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ORIGINAL ARTICLE

Root length, Nodulation and Biological Nitrogen fixation of *Rhizobium* inoculated soybean (*Glycine max* [L.] Merr.) grown under maize (*Zea mays* L.) intercropping systems and P and K fertilization

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ABSTRACT
A two years field trial was carried out to investigate the effects of Rhizobium inoculation supplemented with P and K on root length, nodulation and N_2-fixation in soybean intercropped with maize. The split-split plot design with 2 x 4 x 7 factorial arrangement replicated thrice was used. The main plots had two rhizobial inoculation treatments, while the sub plots comprised of four cropping systems namely Maize (sole crop), Soybean (sole crop) and two intercropping at different spacing. The sub-subplots were assigned to fertilizer levels (kg ha^{-1}): control; 20K; 40K; 26P; 52P; 26P + 20K; 52P + 40K. Dried plant sample were ground for determination of N_2-fixation. N_2-fixation was estimated using total nitrogen difference method where the total nitrogen obtained from none-fixing plants (Maize) was subtracted from total nitrogen obtained from fixing plants (Soybean). The results revealed that cropping systems, Rhizobium inoculation and P and K fertilizers have differently affected the root length, number of nodules and/or nitrogen fixation in soybean. Intercropping increased the number of nodules relative to sole soybean. Inoculated soybean significantly increased root length, number of nodules and nitrogen fixation over un-inoculated. Root lengths were increased by 7.5% and 7.3% in 2015 and 2016 respectively. P and K fertilization also increased the number of nodules and nitrogen fixation over the control. There was also a significant interaction of Rhizobium inoculation and fertilizers on number of nodules and nitrogen fixation in 2015 cropping season. The use of combined fertilizers at lower rates (20K+26P) was generally seen to be better.
Keywords: *Symbiotic relationship, Biological nitrogen fixation, Plant hormones, mixed cropping, Phosphorus, Potassium.*

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INTRODUCTION

All plants need relatively large amounts of nitrogen (N) for proper growth and development [1] Nitrogen element is critical because it is the major component of essential biomolecules such as chlorophyll, an important pigment for photosynthesis; amino acids, which are the key building blocks of proteins and other biomolecules such as ATP and nucleic acids [2]. Nitrogen is added in the soil through addition of industrial nitrogenous fertilizers [3], the decomposition of soil organic matter and redistribution of organic materials, natural processes of converting atmospheric N_2 through lighting and biological nitrogen fixation (BNF).

Several researchers have stated the principal sources of N for crop production are biological N_2 fixation, organic resources recycled within the cropping field or concentrated from a larger area, and mineral N fertilizers [3, 4]. Of these sources of N, mineral fertilizers have raised a global environmental concern resulting from the large amounts of N entering the global food production system [3, 5]. Studies have also

