UNDER PEER REVIEW

EFFECTS OF PHOSPHORUS AND *BRADYRHIZOBIUM JAPONICUM* ON GROWTH AND CHLOROPHYLL CONTENT OF COWPEA (*VIGNA UNGUICULATA* (L) WALP).

ABSTRACT:

Aims: To assess the effects of *Rhizobium* and phosphorus on growth and total leaf chlorophyll content of cowpea.
Study design: The experiment was laid down in split-plot design whereby the main plots comprised of two inoculation treatments (with and without *Rhizobium*), sub-plots included phosphorus(TSP)application at four different levels (0KgP.ha^{-1}, 20KgP.ha^{-1}, 40KgP.ha^{-1}, 80KgP.ha^{-1}). Both experiments were replicated four times.
Place and Duration of Study: The field and screen house experiment was conducted at Tanzania Coffee Research Institute and Seliani Agricultural Research Institute respectively in Tanzania, between March-July 2013.
Methodology: We measured plant growth parameters such as plant height; number of leaves per plant at different stages of plant growth, the stem girth was measured by vernier caliper at physiological maturity. The chlorophyll content was determined at 3, 5, and 7 weeks after planting (WAP). The chlorophyll was extracted by using dimethylsulphoxide (DMSO) and absorbance values were determined at 645 and 663nm, using UV/Visible spectrophotometer.
Results: *Rhizobium* inoculation significantly increased the plant height, number of leaves per plants, and stem girth relative to control. The height was increased by 11.23, 10.43 and 8.99% (screen-house) and by 8.11, 24.05, 9.29% (field) in the measurements taken at 4, 6, and8 WAP respectively. Number of leaves per plant counted at 6 and 8WAP, increased by 14 and 10.8% (screen-house) and 14 and 11.6% (field) respectively. *Rhizobium* also significantly increased chlorophyll content of cowpea by 26% (3 WAP) in screen-house and increased by 52, 37.9 and 13.3% (3, 5 and 7 WAP)in the field. Phosphorus also significantly increased different plant growth parameters and leaf chlorophyll content relative to control.
Conclusion: *Rhizobium* inoculation can function the same as inorganic N fertilizers. Therefore, their combined use with phosphorus boosts plant growth and chlorophyll content of the legumes and eventually legume production is increased.

***Keywords*:** Legumes, Nitrogen, Photosynthesis, Plant Height, *Rhizobium*, Stem Girth

1. INTRODUCTION

Low level of soil P and N are the major constraints to crop growth and production in nutrients depleted sandy soil of Sub-Saharan Africa [1,2]. Phosphorus and Nitrogen are essential elements playingmajor role in plant growth, development and finally they determine the final yield of the crops. Phosphorus (P) is found in every living plant cell and is involved in several

UNDER PEER REVIEW

key plant functions, including energy transfer, photosynthesis, transformation of sugars and starches, nutrient movement within the plant [3,4] nucleic acids, phospholipids, ATP, and it makes about 0.2% of a plant's dry weight [5]. It stimulates root development andgrowth of young plants, giving them a good and vigorous start [6,7] andcontrol key enzyme reactions in the regulation of metabolic pathways [8]. Successful production systems based on legumes therefore requires P inputs [9] either from soil reserves or from added fertilizer [10]. Other workers [11,12,10,4,13] reported that plant height, leaf area,shoot, grain yields, and root dry weights of the legume plants increased with P application. Supplementing legumes with P nutrients enhances symbiotic establishment for increased N_2 fixation [10,14]. Plant cell functions such as photosynthesis, respiration, biosynthesis and the transfer of organic solutes across membranes, requires inorganic phosphate for its formation from ADP. Phosphorus have been reported to increase chlorophyll content and it plays an important role in both energy storage and transfer as ADP and ATP (adenosine di- and tri-phosphate) and DPN and TPN (di- and tri- phosphopyridine nucleotide) and a shortage of inorganic phosphate in the chloroplast reduces photosynthesis [15].

Rhizobium inoculation in legumes is an alternative to the expensive inorganic nitrogen fertilizers and is accredited for stimulating plant growth [16]. *Rhizobium* provides N through symbiotic fixation of atmospheric nitrogen [17,18]. Nitrogen isabuilding blocks of proteins and the amino acids [19] required for cellular synthesis of enzymes, chlorophyll, DNA and RNA, and is therefore important in plant growth [18,15]. The element is involved in many processes of plant growth and development which finally lead to yield and the quality of harvested organs (seeds or shoot biomass) [20]. Several research reports have indicated significant achievements in legume growth and yield in many parts of the world due to inoculation with the appropriate *Rhizobium* inoculants [21,22,23,24,25,26,11,27,28,29,30,12,10,16,17]. Inoculation of legumes with appropriate *Rhizobium* has been reported to increase the leaf Chlorophyll content of legume crops [16]. The increased chlorophyll content results in the increased photosynthesis and as a result plant gets enough carbohydrate for its growth and production.However, there is little information on the effects of phosphorus and *Rhizobium* on growth and chlorophyll content of cowpea in northern Tanzania. Therefore, it is important to study and establish the rolethat could be played by phosphorus and *Rhizobium* on growth and total leaf chlorophyll content of legumes grown under glasshouse and field conditions.

2. MATERIAL AND METHODS

2.1. Description of Location

Field and pot experiments were conducted at two different locations from mid March to late July 2013. A screen house experiments was conducted at Seliani Agricultural Research Institute (SARI) which is situated at an altitude of (1390) meter above sea level in Arusha, Tanzania at a latitude of (3°21'50.08"S) and longitude of (36°38'06.29"E). The field experiment was conducted at the Tanzania Coffee Research Institute which is situated at an altitude of (1268) meter above sea level in Kilimanjaro region, Tanzania at a latitude of (3°14'44'')S and longitude of (37°14'48)E. The area where field experiment was conducted receives a mean annual rainfall of (1200) mm, bimodal rainfall pattern, where short rains begin late October through December and long rains commences in early April and ends in late June.

