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Co-inoculation Potential Impact of PSB and *Rhizobium* on Physico-chemical Properties of Soil and Legume Crop Growth

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ABSTRACT

A field experiment was carried at Botany Department, C.C.S University, Meerut through 2018-19 to observe the potential of *Rhizobium* and PSB on soil properties, growth, yield and nitrogen fixation efficiency of *Lens culinaris L.* (Pusa Ageti). These microbial inoculants were used individually and in dual inoculation. Physiological and biochemical analysis was observed. The results of this field experiment exhibit that the dual inoculated plant significantly increase (30-52%) the biomass, plant height, grain yield, plant nitrogen, phosphorus, carbon, plant and seed protein content of the test crop and also improve the nutrient content of the soil. It can be concluded from the present findings that the dual inoculated (*Rhizobium* + PSB) show high yield and growth of crop whereas the single inoculants show better results in comparison to the control.

Key words: Lentil, *Rhizobium*, Phosphate Solubilizing Bacteria (PSB), Inoculation

Lentil (*Lens culinaris* L.) is an important legume crop native to Southwestern Asia. It is the third most important legume in the world after chickpea and pea (Singh *et al*. 2018), and it is also a second important rabi season legume crop (Khazaei *et al*. 2016). Lentil is an annual and herbaceous plant. The plant is tufted, slender and much branched with tendrils. It has a well-developed taproot system with several lateral branches. Leaves are pinnate with 10 pairs of leaflets. A pair of stipules present at their base which terminate into tendrils. Flowers are white with blue, violet or pink-tinge. The pods are short and broad possessing 1-2 lens-shaped seeds. Seed coat undergoes color change with the age i.e. ranging from white to pale green to gray to brown to the black i.e. age effective. Lentil seeds are consumed because of their higher protein content and also rich in minerals and vitamins. The protein content is equivalent to the meat protein (Joshi *et al*. 2017) that's why lentil is also known as Poor man's meat. The seeds are consumed as Dal, fried or in soups. Flour of lentil is used in purees, making cakes, stews and also used as food for an infant (Matny 2015).

Lentil is able to fix atmospheric nitrogen through the symbiotic association with *Rhizobium*. *Rhizobium* is a gram-negative bacterium found in the rhizospheric zone of the leguminous plants. In this mutual association, they secrete enzymes which convert atmospheric nitrogen into biological nitrogen. This available form serves as a constant source of reduced nitrogen to the host plant by offering nutrients and energy for the bacterial activities. *Rhizobium* fixes atmospheric nitrogen and converts it into available form for plant uptake (Wagh *et al*. 2015). Phosphate Solubilizing Bacteria (PSB) have no direct symbiosis with plants but beneficial in several ways. They solubilize the phosphate so that the plants can absorb them. Phosphate Solubilizing Bacteria secrete different organic acids which lower the pH of the rhizosphere, resulting in the dissociation of the bound form of phosphate (Afzal and Bano 2008). PSB increases the availability of iron, zinc and other micronutrients by the production of plant growth-promoting substances (Kucey *et al*. 1989).

Wilt the population increase, the demand for food getting increased. To meet the food demand of people there

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is a requirement to increase the productivity of crops with the maintenance of the fertility of the soil. Use of chemical fertilizers, pesticides, insecticides, etc. depletes soil fertility. To replenish soil fertility and to replace these chemicals, biofertilizers can be used. Microbial inoculation is an alternative to the chemical fertilizers. They are cost-effective, eco-friendly and renewable sources (Tagore 2005). The current study was designed to evaluate the co-inoculation potential of PSB and *Rhizobium sp.* for improving the growth, nodulation, and yield of lentil crop.

MATERIALS AND METHODS

The field experiment was carried out in the field of Botany Department, Chaudhary Charan Singh University, Campus, Meerut, Uttar Pradesh during Rabi season from 2018 through 2019 on lentil. It was designed in four plots of equal size, three plots for the treatment and one plot for the control. The treatment includes the inoculation of *Rhizobium*, PSB alone and dual inoculation of them.

Material used

Certified seeds of Lentil (*Lens culinaris*) were procured from IARI, New Delhi.

Rhizobium leguminosarum and *Bacillus megaterium* (Phosphate Solubilizing Bacteria) for treatment of lentil were procured from Microbiology Division of Indian Agricultural Research Institute, New Delhi.