shown that excess N has negative effects on water, air, and ecosystem and human health [3, 6, 7]. Apart from environmental effects of mineral N fertilizers, the cost input is high to afford by small holder farmers and it increases the costs of production [8, 9]. To minimize the harmful effects of excessive N form mineral fertilizers and to reduce the costs of production, researchers and farming communities have struggled to maintain soil fertility levels relying mostly on biological N fixation (BNF) [3].
Biological nitrogen fixation (BNF) is the term used for a process whereby atmospheric nitrogen (N=N) is reduced to ammonia in the presence of nitrogenise [10, 11]. Nitrogenase is a biological catalyst found naturally only in certain microorganisms such as the symbiotic *Rhizobium* and *Frankia*, or the free-living *Azospirillum* and *Azotobacter* [3, 12]. The process of BNF is only possible in a select group of plants, with the help of soil microorganisms.
Symbiotic relationship between plants and microbes has been studied and a group of soil dwelling bacteria have long been used to improve the availability of nitrogen through atmospheric nitrogen fixation. These microorganisms stimulate plant growth by a plethora of mechanisms, hence are called plant growth promoting rhizobacteria (PGPR) [13]. Recently, biofertilizers have emerged as a fundamental component for biological nitrogen fixation which provides an ecologically sound and economically attractive way of improving nutrient supply in the soil [14]. Important hosts for these microorganisms to perform biological nitrogen fixation are legumes such as Soybean, Common bean, Lablab, Groundnut, Cowpea, Pigeon pea, Mung bean, Faba bean, Chickpea, Alfalfa, etc. Although BNF has long been a component of many farming systems throughout the world, its importance as a primary source of N for agriculture has diminished as there is increasing use of fertilizer-N for the production of food and cash crops [15].
In this study, we are focused on Soybean (*Glycine max*) as a host plant of nitrogen fixing bacteria. Soybean is a nutritious grain legume grown in diverse parts of the world. It is of economic importance and a nutritious crop which provides human with high proteins [16]. The crop was introduced in Tanzania for the first time at Amani, Tanga by the German traders and has been grown since 1907 [17]. It contains 20% non-cholesterol oil and 45% protein compared to 20 and 13% protein content in meat and egg, respectively [18]. Areas with the greatest potential for soyabean production in Tanzania include Ruvuma, Mbeya, Rukwa, Morogoro and Iringa, all in south-western Tanzania [19] and in northern Tanzania [8]. Apart from its high protein content soybean has a high nitrogen fixing ability [20] for its requirements and contribute to soil N thereby improve soil quality and fertility. None fixing crops growing nearby these legumes or grown in the subsequent season can benefit from nitrogen released out of the fixing plants to the rhizosphere [21].
For effective nitrogen fixation by *Rhizobium* bacteria, there must be favourable conditions similar to those necessary for growth of the host plant. Among of the conditions necessary for plant growth include availability of macro nutrient such as N, P and K. Phosphorus has been reported to influence symbiotic N_2-fixation in leguminous plants by many researchers [8, 22, 23]. Severe deficiency of this element in the soil can significantly impair growth of host plant and symbiotic N_2 fixation [24]. Israel, [24] further pointed out that N_2-fixation has higher phosphorus requirements for optimal functioning than the host plant requires for its growth and nitrate assimilation. Another important element in the process of dinitrogen fixation is Potassium [25]. The process of photosynthesis requires potassium which is essential in maintenance and balance of the electrical charges at ATP production site [26, 27]. Translocation of photosynthetic substances (carbohydrate) to storage organs (fruits or roots) is also mediated under the help of potassium [1, 26]. Under the storage organs such as root nodules, carbohydrate produced by host plant is used by nitrogen fixing bacteria as source energy to fix atmospheric nitrogen [25].
However, currently there is limited information regarding the combined effects of P and K and rhizobia inoculation on root length, nodulation and nitrogen fixation in soybean intercropped with maize. Therefore, the current study aimed to determine the effects of rhizobia inoculation supplemented with P and K on root length, nodulation and nitrogen fixation in soybean intercropped with maize.

MATERIAL AND METHODS

Experimental Design and Treatments

The field experiment was carried out at Tanzania Coffee Research Institute (TaCRI) for two consecutive years (2015 and 2016 cropping seasons). The experiment was laid out in split-split plot design with 2 x 4 x 7 factorial arrangement replicated thrice. The plot size was 3 x 3 m. The main plots had two Rhizobia inoculation treatments, while the sub plots comprised: Maize (sole crop) at a spacing of 75 x 60 cm; Soybean (sole crop) at a spacing of 75 x 40 cm; Maize/soybean (intercropping system) at a spacing of 75 x 60 cm and 75 x 20 cm, Maize and soybean respectively; and the last cropping system was Maize/soybean (intercropping system) at a spacing of 75 x 60 cm and 75 x 40 cm, Maize and soybean respectively. The sub-subplots were assigned the following fertilizer levels (kg ha^{-1}): control; 20 K; 40 K; 26 P; 52 P; 26 P + 20 K; 52 P + 40 K.

Data collection

Plant harvest and sample preparation

At 50% flowering, soybean crop was sampled for nitrogen fixation analysis. Plants were excavated carefully from the soil with their entire root system, washed, nodules were counted and recorded, and root length were also measured and recorded. The above ground part (shoots) of the plants were oven-dried at 70 °C for 48 hrs, weighed and ground into a fine powder for determination of nitrogen fixation.

Estimation of N fixation

Nitrogen fixation was estimated using Total Nitrogen Difference (TND) method where the total nitrogen obtained from none-fixing plants (Maize) was subtracted from total nitrogen obtained from fixing plants (Soybean) [28].