UNDER PEER REVIEW

2.2. Experimental design

The experiment was laid down in split plot design. The main plots comprised of two inoculation treatments viz i) with inoculation of *Rhizobium* and ii) without inoculation of *Rhizobium.* Sub plots included application of Phosphorus at four different levels ie. 0, 20, 40, and 80 KgP.ha^{-1}.The *Rhizobium* inoculants were purchased from MEA Fertilizer Company in Nairobi - Kenya.

2.3 Field and Screen house experiment:

The soil for screen house experiment was collected from the site where field experiment was conducted. The soil was packed into 4 Kg Pots where four seeds were germinated in each pot, and later thinned to two after germination and uniform established. The field was ploughed and harrowed by using tractor before planting. The crop was seeded at a spacing of 50cm by 20cm, where the plot size was 4m by 3m. In the field trial, three seeds were seeded per hill and then thinned to two plants. The plots were weeded twice where the first weeding was done two weeks after emergence and the second weeding was done just before flowering. Each plot comprised of six rows. Data were collected from the four middle rows. The crop plant used for this experiment was Cowpea (*Vigna unguiculata* (L) Walp) supplied by the breeder from Sokoine University of Agriculture, Morogoro, Tanzania. Both screen house and field experiment was planted at the mid of March 2013, and closely monitored from this point until physiological maturity for field, and pod formation for screen house experiment. Both experiments were replicated four times.

2.4 Growth data collection.

The growth parameters measured from 14 days after planting (DAP)to pod formation in both screen house and field experiment were: plant height measured using meter ruler from ground surface to the tip, number of leaves per plant, stem girth measured by using a vernier caliper and leaf area measured according to [31] shown below,

LA= 11.98 + 0.06LW. Where; L= leaf length and W= leaf width.

Leaf length (L) (cm) and leaf width (W) (cm) was measured by direct leaflet area measurements using a meter rule. Length was measured as the distance between the base and the apex of the leaflet; width was measured perpendicular to the length axis at the position on the leaflet yielding the greatest width. Ten randomly selected representative plants from the four central rows in each plot for field experiment were used while for screen house experiment, one plant from each pot was used to collect data. The number of days to 50% flowering and number of days to 50% pod formation was also counted as part of the growth data.

2.5 Chlorophyll content determination.

Extraction of chlorophyll concentrations by dimethylsulphoxide (DMSO) was done as described in [32]. A third of the plant leaves, from the tip was collected from each pot for screen house and five samples of leaves were taken from five randomly selected plants for field experiment. A hundred (100) mg of the middle portion of fresh leaf slices was placed in a 15 mL vial containing 7 mL DMSO and incubated at 4°C for 72 h. After the incubation, the extract was diluted to 10 mL with DMSO. The DMSO technique extracts chlorophyll from shoot tissue without grinding or maceration [32]. A 2 mL sample of chlorophyll extract was then transferred into curvets for absorbance determination. A spectrophotometer (UV/Visible Spectrophotometer) was used to determine absorbance values at 645 and 663 nm, which was then used in the equation proposed by [33] to determine total leaf chlorophyll contents against DMSO blank, expressed as mg L^{-1} as shown below:

UNDER PEER REVIEW

Chlorophyll total (Chlt = $20.2D_{645} + 8.02D_{663}$).

Where "D" is the density values at the respective wavelength which was obtained on the Beckman spectrophotometer.

2.6 Statistical Analysis

The statistical analysis was performed using the 2-way analysis of variance (ANOVA) in factorial arrangement, with the computations being performed with the software program STATISTICA. The fisher least significance difference (L.S.D.) was used to compare treatment means at P=0.05 level of significance [34].

3. RESULTS

3.1 Effects of *Rhizobium* and phosphorus on the height of the cowpea.

The results in table 1 clearly demonstrate that *Rhizobium* inoculation had significant effect on the plant height assessed in this study. *Rhizobium* inoculation significantly improved the plant height measured at four, six and eight weeks after planting (WAP) in both screen house and field experiments relative to control treatment (Table 1). For example in this study, *Rhizobium* inoculation increased the plant height by 11.23, 10.43 and 8.99% in the measurements taken at four, six and eight weeks after planting (WAP) respectively in the screen house experiment where for the field experiment the *Rhizobium* inoculation increased the plant height by 8.11, 24.05, 9.29% in the measurements taken at four, six and eight weeks after planting respectively, compared with un inoculated control.

From this study, phosphorus application significantly increased the plant height measured at four, six and eight weeks after planting in both screen house and field experiment as compared with control (Table 1). In this study, phosphorus application in the screen house at the rate of 20, 40and 80KgP.ha^{-1} significantly increased the plant height by 19, 24 and 30% (4WAP) respectively over the control. The measurements taken in the screen house at 6 and 8WAP shows that the application of phosphorus at the rate of 20, 40and 80KgP.ha^{-1} significantly increased the plant height by 15.18, 16.8, 21.7% (6WAP) and 18, 23, 21.9% (8WAP) respectively over control treatment (Table 1). Table 1also indicated that the plant height measured at 4, 6 and 8WAP in the field experiment had positive response to phosphorus application at all levels. For example application of phosphorus at the rate of 20, 40and 80KgP.Ha^{-1} significantly increased plant height by 12.3, 11.8, 15.8% (4WAP); 9.8, 18.4, 27.2% (6WAP); and 14.9, 21.1, 28.8% (8WAP) respectively over the control.

3.2 Interactive effect of *Rhizobium* and phosphorus on plant height of cowpea.

This study shows significant ($P \leq .01$) interactive effect between *Bradyrhizobium japonicum* and phosphorus on plant height of the cowpeas measured at six weeks after planting in the field experiment. Plant height was increasing with the increase in phosphorus where the greatest height was observed at the rate of 80 KgP.ha^{-1} and the smallest height was seen in the control treatment. *Rhizobium* supplied with P at highest level gave significantly higher values of height compared with other treatments (Figure 1- A).