Seed inoculation

Seeds were inoculated with *Rhizobium* (0.5g) and PSB (0.5g) in the combination as well as individually. 10% jaggery solution was made by adding 10g jaggery into 100 ml distilled water. This jaggery solution was acted as an adhesive to stick the selected biofertilizers on seed coat evenly. Seeds were soaked in this solution until the formation of an even layer of *Rhizobium* and PSB separately. Then the seeds were dried in shade and sowed into the field, as per the experimental design.

Soil parameters

The concentration of hydrogen ions in the soil is known as its pH. It was observed after germination of the seeds. 1g of treated soil was mixed in 10ml of distilled water. The pH of soil suspension was recorded. The cation exchange capacity of the soil is referred to as the holding capacity of the cations by the soil. These cations are held by the anions through electrostatic force. The cations of the soil particles are effectively interchangeable with different cations and thus, they are available for plants. CEC of soil was evaluated according to Jones (1967). For CEC calculation the following formula is used:

$$(\text{pH of soil sample solution} - \text{pH of 1N acetic acid}) \times 22$$
$$\text{m.eq / 100g soil sample}$$

Where the pH of 1N acetic acid = 2.3

Soil bulk density is the mass of dry soil per unit of bulk volume including the air spaces. It is estimated by Cresswell and Hamilton (2002) method. Higher the organic matter in the soil lowers the bulk density and vice versa whereas processes which compact the soil will increase the bulk density of soil. Bulk density of soil was calculated as:

$$\text{Bulk density (g/cm}^3) = \frac{\text{Dry Soil Weight (g)}}{\text{Soil Volume (cm}^3)}$$

Particle density of soil is the mass of individual soil particle/unit volume. It was estimated by Blake (1965) method and calculated by the given formula:

$$\text{Particle Density (Dp)} = \frac{\text{Dw. (Ws)}}{\text{(Ws) - (Wsw - Ww)}}$$

Where;
DW = Density of water (g/cm^3) at temp. observed
Ws = Wt. of oven-dried soil sample
Wsw = Wt. of pycnometer, soil and water
Ww = Wt. of pycnometer and water

The porosity of soil is the number of pores or open space between soil particles. Pore spaces are formed because of the movement of roots, worm, and insects. It was estimated by Piper method (1966). To calculate porosity following formula was used:

$$\text{Porosity} = [1 - (\text{Bulk Density/Particle Density})] \times 100\%$$

Germination

Developing a new plant from seeds refers to seed germination. Seed germination assay ISTA (1976) was used to estimate seed germination rate. It is determined by calculating the difference between the emergent and non-emergent seedlings from the soil. It was calculated as:

$$\frac{\text{Number of Germinated Seeds}}{\text{Total Number of Seeds Sown}} \times 100$$

Physiological parameters

The observation like plant length, biomass and yield were observed after harvesting the crop. The length of root and shoot were measured separately with the help of scale. The whole plant length was the sum-up of the length of root and shoot. The fresh and dry weight of the plant, pods plant^{-1}, seeds pod^{-1} and 10 seeds weight were recorded.

Nodulation

Nodule number, nodule weight, and volume were taken by collecting the three intact plant from each plot. The soil particles adhered to the roots were removed. The nodules were detached and recorded for the number of the nodule, volume of the nodule, and weight (fresh and dry) of nodules per plant of each plot.

Biochemical parameters

Chlorophyll content was estimated by Arnon's method (1949). It was estimated by the following formulas:

$$\text{Chlorophyll a content (mg/g tissue)} = \frac{12.7\,(A_{663}) - 2.69\,(A_{645}) \times V}{1000 \times W}$$

$$\text{Chlorophyll b content (mg/g tissue)} = \frac{22.9\,(A_{645}) - 4.68\,(A_{663}) \times V}{1000 \times W}$$

$$\text{Total Chlorophyll content (mg/g tissue)} = \frac{20.2\,(A_{645}) - 8.02\,(A_{663}) \times V}{1000 \times W}$$

Where,
V = final volume of chlorophyll extract
A = Absorbance at a specific wavelength
W = fresh weight of tissue extracted

Protein content was estimated by the Bradford method (1976) and Proline content was determined by Bates *et al.* (1973). They were calculated as:

$$\frac{\text{OD} \times \text{Factor} \times \text{Dilution (if any)} \times 1000}{100 \times \text{Total Volume}}$$

Nitrate reductase was determined by Hageman and Reed (1980) method and it was calculated by the following formula:

$$\frac{\text{OD} \times \text{Factor} \times \text{Dilution (if any)} \times 1000}{100 \times \text{Total Volume}}$$