Thus

$$N_2 \text{ fixed} = \text{Total N in legume} - \text{Total N in reference crop}$$

$$\text{Total } N_2 \text{ fixed in plants (kg ha}^{-1}) = \frac{(\text{Dry matter weight (kg ha}^{-1}) \text{ X \% N in plants})}{100}$$

Statistical analysis

The collected data was analysed using statistical software called STATISTICA. The statistical analysis was performed using analysis of variance (ANOVA) in factorial arrangement. The fisher's least significance difference (L.S.D.) was used to compare treatment means at $p = 0.05$ level of significance [29]

RESULTS

Root length

The current study showed that cropping systems had no significant effects on the root length of soybean in both cropping seasons. However, this study showed that the roots of soybean were influenced by *Rhizobium* inoculation in all (2015 and 2016) cropping seasons. *Rhizobium* inoculated soybean resulted in significantly longer roots compared with the un-inoculated soybean. In 2015 cropping season, *Rhizobium* inoculation increased root length by 7.5% and in 2016 root length was increased by 7.3%. In this study, P and K fertilization did not show any significant ($p=0.5$) effects on the soybean root length for all two years.

Number of Nodules

The results of this study showed that cropping systems significantly affected the number of nodules in the two cropping seasons (Table 1). Intercropped soybean produced high number of nodules than the pure stand soybean in both cropping seasons. The percentage increase on the number of nodules in intercropped soybeans over sole soybean was 20.41% and 27.36% for 2015 and 2016 cropping seasons respectively. On the other hand, *Rhizobium* inoculation also significantly increased number of nodules over un-inoculated soybean. The percentage increase on the number of nodules in inoculated plots was 95.97% and 78.17% relative to un-inoculated plots in 2015 and 2016 cropping seasons respectively. Likewise, P and K fertilization significantly increased the number of nodules over the control in both cropping seasons. The highest mean number of nodules (13.25 and 20.46) was recorded in 20 K+26 P (kg ha^{-1}) for 2015 and 2016 cropping seasons respectively, while the lowest mean number of nodules (6.65 and 12.11) of was recorded control plots for both 2015 and 2016 respectively (Table 1).

Interactive effects of *Rhizobium* inoculation and P and K fertilization on number of nodule in 2015 cropping seasons

There were significant interactions between *Rhozobium* inoculation and fertilizers in the cropping year 2015. In this study, *Rhizobium* inoculation interacted well with fertilizers leading to increased number of nodule. Un-inoculated plots produced nodules that were below five, and the fertilizer level of 20 K and 40K+52P (kg ha^{-1}) produced higher number of nodules compared with other fertilizer levels. *Rhizobium* inoculation significantly increased the number of nodules regardless of whether it was fertilized or not. However, the plots treated with 26 P resulted in high number of nodules over all other treatments followed by 40K+52P (kg ha^{-1}) (Fig. 1)

Table 1. Effects of cropping systems, Rhizobium inoculation supplemented with P and K on soybean root length and number of nodules in 2015 and 2016

Treatments	Root Length		Number of nodules	
	Season one (2015)	Season two (2016)	Season one (2015)	Season two (2016)
Cropping System				
SB	19.64±0.37a	20.30±0.43a	8.58±1.60b	14.60±2.10b
M+B(A)	18.60±0.37a	20.50±0.74a	10.44±1.79a	16.45±2.65ab
M+B(B)	18.81±0.44a	20.52±0.53a	10.78±1.77a	20.10±2.46a
Rhizobia				
With	19.71±0.32a	21.16±0.53a	19.09±1.03a	27.99±1.66a
With out	18.33±0.31b	19.72±0.38b	0.77±0.43b	6.11±1.13b
Fertilizers				
Control	19.36±0.46a	20.02±0.79a	6.65±2.11d	12.11±2.50b
20K	18.30±0.45a	21.41±0.63a	7.79±1.97cd	14.78±3.33ab
40K	18.40±0.67a	19.59±0.35a	9.41±2.82bcd	16.11±4.01ab
26P	19.95±0.58a	22.02±1.01a	9.68±2.43abcd	18.37±4.55ab
52P	19.74±0.58a	19.44±1.46a	11.20±2.49abc	18.57±3.64ab
20K+26P	18.54±0.66a	20.56±0.82a	13.25±3.41a	20.46±3.94a
40K+52P	18.84±0.79a	20.06±0.65a	11.52±2.93ab	18.94±3.85ab
3-Way ANOVA F-statistics				
CroSyt	2.162ns	0.046ns	1.9149*	2.45*
Rhiz	10.154**	4.884*	345.4335***	111.90***
Fert	1.355ns	1.221ns	3.0209**	1.11*
CroSyt*Rhiz	0.989ns	1.037ns	0.9663ns	0.84ns
CroSyt*Fert	1.022ns	1.161ns	1.3582ns	0.26ns
Rhiz*Fert	1.688ns	0.601ns	2.6283*	1.46ns
CroSyt*Rhiz*Fert	0.980ns	1.188ns	1.5647ns	0.65ns

