost early in the root nodule development Groten *et al*., (2005). This finding could suggest that the common bean might regulate key aspects of nodule metabolism through the transport of ascorbate from the shoot to the nodule via roots. In addition carbohydrate pool present in the nodule might serve as precursor for AsA production during plant microbe interaction and N_2 fixation as reported by Ghosh and Maiti (2014).

In our study, the inoculated plants exhibited higher hydrogen peroxide levels than untreated control, suggesting that these plants lacked an adequate system to control the levels of H_2O_2 during nodulation stage. Hydrogen peroxide has been highlighted as a powerful signaling molecule for several cellular events and detected higher during the infection, development and senescence of bacteroids as described by Becana *et al.* (2010). Same trends was followed with lipid peroxidation production and this observation indicates an adequate accumulation of free radicals mainly hydrogen peroxide (H_2O_2) and superoxide radical (O_2•−) causing oxidative burst during microbe-host interactions. The high levels of lipid peroxidation might be associated with the generation of hydrogen peroxide and other free radicals during the nodulation stage. Our results are in accordance with Mohammadi and Karr (2001) who studied lipid peroxidation, nitrogen fixation and leghaemoglobin content in root nodules of soybean. In conclusion, we demonstrated that there was constant increase in oxidative stress indices such as ascorbic acid, hydrogen peroxide (H_2O_2), and lipid peroxidation during nodulation stage which might attributes to generation of free radicals during rhizobacterial-common bean interactions. However, the effect of

Bacillus was more prominent when co-inoculated with *Rhizobium* spp. for most of the oxidative stress indices in different cultivars of *Phaseolus vulgaris* L. subjected to bacterial inoculation.

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Conflict of Interest. None.

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