3.3 Effects of *Rhizobium* inoculation and phosphorus on number of cowpea leaves per plant

In this study, *Rhizobium* inoculation significantly increased the number of leaves of cowpeas measured at 6 and 8WAP in both the screen house and the field experiment (Table 2) as compared with un-inoculated control treatments. *Rhizobium* did not show significant different in the number of leaves measured from 2 and 4 WAP. The results in Table 2 shows that in the screen house experiment, supplying P at 40 and 80 KgP.ha^{-1} resulted into significant number of leaves measured at 6 and 8 WAP compared with other treatments. In field

UNDER PEER REVIEW

experiment, measurements taken at 6 WAP showed that P supplementation at 20, 40 and 80 KgP.ha^{-1} resulted into significantly higher number of leaves per plant as compared with control treatment. However, at 8 WAP, supplying P at 40 KgP.Ha^{-1} and 80 KgP.Ha^{-1} resulted into significantly higher number of leaves relative to other treatments.

3.4 Interactive effect of *Rhizobium* and phosphorus on number of leaves per plant.

There was significant (*P*= .05) interactive effect between *Bradyrhizobium japonicum* and phosphorus on number of leaves per plant of the cowpeas measured at six weeks after planting in the field experiment (Figure 1-B). *Rhizobium* inoculation supplied with P at any level significantly gave the higher number of leaves per plant compared withthe control.

3.5 Effects of *Rhizobium* inoculation and phosphorus application on the stem girth, leaf area, number of days to 50% flowering and number of days to 50% pod formation.

Table 3 clearly indicates that *Rhizobium* inoculation significantly affected almost all growth parameters measured in this study as compared with un-inoculated control. For example plant growth parameter measured and presented in table 3 were stem girth, leaf area, number of days to 50% flowering, and number of days to 50% pod formation. All parameter were significantly affected by inoculation of *Rhizobium,* compared with un-inoculated control in both screen house and field experiment, except leaf area in the screen house did not show significant difference with or without *Rhizobium* inoculation (Table 3). Inoculation of cowpeas with *Rhizobium* lowered the number of days to 50% flowering and pod formation in the screen house by 4.5 and 4% respectively, while it was vice versa in the field experiment where inoculation of cowpea seeds with *Rhizobium* increased the number of days to 50% flowering and pod formation by 2.32 and 2.4% respectively. Table 3 also shows that phosphorus supplementation at any level significantly affected the stem girt hand leaf area of cowpea in both screen house and field experiment relative to control treatment. In addition, phosphorus supplementation at 20 Kg.ha^{-1} showed significant effects on number of days to 50% flowering and 50% pod formation in the screen house experiment compared withall other treatments. Supplying P at 20 Kg.ha^{-1} resulted in early flowering and pod formation in screen house experiment. However, in the field study, the results shows that supplying P at any level significantly affected the number of days to 50% flowering and pod formation compared with control.

3.6 Effects of *Rhizobium* inoculation and phosphorus on total leaf chlorophyll content of cowpea.

The results in table 4 indicated that the *Rhizobium* inoculation had positive effects on the total leaf chlorophyll content of cowpeas measured from three weeks after planting and taken three times at two weeks interval. The total leaf chlorophyll content measured at 3 WAP significantly increased with *Rhizobium* inoculation in both screen house and field experiment compared with un-inoculated pots and plots (Table 4). The total leaf chlorophyll content of cowpea measured at five and seven weeks after planting were also significantly increased with inoculation of *Rhizobium* in the field experiment. However, the total leaf chlorophyll content measured at the same time from the screen house experiment did not show significant different from *Rhizobia* inoculated and un-inoculated control (Table 4). In the screen house, P supplementation at all levels did not show significant effect on the chlorophyll content of the cowpeas measured at 3, 5 and 7 WAP. Table 4 also shows that supplementation of phosphorus at all levels significantly increased the chlorophyll content of cowpea measured at 5 and 7 WAP compared to control. Supplementation of P at 80 Kg.ha^{-1} (5 WAP) and 40 Kg.ha^{-1} (7 WAP) resulted in greater values of chlorophyll content compared to other treatments

UNDER PEER REVIEW

3.7 Interactive effect of *Rhizobium* and phosphorus on the total leaf chlorophyll content of cowpea.

There was significant (*P*= .05) interactive effect between *Rhizobium* and phosphorus on the total leaf chlorophyll content of cowpeas measured at three weeks after planting in the screen house and seven weeks after planting in the field experiment. (Figure 2 A and B).