Leghemoglobin concentration in mM was determined by Bergersen and Turner (1980) method and it was estimated by the given formula:

$$\text{Lb concentration (mM)} = \frac{A_{556} - A_{539} \times 2D \times 100}{23.4}$$

Nutrient content

The observation like total nitrogen, phosphorus and organic carbon content of plant as well as of soil were observed. Total nitrogen was estimated by the Kjeldahl (1883) method. The formula used to calculate total nitrogen (%) is as follows:

$$\frac{14 \times \text{Normality of acid} \times \text{Actual titrant value} \times 100}{\text{Sample weight} \times 1000}$$

Estimation of phosphorus was carried out by the Olsen (1954) method and Organic carbon content was estimated by using Datta *et al.* (1962) method. The amount of phosphorus and organic carbon was calculated individually by the given formula:

$$\frac{\text{OD} \times \text{Factor} \times \text{Dilution (if any)} \times 1000}{100 \times \text{Total Volume}}$$

Statistical analysis was carried out for each parameter of the experiment. To determine the significant difference within the treatments the single-way ANOVA was performed by using the statistical tool IBM SPSS 20.0.

RESULTS AND DISCUSSION

Germination percentage

Germination is the process of emergence and development of seedlings from the seeds after breaking off the dormancy under favorable conditions. Seeds treated with dual inoculation of *Rhizobium* and Phosphate Solubilizing Bacteria (PSB) show quick emerging of seedlings from the soil as compared to the individual treatment of the seeds with *Rhizobium* and PSB. Least germination of seeds was seen in the control. Increase in germination percentage with the inoculants was because of their ability to suppress the growth of antagonists present in soil and by releasing plant growth-promoting substances around seeds sown. Rudresh *et al.* (2005) reported the effect of combined inoculation of salt-tolerant *Rhizobium* and Phosphate Solubilizing Bacteria in increased germination of chickpea. Similar results were observed by Afzal and Bano (2008), Dastager *et al.* (2010), Muhammad *et al.* (2013), Pawar *et al.* (2018).

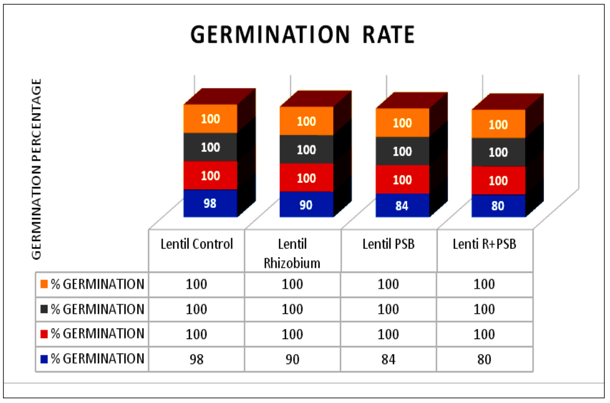

Fig 1 Impact of combined inoculation of *Rhizobium* and PSB on the germination rate of *Lens culinaris* seeds

Plant growth

Growth of plant depicts the increase in the size of the plant. It is an irreversible process. Growth is the result of cell division, cell enlargement, and differentiation. Higher the cell division and cell differentiation rate more will be the plant growth. The co-inoculated treated plants attain utmost growth, while least plant growth was observed in the untreated plants. Dual inoculation of *Rhizobium* and PSB significantly increased plant height over their sole application and control. The soil has natural reserves of plant nutrients but these reserves are mostly in unavailable forms for the uptake of plants. The most constraining nutrients for plant development and production are nitrogen and phosphorus. Soil microbes play a significant role in providing nutrients for plant growth and yield production (Adesemoye *et al.* 2008, Berg 2009). Co-inoculation of microbes is more effective than single inoculation. Rudresh *et al.* (2005), Afzal and Bano (2008), Dastager *et al.* (2010), Muhammad *et al.* (2013) recorded that combined inoculation of *Rhizobium* and PSB increased plant height in chickpea. The present results are also in harmony with the findings reported by Tagore *et al.* (2013), Pawar *et al.* (2018).