CroSyt: Cropping Systems; Fert: Fertilizers; Rhiz: Rhizobium; SB: Sole soybean; M+B (A): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 20 cm, maize and soybean respectively; M+B (B): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 40 cm, maize and soybean respectively; Values presented are means ± SE; *,**, ***: significant at p ≤ 0.05, p ≤ 0.01, p ≤ 0.001 respectively, ns = not significant, SE = standard error. Means followed by dissimilar letter(s) in a column are significantly different from each other at p=0.05 according to Fischer least significance difference (LSD).

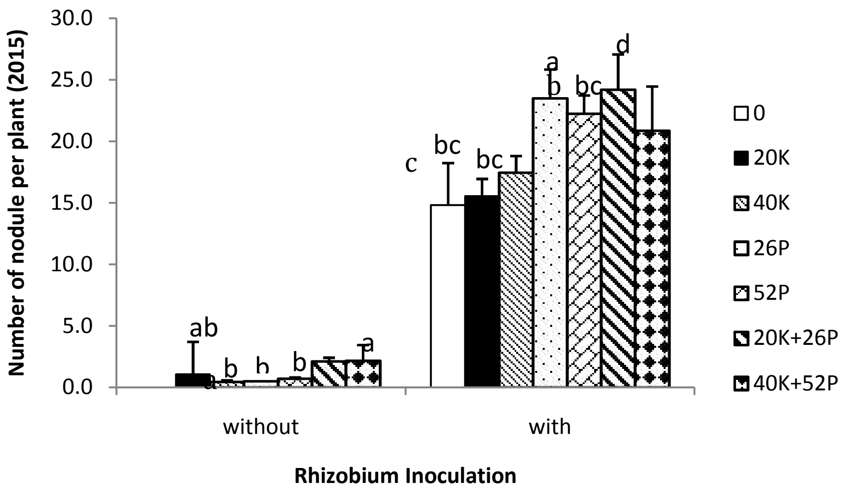

Figure 1. Interactive effects of rhizobia inoculation and P and K fertilization on number of nodule in 2015

Nitrogen fixation

The results of the current study indicated that cropping systems had no significant effects on nitrogen fixation for the two cropping seasons. However, numerically there was slight increase in nitrogen fixation under intercropping relative to the sole cropped soybean (Table 2). Rhizobium inoculation showed a highly significant effect on nitrogen fixation over un-inoculated treatments with an increase of 63 and 55.16 (kg ha^{-1}) in 2015 and 2016 respectively. In both cropping seasons, P and K fertilization significantly improved nitrogen fixation over the control. The highest value of fixed nitrogen was found in the plots fertilized with 52 P (kg h^{-1}) while the lowest values were recorded in the control plots for the two cropping seasons (Table 2).

Table 2: Effects of cropping systems, Rhizobium inoculation supplemented with P and K on nitrogen fixation in 2015 and 2016

	N Fixed (kg/ha)	
Level of Factor	Season one (2015)	Season two (2016)
Cropping Systems		
SB	81.92±8.27a	127.25±9.53a
M+B(A)	84.96±6.90a	129.32±9.33a
M+B(B)	88.40±10.48a	130.63±8.18a
Rhizobium inoculation		
With	116.97±7.51a	156.66±7.29a
With out	53.21±3.25b	101.47±5.50b
Fertilization		
Control	57.85±8.51c	79.51±9.29c
20K	78.32±9.38bc	118.51±7.95b
40K	77.83±8.10bc	117.05±9.05b
26P	89.53±14.24ab	146.70±16.36ab
52P	108.10±22.05a	158.23±15.04a
20K+26P	88.67±7.45ab	142.68±14.70ab
40K+52P	95.35±14.64ab	140.76±13.77ab
3-way ANOVA (F-statistics)		
CropSystem	0.25ns	0.06ns
Rhizobia	71.78***	46.11***
Fertilizer	2.54***	6.05***
CropSystem*Rhizobia	0.63ns	1.32ns
CropSystem*Fertilizer	1.08ns	0.90ns
Rhizobia*Fertilizer	2.98**	1.46ns
CropSystem*Rhizobia*Fertilizer	1.23ns	1.16ns