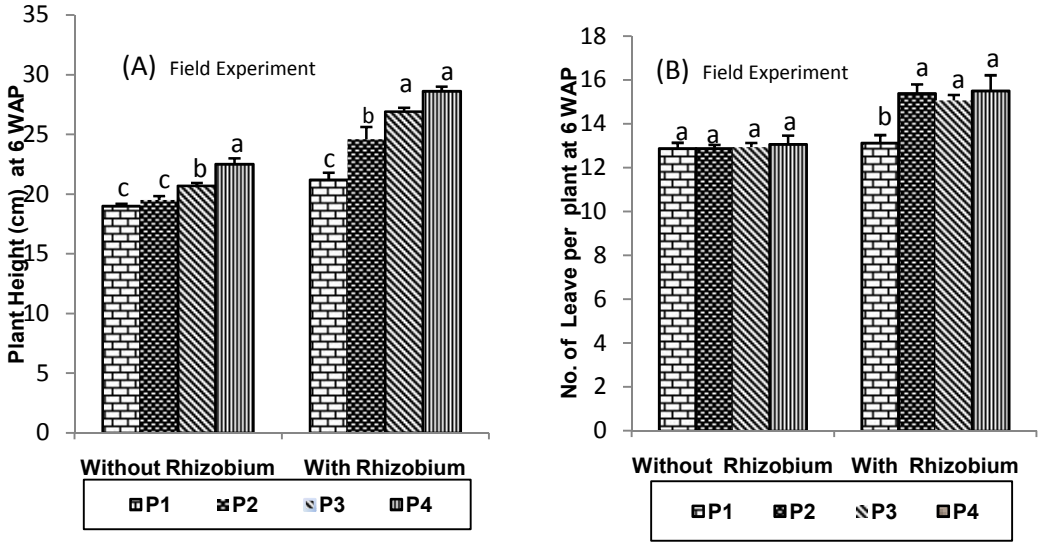
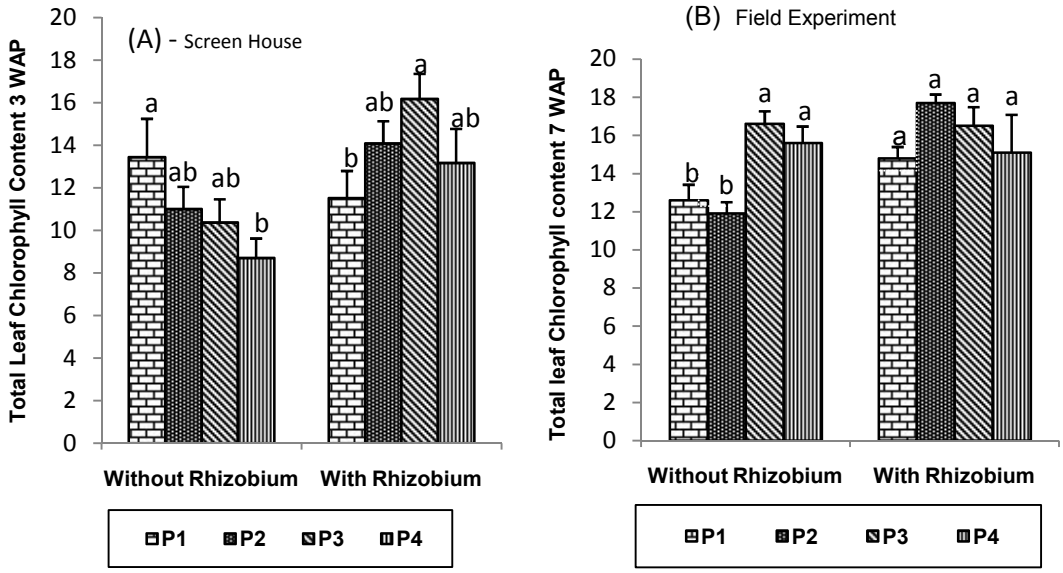


Figure 1. Interactive effects of *Rhizobia* and Phosphorus (P) on: (A) Plant height (cm) measured at 6 WAP, (B) Number of leaves per plant counted at 6 WAP.

P1 = Control, P2 = 20 KgP.ha^{-1}, P3 = 40 KgP.ha^{-1}, P4 = 80 KgP.ha^{-1}, WAP = Weeks After Planting. Bars followed by similar letter are not significantly different.



UNDER PEER REVIEW

Figure 2. Interactive effects of *Rhizobia* and Phosphorus (P) on: (A) Total Leaf Chlorophyll Content at 3 WAP, (B) Total leaf chlorophyll content at 7 WAP.
P1 = Control, P2 = 20 $KgP.ha^{-1}$, P3 = 40 $KgP.ha^{-1}$, P4 = 80 $KgP.ha^{-1}$, WAP = Weeks After Planting. Bars followed by similar letter(s) are not significantly different

.

Table 1: Effects of *Rhizobium* and phosphorus on plant height of cowpea grown under screen house and field condition.

	Screen House				Field			
	Plant Height (cm)				Plant Height (cm)			
Treatments	**2 weeks after planting**	**4 weeks after planting**	**6 weeks after planting**	**8 weeks after planting**	**2 weeks after planting**	**4 weeks after planting**	**6 weeks after planting**	**8 weeks after planting**
Rhizobium								
-R	16.78±0.42a	22.08±0.58b	32.88±0.83b	120.88±3.59b	6.41±0.12a	10.23±0.14b	20.42±0.39b	49.94±1.71b
+R	17.18±0.36a	24.56±0.74a	36.31±0.73a	131.75±2.52a	6.77±0.19a	11.06±0.32a	25.33±0.77a	54.58±2.05a
P Levels (Kg.ha^{-1})								
0	15.13±0.45b	19.69±0.43c	30.50±0.91c	109.13±3.80c	6.33±0.15a	9.67±0.15b	20.09±0.50d	44.98±2.12c
20	17.56±0.38a	23.50±0.82b	35.13±1.26b	128.88±3.22b	6.71±0.25a	10.86±0.36a	22.06±1.09c	51.66±2.35b
40	17.39±0.42a	24.50±0.73ab	35.63±0.91ab	134.25±2.82a	6.59±0.26a	10.81±0.31a	23.78±1.18b	54.48±2.20ab
80	17.84±0.34a	25.60±0.66a	37.13±0.48a	133.0±2.87ab	6.72±0.27a	11.23±0.41a	25.56±1.19a	57.93±2.39a
2 – Way ANOVA (F-Statistics)								
R	1.04ns	23.80***	27.58***	17.90***	2.21 ns	8.76**	181.91***	4.24*
P	10.43***	25.54***	19.07***	20.67***	0.58 ns	5.76**	41.41***	85.96***
R*P	1.53ns	0.6 ns	1.64ns	0.5 ns	0.35 ns	1.42 ns	6.65**	0.09 ns

***+R**: With Rhizobium; **-R:** Without Rhizobium; **R:**Rhizobium; **P:** Phosphorus; Values presented are means ± SE; *; **; ***; significant at P= .05, P≤ .01, P≤ .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= .05 according to Fischer least significance difference (LSD).*

Table 2: Effects of *Rhizobium* and phosphorus on number of leaves of cowpea grown under screen house and field condition.