Plant biomass

The total quantity of living plant material comprises of root and shoot of the plant in a specific area at a particular time period. The selected legume crop shows the stimulatory effect of the application of *Rhizobium* and PSB individually as well as their co-inoculation on the biomass (fresh and dry). The shoot and root of dual inoculated plants show utter biomass followed by the sole application of *Rhizobium* and untreated plants exhibit infinitesimal plant biomass. Nitrogen and Phosphorus are major macronutrients of plants. In the environment, only a limited amount of these nutrients is available for the uptake of plants. *Rhizobium* and PSB benefit plant growth by increasing soil nutrients availability (Sayed 1999). *Rhizobium* is well known for its biological nitrogen fixation activity in leguminous crops. On the other hand, PSB solubilizes the bounded form of Phosphorus and make it available for the plant uptake. In the last few years' uses of biofertilizers instead of chemical fertilizers or in combination with chemical fertilizers increased due to their beneficial role and harmless activity towards nature. Phosphate Solubilizing Bacteria (PSB) and *Rhizobium* not only increase the P and N uptake of plants but, these also can improve the plant health by increasing their productivity and metabolism. Similar kind of results has also been observed by Abid *et al*. (2016), Rasool and Singh (2016), Pawar *et al*. (2018).

Fig 3 Impact of Co-inoculation of *Rhizobium* and PSB on the growth of lentil (A) Untreated plant (B) Treated with *Rhizobium* (C) Treated with PSB and (D) Treated with *Rhizobium* and PSB

Nodulation parameters

Nodulation is the process of formation of nodules on plant roots it is the site of the nitrogen fixation with the help of nitrogen-fixing bacteria such as *Rhizobium.* Dual inoculation of *Rhizobium* and PSB depicts the superior results for the parameters of nodulation. In comparison to the control, all the treatments have higher nodule number, nodule volume and nodular biomass per plant. After dual inoculated treatment *Rhizobium* inoculated plants show maximum nodulation parameters. Lentil shows the stimulatory effect of dual inoculation. However, the sole application of *Rhizobium* and PSB also have a positive impact on nodulation in comparison with untreated plants. *Rhizobium* plays an important role in nitrogen fixation in legumes. This unique ability to fix nitrogen biologically is possible due to the symbiosis between *Rhizobium* and their specific host legume plant. Nodule formation is the result of the symbiotic association of *Rhizobium* and Legumes. It can be used as a direct indicator of plant health and bacterial-legume relationship as the nodulation increased more nitrogen would be fixed by *Rhizobium* and it can be available for the further uses such as protein formation, genetic material synthesis, and other metabolic processes. Similarly, Phosphorus plays an important role in nutrients condition of legumes. Nodule development indirectly influenced by Phosphorus because of its basic function as an energy source. Limited amount of Phosphorus inhibits the growth of roots, photosynthesis, translocation of photosynthates, and other such functions which influence N fixation in leguminous plants. There are lots of results available on the impact of phosphorus on legume *Rhizobium* symbiosis. It was observed that nodulation of legumes, significantly stimulated by P application (Tang *et al*. 2001, Chhonkar and Rao 1967). Leguminous plants require large amounts of Phosphorus for nodulation and for *Rhizobium* activity. In the present study, enhancement in nodulation with inoculation of PSB and *Rhizobium* suggests the stimulatory effect of Phosphorus and Nitrogen in the plants. Consequently, P and N availability affect nodulation in legumes (Leidi and Rodriguez-Navarro 2000, Zaman-Allah *et al*. 2007, Vikram and Hamzehzarghan 2008). Results have harmony with those of Tagore *et al*. (2013), Pawar *et al*. (2018).

Yield attributes

Crop yield is the measure of the seeds produced by the plant at a particular land. The highest number and weight of seeds per pod and pods per plant were recorded with dual inoculation of PSB + *Rhizobium*, but the results are statistically insignificant. All the yield attributes are found least in untreated plants. The individual effect of *Rhizobium* and PSB show that *Rhizobium* treatment has an advantage over the sole application of PSB. It can be due to the reason that plants require nitrogen in large amount during seed formation. During this period, protein formation takes place and nitrogen plays a major role in amino acid synthesis. The invigorating impact of Phosphate Solubilizing Bacteria on the yield of selected legume may be due to an adequate amount of Phosphorus, which further results in increased synthesis of biomolecules. The role of P in carbohydrate metabolism is well known, it acts as an energy carrier and stored as phosphate molecules for subsequent use in overall growth and development of plants. When PSB and *Rhizobium* were applied in combination, their synergistic effect increased the yield of Lentil. Sayed (1999) also observed a similar kind of effect. Such results were also observed by Patil *et al*. (2004), Rasool *et al*. (2016), Heisnam (2017), Pawar *et al*. (2018).