CropSystem: Cropping Systems; SB: Sole soybean; M+B (A): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 20 cm, maize and soybean respectively; M+B (B): Maize/soybean intercropped at a spacing of 75 x 60 cm and 75 x 40 cm, maize and soybean respectively; Values presented are means ± SE; **, ***: significant at p ≤ 0.01, p ≤ 0.001 respectively, ns = not significant, SE = standard error. Means followed by dissimilar letter(s) in a column are significantly different from each other at p=0.05 according to Fischer least significance difference (LSD).

Interactive effects of *Rhizobium* inoculation and P and K fertilization on nitrogen fixation in 2015 cropping season

The results of this study also showed a significant interactions between *Rhizobium* inoculation and P and K fertilizers for the first (2015) cropping season on nitrogen fixation. The combination of fertilizers and *Rhizobium* showed a good performance in nitrogen fixation where the best combination was observed in plots which received 52 kg of phosphorus and 20 K + 26 P (kg ha^{-1}). Inoculation alone without fertilizers resulted in lower nitrogen fixation compared with the *Rhizobium* plus fertilizers (Fig. 2). Compared with *Rhizobium* inoculated, un-inoculated plots recorded significantly lower amount of fixed nitrogen with fertilizer application. Under un-inoculated plots, the combined lower rates of fertilizers (20 K + 26 P (kg/ha)) improved the nitrogen fixation over all other treatments.

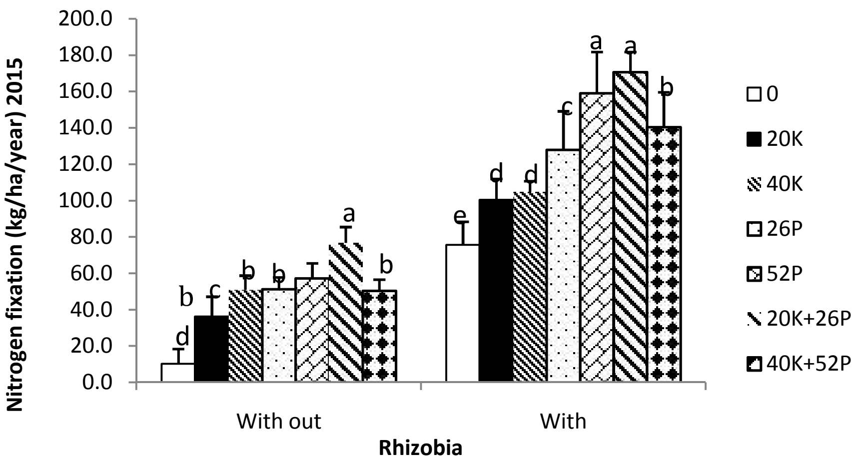

Figure 2. Interactive effects of rhizobia inoculation and P and K fertilization on nitrogen fixation in 2015

DISCUSSION

The current study showed that cropping systems, *Rhizobium* inoculation and fertilizers improved the root length, number of nodules and nitrogen fixation. In this study, cropping systems and fertilizers did not show significant effects on root length for the two cropping season. However, roots length was significantly improved with *Rhizobium* inoculation. The improved root length in *Rhizobium* inoculated plots could have been caused by nitrogen fixation, which eventually resulted into available nitrogen for plant growth. Furthermore, the increased root length in the *Rhizobium* inoculated plots could have been attributed by Plant Growth promoting Rhizobacteria (PGPR) which functions through production of plant hormones such as auxins, and cytokinins [30-32].