Treatments	Screen House				Field			
	Number of leaves (counted at)				Number of leaves (counted at)			
	2 weeks after planting	4 weeks after planting	6 weeks after planting	8 weeks after planting	2 weeks after planting	4 weeks after planting	6 weeks after planting	8 weeks after planting
Rhizobium								
-R	3±0.00a	4.75±0.14a	9.56±0.38b	19.69±0.41b	3.49±0.05a	6.13±0.21a	12.94±0.12b	26.85±0.89b
+R	2.94±0.06a	4.56±0.13a	10.94±0.35a	21.81±0.49a	3.48±0.05a	6.58±1.58a	14.77±0.33a	29.96±1.16a
P Levels (Kg.ha^{-1})								
0	3±0.00a	4.63±0.18a	8.75±0.37c	18.88±0.35b	3.61±0.07a	6.10±0.13a	13.00±0.21b	25.29±0.70c
20	3±0.00a	4.75±0.25a	10.00±0.42b	20.00±0.60b	3.49±0.06ab	6.21±0.31a	14.13±0.52a	27.14±1.36bc
40	3±0.00a	4.50±0.19a	10.75±0.56ab	21.63±0.63a	3.48±0.08ab	6.95±2.21a	14.00±0.43a	29.53±1.58ab
80	2.9±0.13a	4.75±0.16a	11.5±0.42a	22.50±0.63a	3.36±0.08b	6.15±0.33a	14.28±0.60a	31.68±1.54a
2 – Way ANOVA (F-Statistics)								
R	ns	0.82 ns	13.2**	26.68***	0.0 ns	1.28 ns	45.38***	5.89*
P	ns	0.33 ns	9.6***	15.57***	1.8 ns	0.99 ns	4.56*	4.72*
R*P	ns	0.33 ns	0.69 ns	0.52 ns	0.3 ns	0.76 ns	3.85*	0.26 ns

***+R:** With Rhizobium; **-R:** Without Rhizobium; **R:**Rhizobium; **P:** Phosphorus; Values presented are means ± SE; *; **; ***; significant at P= .05, P≤ .01, P≤ .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= .05 according to Fischer least significance difference (LSD).*

Table 3: Effects of *Rhizobium* and Phosphorus on growth parameters of cowpea grown under screen house and field condition.

	Screen House				Field			
Treatments	**Stem Girth (mm)**	**Leaf Area (cm^2)**	**Days to 50% flowering**	**Days to 50% Pod formation**	**Stem Girth (mm)**	**Leaf Area (cm^2)**	**Days to 50% Flowering**	**Days to 50% Pod formation**
Rhizobium								
-R	0.47±0.01b	28.46±071a	65.31±0.28a	68.81±0.29a	7.64±0.12b	29.17±0.39b	70.25±0.64a	75.63±0.51b
+R	0.53±0.02a	29.90±0.81a	62.50±0.70b	66.19±0.66b	8.31±0.14a	30.49±0.55a	71.88±0.49b	77.44±0.48a
P Levels $K.gha^{-1}$								
0	0.45±0.02b	26.83±0.66b	64.88±0.77a	68.63±0.63a	7.48±0.18b	28.21±0.42b	69.63±0.73b	75.25±0.62b
20	0.52±0.02a	30.31±1.00a	62.13±1.14b	65.75±1.01b	7.95±0.26a	29.88±0.64ab	71.75±0.56a	76.63±0.42ab
40	0.50±0.02a	29.79±1.18a	64.38±0.56a	67.75±0.56a	8.22±0.20a	30.36±0,53a	72.00±1.10a	77.5±1.00a
80	0.54±0.02a	29.79±1.18a	64.25±0.84a	67.88±0.91a	8.25±0.10a	30.88±0.87a	70.88±0.77ab	76.75±0.80ab
2 – Way ANOVA (F-Statistics)								
R	9.97**	1.84 ns	20.18***	18.77***	20.44***	4.58*	4.47*	7.40*
P	4.66*	2.22 ns	3.78*	4.11*	5.67**	3.52*	1.95 ns	1.98 ns
R*P	0.08 ns	0.07 ns	2.61 ns	2.06 ns	1.46 ns	0.42 ns	0.96 ns	1.06 ns

***+R**: With Rhizobium; **-R:** Without Rhizobium; **R:**Rhizobium; **P:** Phosphorus; Values presented are means ± SE; *; **; ***; significant at P= .05, P≤ .01, P≤ .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= .05 according to Fischer least significance difference (LSD).*

Table 4: Effects of *Rhizobium* and phosphorus on total Leaf Chlorophyll content of cowpea grown under screen house and field condition

	Screen House			Field		
	Total Leaf Chlorophyll Content (mgL^{-1})			Total Leaf ChlorophyllContent (mgL^{-1})		
Treatments	**3 weeks after planting**	**5 weeks after planting**	**7 weeks after planting**	**3 weeks after planting**	**5 weeks after planting**	**7 weeks after planting**
Rhizobium						
-R	10.88±0.72b	13.30±0.71a	13.54±0.51a	7.65±0.79b	11.61±0.76b	14.17±0.61b
+R	13.74±0.72a	12.49±0.81a	13.76±0.44a	11.17±0.42a	16.01±0.65a	16.05±0.60a
P Levels ($Kg.ha^{-1}$)						
0	12.48±1.08a	12.73±1.30a	14.59±0.71a	8.90±1.34a	12.39±1.58b	13.70±0.63b
20	12.54±0.90a	13.63±1.24a	13.93±0.48a	10.34±1.03a	13.92±1.22ab	14.81±1.16ab
40	13.27±1.33a	13.00±1.01a	13.01±0.66a	8.41±0.99a	13.55±0.82ab	16.55±0.55a
80	10.93±1.20a	12.23±0.83a	13.07±0.72a	10.00±0.98a	15.38±1.33a	15.37±0.99ab
2 – Way ANOVA (F-Statistics)						
R	10.03**	0.62 ns	0.10 ns	14.33***	21.20***	7.50*
P	1.19 ns	0.32 ns	1.23 ns	0.96 ns	1.67*	2.97*
R*P	3.50*	2.79 ns	0.56 ns	0.30 ns	1.38 ns	4.45*