Table 1 Impact of combined inoculation of *Rhizobium* and Phosphate Solubilizing Bacteria on the selected growth attributes of *Lens culinaris*

Treatment	Root length (cm)	Shoot length (cm)	Plant length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot fresh weight (g)	Shoot dry weight (g)	No. of nodules / plant	Volume of nodules (ml)	Fresh weight of nodules (g)	Dry weight of nodules (g)
Control	11.83	32.17	44.07	0.49	0.15	2.88	0.71	6.67	0.10	0.021	0.005
Rhizobium	12.17	43.40	55.57	0.56	0.18	15.85	4.21	27.33	0.67	0.131	0.011
PSB	12.17	37.50	50.00	0.50	0.18	5.04	1.37	8.33	0.37	0.044	0.006
R+ PSB	12.90	54.33	67.23	0.64	0.23	22.11	5.88	36.00	1.37	0.143	0.020

Total nitrogen content

Nitrogen plays a vital role in the growth, development and reproduction. As the nitrogen is a major component of chlorophyll, amino acids, energy transfer compound (such as ATP), and nucleic acid (DNA). Higher the nitrogen amount the more growth of the plant and vice versa. In comparison to control the dual inoculated plants shows the highest nitrogen content followed by their individual treatments. The nitrogen content of plants got increased significantly over the control due to the seed inoculation with *Rhizobium* and PSB isolates. Seed inoculation with *Rhizobium* and PSB isolates increase the nitrogen fixation which increased the nitrogen content in plant biomass and reflected its uptake in lentil. Phosphorus plays a crucial role in electron transfer during nitrogenase activity, therefore increase in phosphorus significantly increases the nitrogen fixation by *Rhizobium*. Consequently, *Rhizobium* coupled with PSB increased the nitrogen fixation thereby plant biomass. Rashid *et al.* (1999) reported the increase in nitrogen uptake by the plant due to inoculation of *Rhizobium* and PSB. Govindan and Thirumurugan (2005), Rudresh *et al*. (2005) observed that the effect of the selected microorganisms significantly increases the NPK uptake. Similar observations were reported by Pawar *et al*. (2018) when they applied the PSB and *Rhizobium* in combination as well as individually on cowpea.

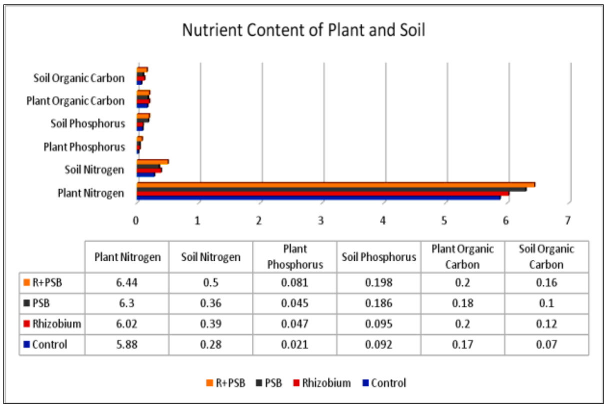

Fig 2 Impact of combined inoculation of *Rhizobium* and PSB on the nutrient content of *Lens culinaris* and soil

Total phosphorus content

Phosphorus is an important macronutrient for the overall development of the plant. Phosphorus is essential for sunlight capturing and convert it into useful compounds. Phosphorus stimulates the root development and also increase the nitrogen-fixing capacity of legume. Like nitrogen content, total phosphorus content was found maximum with dual inoculation of selected biofertilizers (PSB and *Rhizobium*) followed by individual application of

PSB. Phosphorus is present in the unavailable form in the soil reservoir, but plants can only uptake the anionic form of phosphorus. PSB solubilize the unavailable phosphate and enhance its availability to plants resulted in the better root development and nodulation efficiency. So, increased availability of N and P content have resulted in greater uptake of these macronutrients by plants. Co-inoculation of PSB and *Rhizobium* found more beneficial as compared with their individual application and control. All the selected parameters also increased due to solubilization and mineralization of N and P content.

Total carbon content

Plants use carbon dioxide for photosynthesis. The carbon comprises fat, carbohydrate, protein, etc. Once the plant complete its life cycle carbon will release into the atmosphere. The total carbon content of lentil exhibits a stimulatory response of PSB and *Rhizobium* inoculation. In comparison to the untreated plants, co-inoculation of Phosphate Solubilizing Bacteria and *Rhizobium* shows maximum carbon content followed by *Rhizobium* and PSB individually. Least carbon content was observed in untreated plants. Soil carbon content was also found maximum with the combined application of *Rhizobium* and PSB. These results can be due to the synergistic effect of *Rhizobium* and PSB on the microflora associated with carbon cycling in the rhizosphere (Raja and Takankhar 2017). Decomposition of organic compounds takes place by various organisms which help in the assimilation of carbon to plants. On the other hand dual inoculation of PSB + *Rhizobium* have a stimulatory effect on chlorophyll and protein content of plants which indirectly increase the carbon assimilation because chlorophyll content increases the photosynthetic activity of plants through which much carbon is utilized and accumulate as photosynthate in the form of sugar. The results have harmony with Tagore *et al*. (2013).