The number of nodules was significantly increased with Cropping systems, *Rhizobium* inoculation and fertilization with P and K. The intercropped plots produced more nodules compared with sole grown soybean. The increased number of nodules in intercropped soybean could have been attributed by and enhancement with flavonoids found in root exudates of both soybean and maize. The similar findings were obtained by Liu et al. [33] who conducted the study on "Intercropping influences component and content change of flavonoids in root exudates and nodulation of Faba bean". In their study, they found the increased number of nodules and nodules dry weight in Faba bean intercropped with wheat compared with those found in monocropping and attributed it to the enhancement with flavonol, isoflavone, chalcone and hesperetin from their root exudates. The increased number of nodules in intercropped legumes over the intercropped one is in agreement with the previous related studies [34, 35]. The concept behind these finding is that root exudates contains flavonoids which are signal molecules acting as nod gene inducers for the nodules forming symbiotic *Rhizobium*, hence increased number of nodules over the pure stand legumes [33]. Apart from intercropping, P and K fertilization also increased number of nodules over the control. This study revealed unfertilized plots (control plots) significantly lowered the number of nodules as compared with any level of P and K whether singly or applied in combination. The combine application was superior in enhancing nodule formation relative to single application. These results showed that P and K are important elements for nodules formation, which finally enhance nitrogen fixation in soybean. Other studies have similarly reported the increase in nodule number under P sufficient treatments relative to P deficient treatments [36-40]. Potassium was reported to contribute to good root growth and has been shown to improve the number and size of nodules on roots [26, 32, 40-42] In another study on the influence of potassium supply on nodulation, nitrogenase activity and nitrogen accumulation of soybean (*Glycine max* L. Merrill) grown in nutrient solution it was found that nodule parameters (nodule number and fresh weight of nodule per plant, average weight of nodule) increased with increasing K-supply [43].

Following the effects of nodulation in soybean, the study also determined the effects of cropping systems, *Rhizobium* inoculation and P and K fertilization on nitrogen fixation through N difference method. Except for the cropping systems, *Rhizobium* inoculation and P and K fertilization increased nitrogen fixation for the two cropping seasons. Although the nodulation was increased in intercropped soybean this increase was not reflected in nitrogen fixation. However, there was a numerical increase of nitrogen in intercropped soybean compared with sole soybean which correlates with the number of nodules in intercropping system. The increased nitrogen fixation in *Rhizobium* inoculated plots is an indication of effective legume-microbes symbiosis in which legumes confer sources of carbon to the bacteria and in turn the bacteria fix atmospheric nitrogen for the host plant. Similar to our findings, several researchers have reported an increase in nitrogen fixation following *Rhizobium* inoculation in legumes [15, 44-46]. As previously reported in this study, the increase in number of nodules in P and K fertilized plots, was positively reflected in nitrogen fixation. Phosphorus and potassium significantly enhanced biological nitrogen fixation in this study relative to un-fertilized treatments. A number of studies [8, 22-24] have reported the increased nitrogen fixation in different legumes following P fertilization and that P deficient reduced nitrogen fixation. Potassium also has been similarly reported to increase nitrogen fixation in different legumes [41, 42]. These results suggest the importance of these mineral elements in enhancing nitrogen fixation, eventually growth and development of crops.

CONCLUSION

This study revealed that cropping systems, *Rhizobium* inoculation and P and K fertilizers have differently affected the root length, number of nodules and/or nitrogen fixation in soybean. In this study, intercropping significantly increased the number of nodules relative to sole soybean. The inoculation of soybean with *Bradyrhizobium japonicum* significantly increased the soybean root length, number of nodules per plant and nitrogen fixation over un-inoculated soybean. P and K fertilization significantly increased the number of nodules per plant and nitrogen fixation over the control. The best combination of fertilizers which increased the number of nodules in this study was 20 K + 26 P (kg/ha). For the nitrogen fixation, supplying 52 kg of P resulted in higher values compared with other treatments. The amount of nitrogen fixed in plots supplied with 52 kg of P was statistically similar to 26P, 20K+26P and 40K+52P (kg ha^{-1}). There was also a significant interaction of *Rhizobium* inoculation and fertilizers on number of nodules and nitrogen fixation in 2015 cropping seasons. Since all elements are important in nodulation and nitrogen fixation, we can conclude by recommending the use of combined fertilizers at lower rates (20K+26P) in areas with similar characteristics. Doubling of the combined fertilizers may not significantly increase nodulation and nitrogen fixation but rather a cost burden to a farmer.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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