***+R**: With Rhizobium; **-R**: Without Rhizobium; **R:**Rhizobium; **P:** Phosphorus; Values presented are means ± SE; *; **; ***; significant at P= .05, P≤ .01, P≤ .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= .05 according to Fischer least significance difference (LSD).*

UNDER PEER REVIEW

4. DISCUSSION

The field and Screen house experiment was carried out in 2013 at northern Tanzania to assess the effects of Rhizobium (*Bradyrhizobium japonium)* on growth and total leaf chlorophyll content of cowpeas (*Vigna unguiculata* (L) Walp). The results of this study revealed that *Rhizobium* inoculation had significant effect on different plant growth parameters. For example, *Rhizobium* inoculation significantly increased the plant height, number of leaves per plant, stem girth and leaf area of cowpea (Table 1, 2 and 3 respectively) in both screen house and field experiment over un-inoculated control. The positive plant height, stem girth, leaf area and maximum number of leaves per plant observed in the inoculated pots and plots might be due to symbiotic relationship between *Rhizobium* and nodulated roots of legumes which fixes atmospheric nitrogen into a usable form by plants [10,35,36] and ultimately results in the plant growth over the control. The fixed nitrogen is an important input in the plant growth [37,38,39]. Therefore, if there is reduced nitrogen fixation due to different factors in nutrient depleted soils, there will be reduced plant growth [17]. Similar to our study, other workers [36,40,4] have reported that *Rhizobia* inoculation significantly increased the plant height, number of leaves per plant and biomass of Groundnuts (*Arachis hypogaea*) and Soybean (*Glycine max*).

This study also showed significant improvement onthe total leaf chlorophyll content of cowpeas as a result of *Rhizobium* inoculation in both screen house and field experiment relative to un-inoculated treatments. These findings concur with the results found by [16] in which *Rhizobium* inoculation improved chlorophyll content in *Phaseolus vulgaris*. Furthermore, inoculation with *Rhizobium* significantly increased the number of days to 50% flowering and pod formationin the field experiment.The late maturity of legumes in the field experiment may be attributed to biological nitrogen fixation by *Rhizobium* inoculation on cowpea seeds which produce adequate nitrogen and make the plant to grow luxuriously vegetative. These results differs from the study by [41] on the effect of *Rhizobium* inoculant on nodulation, yield and yield traits of chickpea (*Cicer arietinum* L) who found that *Rhizobium* inoculation resulted in the early maturity of chickpea.
The improved plant growth parameters in the inoculated treatments over the control is an indication that *Rhizobium* were efficient in fixing nitrogen which is a building block of plant proteins many of which determines the size and structure of plant tissues and consequently plant growth is enhanced.

In this study, phosphorus supplementation significantly increased the plant height, number of leaves per plant and stems girth (Table 1, 2 and 3 respectively) of cowpea in both screen house and field experiment. These findings are supported by other workers [42] who concluded that supplying P irrespective of the rate significantly increase the plant height, the number of leaf per plant, stem girth and leaf area. These results suggestthat P is an important element for plant growth and development. In relation to our findings, other workers [43,44] have reported that supplying P to legume and cereal crops significantly improvedthe plant height. The improvement ingrowth parameters and chlorophyll content observed in this experiment might have been caused by the activity of phosphorus which plays major role in structural and metabolic function in the plant leading to growth and development [45].Therefore, an adequate supply of phosphorus is needed for the promotion of early root formation and plant growth because phosphorus is a component of numerous cell constituents and performs major roles in several key processes, such as photosynthesis, respiration, energy storage and transfer, cell division, and cell enlargement [46,47].

This study also showed significant interactive effects between *Rhizobium* and phosphorus on plant height, number of leaves.plant^{-1} (Figure 1-A & B) and chlorophyll content of cowpeas (Figure 2-A & B). For instance, whether supplied alone or with Rhizobium, P significantly gave thegreater plant height at its highest level of 80kg P.Ha^{-1} (Figure 1-A). The maximum numbers of leaves per plant were also observed where *Rhizobium* was supplied with phosphorus relative to control (Figure 1-B).There was also significant interactive effect between *Rhizobium* and phosphorus on the chlorophyll content of cowpea where the greatest value of chlorophyll content was recorded in *Rhizobium* supplied with 40 KgP.ha^{-1} relative to the control. P supplementation alone without *Rhizobium* decreased chlorophyll content in the screen house compared with control (Figure 2-A). The decreasing values of chlorophyll content with increasing P without *Rhizobium* inoculation suggests that increasing P alone does not increase chlorophyll concentration in the leaves, but rather nitrogen is a necessary building blocks of chlorophyll molecule in the plant and therefore, it influences the formation of green pigment in the plant leaves. These results also suggest that there was limited amount of nitrogen in the small volume of soil in the pots compared with the field experiment where the plant roots can move far searching nitrogen and consequently chlorophyll content of plant leaves was improved in both inoculated and un-inoculated treatments in the field trial. Figure 2- B shows interactive effects between *Rhizobium* and phosphorus on the total leaf chlorophyll content of cowpea in which P supplementation alone produced greater values of chlorophyll content at 40 and 80 Kg.ha^{-1} relative to other treatments while both *Rhizobium* inoculation and P supplementation had no significant different over the control. However, treatments which received *Rhizobium* inoculation and supplemented with P produced greater values of

UNDER PEER REVIEW

chlorophyll content compared with the treatments received P alone (Figure 2-B).The increased plant height, number of leaves and chlorophyll content of cowpea might have been contributed by combination of *Rhizobium* and phosphorus which enhance root formation, nodulation, N-fixation and finally plant grow with dark green color for food production [28,30,11, 12].