Chlorophyll content

Chlorophyll provides green color to the plant. It is the most abundant pigment among all biologically synthesized pigments. Chlorophyll plays the most important role as a food synthesizing molecule for photosynthetic plants because it is the main molecule which captures the solar energy and further reaction takes place in the conversion of solar energy to chemical energy. Co-inoculation of *Rhizobium* sp. with PSB showed non-significant differences for chlorophyll content as compared to *Rhizobium* sp. alone. Significantly higher chlorophyll content was observed in *Rhizobium* + PSB. The stimulatory effect on chlorophyll content may be due to increased availability of N and its uptake by a larger root surface areas associated with additional root hairs and lateral root development. Amir et al. (2001) reported the beneficial bio-enhancing effect of rhizobacteria through higher photosynthetic activity and more nutrients (P, K, Ca and Mg) uptake in oil palm seedlings. Sarig *et al*. (1990) reported Phosphate solubilizing bacteria as delaying leaf senescence thereby bio-enhancing photosynthesis. Such results were also observed by Mfilinge *et al*. (2014), Kaur *et al*. (2015).

Protein content (Leaf and Seed)

Protein is made up of the chain of amino acids. It is an important component of all cell and an essential macronutrient. These are highly evolved class of molecules. Protein is essential for the formation of hormones, enzymes, membrane channels and pumps. The protein content of leaves and seeds exhibit the positive impact of PSB + *Rhizobium* as well as their sole applications. Dual inoculation of PSB and *Rhizobium* depicts the higher production of protein followed by individual applications of *Rhizobium* and PSB respectively, and the least amount of protein was produced by untreated plants of lentil. It is well known that nitrogen and phosphorus are the major macronutrients which play a key role in various metabolic processes of plants including protein synthesis. Nitrogen metabolism involves the synthesis of amino acids directly while phosphate plays a major role in energy kinetics and provides energy for amino acid polymerization as well as other associated processes. So the role of these two macronutrients cannot be avoided. Phosphorus and Nitrogen are required by plants in large amount and their availability is always deficit into the soil. Only a few forms of these macronutrients can be up taken by plants, so PSB and *Rhizobium* play the important role of solubilization of these macronutrients. As the sole application of these biofertilizers increases the protein synthesis, their combined effect also increases the protein content of leguminous crops. Similar results were observed by Diep *et al*. (2016).

Table 2 Impact of combined inoculation of *Rhizobium* and Phosphate Solubilizing Bacteria on the yield and selected biochemical properties of *Lens culinaris*

Treatment	No. of pods / plant	No. of seeds / pod	Weight of 10 seeds / plant	Chlorophyll content (mg/g tissue)	Protein content in plant	Protein content in seed	Proline content in plant	Nitrate reductase	Leghaemoglobin concentration
Control	22.00	1.47	0.18	0.0045	0.18	0.43	0.06	7.29	0.15
Rhizobium	30.33	1.73	0.25	0.0056	0.23	0.50	0.03	9.16	2.04
PSB	27.33	1.67	0.22	0.0051	0.22	0.49	0.04	7.78	1.88
R+ PSB	107.00	1.87	0.30	0.0061	0.25	0.60	0.01	10.90	2.68

Leghaemoglobin content

Leghaemoglobin is a red color pigment found in the root nodules of leguminous plants. It is an oxygen-carrying protein, which helps to maintain the low oxygen tension necessary for the nitrogen fixation. All the treatments of microbial inoculants as given in the combination or their individual applications have a positive impact on the leghaemoglobin content of lentil. In the present study, co-inoculation of *Rhizobium* and PSB shows the more effective impact on the Leghaemoglobin content in the nodular tissues of lentil, followed by *Rhizobium* and PSB alone and the least leghemoglobin concentration was observed in the untreated plants. Leghaemoglobin is the most important protein synthesized due to the symbiosis of legume and their specific *Rhizobium* species. It works as a reservoir of oxygen and provides the conditions to the *Rhizobium* for the biological nitrogen fixation. *Rhizobium* has a direct positive impact on the leghaemoglobin content because it takes part in the synthesis of this protein by synthesizing haem (iron-containing) part of leghaemoglobin. On the other hand, phosphorus helps in the nodulation process of legumes and indirectly helps in the legume-*Rhizobium* symbiosis. Increased nodulation efficiency with the application of P solubilizers and P fertilizers was also noticed in gram and other legumes (Alagawadi and Gaur 1988, Tiwari *et al*. 1989, Nagaraju *et al*. 1995). Results have similarity with the findings of Tagore *et al*. (2013).

Proline content

Proline is a cyclic α-amino acid, which has an important function as an antioxidant and osmolyte (Siripornadulsil *et al*. 2002). Proline content is inversely proportional to the growth of the leguminous crops. As the growth attributes show the stimulatory effect of dual inoculation as well as individual applications of PSB and *Rhizobium*, proline content exhibit inhibitory effect of applications of these biofertilizers. The reason behind this is, proline is cyclic amino acids which produced in the plants during stress condition. It is one of the scavenging methods of plants to combat the stress conditions. So an increased amount of proline directly indicates the stress condition of plants. In the present investigation, the highest amount of proline was recorded in untreated plants and the minimum amount was observed with plants treated with dual inoculation of PSB and *Rhizobium*. So these results depict that the plants treated with dual inoculation have minimum stress condition and these biofertilizers can be used for the improvement of plant health and yield of leguminous crops. Results have harmony with Ziyaul et al. (2018), they observed the impact of arbuscular mycorrhyza and *Rhizobium* on *Vigna radiata* and found that proline content and observed that plants treated with the dual inoculation and sole application of *Rhizobium* have a minimum amount of proline.

Nitrate-reductase activity

Nitrate reductase is an important enzyme of Nitrogen metabolism (Solomonson and Barber 1990). Nitrate reductase catalyzes the first step of nitrogen fixation through the assimilation of the nitrate absorbed from the soil by the reduction of nitrate to nitrite (Sung and Sun 1990, Chamber-Perez *et al*. 1997, Caires and Rosolem 2000, Quaggio *et al*. 2004). Maximum Nitrate reductase content was found with dual inoculation of PSB and *Rhizobium* while minimum nitrate content was observed in control plants. As we know that phosphorus and nitrogen are available in the insoluble form in soil reservoir and microbes avail these macronutrients to the plants through various activities, so there is enough substrate available for these microorganisms to convert the insoluble to a soluble form. So, dual inoculation, as well as sole application, has a positive impact on the Nitrate reductase activity. However, there is little research available on dual inoculation effect on nitrate reductase activity, but their individual application impact was observed by many researchers. The stimulatory effect of PSB nitrate reductase activity of lentil was observed by Singh *et al*. (2015, 2018) as well as *Rhizobium* application also increases the nitrate reductase and nitrogenase activity (Karumey 2009, Crusciol *et al*. 2019). Utilization of phosphate solubilizing microorganisms is considered as an important bio-inoculant to convert soil insoluble phosphate to soluble phosphate in natural and agriculture ecosystem, which also helps to improve soil fertility, plant health and ultimately leads to better plant growth and crop yield.

Soil properties

Soil phosphorus content

Phosphorus is an essential macronutrient for plant nutrition. It is found in the soil in organic and mineral form. Nevertheless, the amount of phosphorus which is easily available for the plants is very low in comparison to the total amount of the phosphorus in the soil. This is the reason that most of the plants have a deficiency of the phosphorus. Dual application of PSB and *Rhizobium* increases the phosphorus content of plants as well as soil. The minimum amount of phosphorus was observed in control soils while phosphorus content increases gradually with the application of *Rhizobium*, PSB and *Rhizobium* + PSB in increasing order respectively. This may be due to fact that biofertilizers enhanced soil fertility by solubilizing unavailable sources of elemental nitrogen, bound phosphorus and non-exchangeable potassium into forms to facilitate the plant to absorb them (Goud and Kale 2011). Similar results were observed by Heisnam *et al*. (2017).

Soil nitrogen content

Plant require more nitrogen than any other nutrients but only a small proportion of nitrogen in the soil is available for plants. Generally, soil nitrogen is found in the organic form which cannot be taken up by the plants. Total nitrogen content of soil found higher in the soil inoculated with combined treatment of PSB + *Rhizobium* followed by the individual treatment of *Rhizobium* and PSB whereas the minimum nitrogen content was found in the untreated soil. Microorganisms play a major role in the nutrient cycling of soil reservoir. PSB and *Rhizobium* are well known for their role in Phosphate and Nitrogen solubilization, respectively. Pandey *et al*. (2015) also reported that combined inoculation of *Rhizobium*, PSB, and PGPR improved the nutrient status of soil through their synergistic effect on nitrogen fixation and solubilization of native soil phosphorus which increased availability and uptake of these nutrients by the crop plant (Tagore *et al*. (2013).