5. CONCLUSION

In conclusion, our results showed the importance of *rhizobium* inoculation and phosphorus on the growth and total leaf chlorophyll content of legumes crops in the nutrient depleted soils. The growth parameters such as plant height, number of leaves per plant, leaf area, stem girth, were significantly improved with *Rhizobium* inoculation and application of P over the control treatments. Inoculation with *Rhizobium* also significantly improved the total leaf chlorophyll content of the cowpea. There was also significant interaction between *Rhizobium* and phosphorus on the plant height, number of leaves per plant, and total leaf chlorophyll content of cowpeas. Therefore, for improved plant growth, number of leaves and chlorophyll content in the legume production system, it is important to supplement the depleted soils of sub-Saharan Africa with phosphorus and *Rhizobium* inoculation.

6. ACKNOWLEDGEMENTS

Special thanks to the Nelson Mandela African Institution of Science and Technology (NM-AIST) and the Commission for Science and Technology (COSTECH) of Tanzania who supported this study, Tanzania Coffee Research Institute (TaCRI) and Seliani Agricultural Research Institute (SARI) for providing study sites.

REFERENCES

1. Buerkert A, Bationo A, Piepho HP. Efficient phosphorus application strategies for increased crop production in sub-Saharan West Africa. Field Crops Res.2001;72:1-15.
2. Nekesa ECR, Thuita M, Ndungu K, Kifuko M, Bationo A. The potential of underutilized phosphate rocks for soil fertility replenishment in Africa: case studies in western Kenya, 2007;1589-1598.
3. Brady NC. Phosphorus and potassium. In: The nature and properties of soils. Prentice-Hall of India, Delhi.pp, 2002;352.
4. Shahid MQ, Saleem MF, Khan HZ, Anjum SA. Performance of soybean (*Glycine max* L.) under different phosphorus levels and inoculation. Pak. J. Agr. Sci. 2009;46:237-241.
5. Daniel PS, Robert JR and Ayling SM. Phosphorus uptake by plants: From soil to cell. Plant Physiology, 1998;116: 447-453
6. Abdel-Wahab SM, HassmanME, Elwarraky K and Safwat MSA. Evaluation of N_2 – fixation of faba bean, chickpea and lentil as affected by phosphorus fertilization. African Crop Science Conference Proceedings, 1994;1:71-73.
7. Luyindula N, Kabinda PP, Mbaya N, Nwange K and Babela K.Effect of soil *bradyrhizobium* populations on nodulation and growth of *Glycin max* (L.) Merrill. African Crop Science Conference Proceedings, 1994;1: 68-70.
8. Theodorou ME, Plaxton WC. Metabolic adaptations of plant respiration to nutritional phosphate deprivation. Plant Physiol. 1993;101: 339–344
9. Rubio V, Linhares F, Solano R, Martín AC, Iglesias J, Leyva A, Paz-Ares J. A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev. 2001;15: 2122-2133.
10. Ndakidemi PA, Dakora FD, Nkonya E, Ringo D, Mansoor H. Yield and economic benefits of common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) inoculation in northern Tanzania. Aust. J. Exp. Agric. 2006;46:571-578.
11. Rani BP.Response of soybean to nitrogen and phosphorus application in the black soils of Krishna-Godavari zone of Andhra Pradesh. Ann. Agr. Res. 1999;20(3):367-368
12. Tomar SS, Singh R and Singh PS.Response of phosphorus, sulphur and *Rhizobium* inoculation on growth, yield and quality of soybean.Prog. Agric. 2004;4(1):72-73.
13. Singh A, Baoule A, Ahmed H, Dikko A, Aliyu U, Sokoto M, AlhassanJ, Musa M,Haliru B. Influence of phosphorus on the performance of cowpea (*Vignaunguiculata* (L) Walp.) varieties in the Sudan savanna of Nigeria. Agricultural Sciences, 2011;2:313-317.
14. Muleba N, Ezumah HC. Optimizing cultural practices for cowpea in Africa. Pages 289–295 in Cowpea research production and utilization, edited by S.R. Singh and K.O. Rachie. John Wiley and Sons Ltd., Chichester, UK.1985

UNDER PEER REVIEW

15. Uchida RS. Essential nutrients for plant growth: nutrient functions and deficiency symptoms. In: Silva J. A. and R. S. Uchida. (eds.). Plant nutrient management in Hawaii's soils. ManoaCollege of Tropical Agriculture and Human Resources, University of Hawaii at Manoa.2000
16. Bambara S, Ndakidemi PA. Effects of Rhizobium inoculation, lime and molybdenum on photosynthesis and chlorophyll content of *Phaseolus vulgaris* L. Afr. J. Microbiol. Res.2009;3: 791-798.
17. Bambara S, Ndakidemi PA. *Phaseolus vulgaris* response to *Rhizobium* inoculation, lime and molybdenum in selected low pH soil in Western Cape, South Africa.Afr. J. Agric. Res. 2010;5(14),1804-1811.
18. Hayat R, Ali S, Amara U, Khalid R, Ahmed I. Soil beneficial bacteria and their role in plant growth promotion: a review. Ann. Microbiol. 2010;60:579-598.
19. Dutta AC. Botany for degree students.Oxford University Press. p.708.2004
20. Salon C, Avice JC, Larmure A, Ourry A, Prudent M and Voisin AS. Plant N fluxes and modulation by nitrogen, heat and water stresses: A review based on comparison of legumes and non legume plants. In. Abiotic Stress in Plants- Mechanism and Adaptation (ed A. S. A. B. Venkateswarlu). 2011.
21. Hardarson G. Methods for enhancing symbiotic nitrogen fixation.Plant Soil. 1993;152(1): 1-17
22. Carter JM, Gardner WK, Gibson AH.Improved growth and yield of faba beans (*Viciafaba*cv. Fiord) by inoculation with strains of *Rhizobium leguminosarum*biovarviciaein acid soils in south-west Victoria. Aust. J. Agric. Res. 1994;45: 613-623.
23. Brockwell J, Bottomley PJ,Thies JE. Manipulation of *rhizobia*microflora for improving legume productivity and soil fertility: a critical assessment. Plant Soil. 1995;174: 143-180.
24. Wani SP, Rupela OP, Lee KK. Sustainable agriculture in the semi-arid tropics through biological nitrogen fixation in grain legumes.Plant Soil. 1995;174:29-49.
25. Dakora FD, Keya SO.Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. Soil Biol. Biochem. 1997;29: 809-817.
26. Popescu A. Contributions and limitations to symbiotic nitrogen fixation in common bean (*Phaseolus vulgaris*L.) in Romania. Plant Soil, 1998;204(1): 117-125.
27. Zahran HH. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate.Microbiol. Mol. Biol. Rev. 1999;63(4):968-989.
28. Vargas MAT, Mendes IC, Hungria M. Response of field-grown bean (*Phaseolusvulgaris*L.) to *Rhizobium*inoculation and nitrogen fertilization in two Cerrados soils. Biol. Fertil. Soils. 2000;32:228-233.
29. Hernandez M, Cuevas F. The effect of inoculating with *Arbuscularmycorrhiza*and*Bradyrhizobium*strains on soybean (*Glycine max*(L.) Merrill) crop development.CultivosTropicales. 2003;24(2):19-21.
30. Menaria BL, Nagar RK, Singh P.Effect of nutrients and microbial inoculants on growth and yield of soybean (*Glycine max* L.). Journal of Soils and Crops, 2003;13(1): 14-17.
31. Bhatt M, Chanda SV. Prediction of leaf area in *Phaseolus vulgaris* by non-destructive method. Bulg. J. Plant physiol. 2003;29(1–2):96- 00.
32. Hiscox J, Israelstam G. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. of Bot. 1979;57:1332-1334.
33. Arnon DI. Copper enzymes in isolated chloroplasts, polyphenoxidase in Beta vulgaris. Plant physiology, 1949;24:1-15
34. Steel RGD, Torrie JH, Dickey DA. Principles and procedures of statistics: a biometrical approach. McGraw-Hill Inc.: New York.1980
35. Rahman MM, Bhuiyan, MMH, Sutradhar, GNC, Paul, AK. Effect of phosphorus, molybdenum and *Rhizobium* inoculation on yield and yield attributes of mungbean. International Journal of Sustainable Crop Production. 2008;3(6): 26-33.
36. Sajid M, Rab A, Wahid F, Shah SNM, Jan I, Khan MA, Hussain SA, Khan MA and Iqbal Z. Influence of *rhizobium* inoculation on growth and yield of groundnut cultivars. Sarhad J. Agric. 2011;27(4): 573-576
37. Verhoeven JTA, Koerselman W, Meuleman AFM. Nitrogen- or phosphorus-limited growth in herbaceous, wet vegetation: relations with atmospheric inputs and management regimes. Trends Ecol. Evol. 1996;11(12):494-497.
38. Bambara S, Ndakidemi PA. The potential roles of lime and molybdenum on the growth, nitrogen fixation and assimilation of metabolites in nodulated legume: A special reference to *Phaseolus vulgaris* L. Afr. J. Biotechnol. 2010;9:2482-2489.
39. Bambara S, Ndakidemi PA. Effects of Rhizobium innoculation, lime and molybdenum on nitrogen fixation of nodulated*Phaseolus vulgaris*L. Afr. J. Microbiol. Res. 2010;4:682-696.
40. Saharan B and Nehra V. Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. 2011;21: 1-30.

UNDER PEER REVIEW

41. Zaman S, Mazid MA, Kabir G. Effect f*Rhizobium* Inoculants on Nodulation, yield and yield traits of Chickpea (*Cicerarietinum L.*)In four different soils of greater rajshahi. Journal of Life and Earth Science 2011;6:45-50.
42. Amhakhian SO, Osemwota IO. Effects of Different Levels of phosphorus on the Performance of Maize (*Zea mays* L.) in Anyigba, North central, Kogi State, Nigeria.Int. J. Agr. and Rural Dev. 2012;15(2):1049-1058.
43. Abayomi Y, Ajibade T, Sammuel O,Saadudeen B. Growth and yield responses of cowpea (*Vignaunguiculata* (L.) Walp) genotypes to nitrogen fertilizer (NPK) application in the Southern Guinea Savanna Zone of Nigeria. Asian J. Plant Sci. 2008;7:170-176
44. Masood TR, Gul F, Munsif F, Jalal Z, Hussain N, Noreen H, Khan ND and Khan H. Effect of different phosphorus levels on the yield and yield components of maize.Sarhad J. Agric. 2011;27(2):167-170
45. Bibi A, Oosterhuis DM, Gonias E, and Mozaffari M. Effect of Phosphorus Deficiency on Cotton Growth. In N.A. slaton (ed) W. E. SabbeArkansas Soil Fertility Studies. 2005. AR Ag. Exp. Stn Res. Ser. 537.Fayetteville, AR. 2006
46. Awasthi R, Tewari R, Nayyar H. "Synergy between plants and P-Solubilizing microbes in soils: Effects on growth and physiology of crops." International Research Journal of Microbiology, 2011;2(12):484-503.
47. Griffiths BS, Ritz K, Dobson G. Soil microbial community structure: effects of substrate loading rates. Soil Biol. Biochem. 1999;31:145–153