Soil carbon content

Soil carbon plays a vital role in the stability and fertility of the soil (Milne *et al*. 2015). Soil is an important sink of the carbon. Soil organic carbon is the largest carbon stock in the most terrestrial ecosystem (Lal 2008). The results of the present study show that the organic carbon content in soil was influenced significantly due to individual seed treatment with *Rhizobium* and PSB. Maximum carbon content was observed in dual inoculation treated soil followed by the sole application of *Rhizobium* and PSB, respectively. Least amount of carbon was recorded in untreated soil. The increase in carbon content might be due to seed treatment with *Rhizobium* and PSB increased the activity of microbes in the soil which can decompose the unavailable carbon and facilitate it to the plants. Gupta and Thomas (2003) reported a significant increase in organic carbon content with *Rhizobium* as compared with control. Iraj *et al*. (2009) reported a significant increase in organic carbon content with *Rhizobium* and PSB as compared with control. Similar results were observed with Tagore *et al*. (2013), Nissa *et al*. (2017), Raja and Takankhar (2017).

Physico-chemical properties of soil

Soil pH

Soil pH is the measure of the acidic and alkaline nature of the soil. The optimal pH ranges from 5.5-7.0 for most of the plants whereas some plants are adapted to grow in the pH value outside of this range. In the present investigation, there is no significant variation in the pH of soil. The soil pH ranged between 7.67-8.45 for lentil. The soil in which lentil crop was planted slightly alkaline. However soil pH decreased in the *Rhizobium* and PSB inoculated soil. Minimum soil pH which came into the range of optimum pH was observed with dual inoculated soil followed by *Rhizobium* treatments in both the crops. It can be stated from the above results that these microorganisms have the capability to maintain the pH at an optimum level. Results have harmony with Tagore *et al*. (2013), Jangir *et al*. (2017).

Table 3 Impact of combined inoculation of *Rhizobium* and Phosphate Solubilizing Bacteria on the Physico-chemical properties of Soil

Treatment	pH	CEC	Bulk density	Particle density	Porosity (%)
Control	8.45	10.34	0.753	3.4860	73.63
Rhizobium	7.97	11.00	0.823	3.3010	74.04
PSB	7.74	11.22	0.888	3.3680	73.86
R+ PSB	7.67	12.54	0.905	2.8810	75.07

Cation exchange capacity

Cation exchange capacity is the measure of the soil ability to hold positively charged ions. It affects the soil pH, soil structure, stability, nutrient availability and soil reaction to fertilizers and other ameliorant (Hazleton and Murphy 2007). Maximum cation exchange capacity was observed in *Rhizobium* + PSB treated soil. Contrarily minimum CEC was observed in untreated soil. CEC is directly proportional to the soil pH. As the pH increases, CEC values decrease and maximum CEC was recorded at optimum pH. CEC avail the nutrients of soil to the plants so it has a positive impact on soil fertility. As the CEC increase soil nutrient conditions improve. However, the effect of these biofertilizers is not significant. Similar results are also observed by Jangir *et al*. (2017).

Bulk density, particle density, soil porosity

Bulk density is the indicator of the soil compaction. It shows the capability of soil for structural support, aeration, solvent (water) and solute movement. Particle density is the density of the solid particles which forms the soil. Porosity refers to the number of pores a soil have. Soil pores determine the amount of water holds by the specific volume of soil. There is no significant difference between the different inoculation of PSB and *Rhizobium* on Bulk density, Particle density, and porosity. However, maximum bulk density and porosity was observed in PSB and *Rhizobium* combined inoculation and minimum values are recorded in untreated plants. Particle density has the opposite influence of these treatments because particle density is inversely proportional to the bulk density. So, minimum particle density was observed in the soil treated with dual inoculation of PSB + *Rhizobium* and the maximum particle density was observed in untreated plants.

The present study revealed that the combined inoculation of *Rhizobium* and PSB influence the growth and yield of lentil positively. Finding also confirmed that the dual inoculation approach increases the biomass, nutrient content (N, P, C) of lentil as compared to individual treatment of the *Rhizobium* or PSB. The physiological parameters of soil do not have much difference due to the microbial inoculation treatment.

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