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Research Article

Nodulation, Nitrogen Fixation and Growth of Rhizobia-Inoculated Cowpea (*Vignaunguiculata L. Walp*) In Relation with External Nitrogen and Light Intensity

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Abstract

Cowpea is a legume crop that fixes atmospheric nitrogen with soil rhizobia. This study investigated the effects of nitrogen supply and light intensity on N_2 fixation and growth of rhizobia-inoculated cowpea. The hydroponic experiment designed to evaluate the response of cowpea inoculated with three *Bradyrhizobium* strains (TSC7, TSB2, TTC9) under different N concentrations (0, 1, 2.5, 5, 7.5, 10 mM) showed that regardless to the used inoculant, N ≥ 2.5 mM inhibit the nodulation and nitrogen fixation of cowpea at early stages (2-4 WAT). While the three strains similarly affect cowpea nodulation at 2 WAT, at later stages (4-6 WAT), the nodulation phenotype varied among them. At 6 WAT, N ≥ 2.5 mM did not repress the nodulation of TSC7- and TTC9-inoculated but rather improved it. This study shows that the supply of 1 mM N is beneficial in stimulating the nitrogen fixation of TTC9- and TSB2-inoculated cowpea, and at later stages, the nitrogen fixation inhibitory effect of N 2.5 mM and N 5 mMis decreased. In the second experiment, conditions of100, 75, 50and 25%light intensities of the natural light were set up in a glasshouse to study their effect on N_2 fixation and growth of cowpea inoculated with *B. yuanmingense* DTC8, TSC7, and TTC9 strains. The 100% and 75% light intensities led to better cowpea growth and production. The study shows that when the light intensity is decreased to 75% of the natural light (about 535µ $molm^{-2}$ sec^{-1}PPFD), cowpea seed production and nitrogen content is improved. Appropriate lightning would therefore greatly improve the quantity and quality (protein) of cowpea seeds.

ABBREVIATIONS

WAT: Weeks After Transplanting; WAS: Weeks After Sowing; PFD: Photon Flux Density; PPFD: Photosynthetic Photon Flux Density; R: Red Light Ratio; FR: Far-Red Light Ratio; GS/GOGAT: Glutamine Synthetase/ Glutamine-2-Oxoglutarate-Amino-Transferase; ARA: Acetylene Reduction Activity; NA: Nitrogenase Activity; ANOVA: Analysis Of Variance

INTRODUCTION

The Leguminosae are unique in their ability to form N_2-fixing symbioses with members of the Rhizobiaceae (or rhizobia). Inside root nodules these rhizobial bacteria are able to reduce atmospheric N_2 into ammonia by the nitrogenase enzyme. The ammonia as the NH_4^+ ion is assimilated into glutamate, and in both bacteria and plants this occurs via the joint action of the enzymes glutamine synthetase (GS) and glutamine-2-oxoglutarate-amino-transferase (GOGAT). The further assimilated fixed nitrogen is transported predominantly as the amino acids asparagine and glutamine by amide-exporters, or as the ureidesallantoin and allantoic acid via the ureide-exporters, and exchanged for photosynthates from the host plant [1]. The mutualistic

relationship between the Leguminosae and Rhizobiaceae forms the basis for the ecological importance of legumes in natural and agricultural ecosystems in promoting increased crop yields. A common approach to improve symbiotic nitrogen fixation and legume productivity has been the reliance on superior and very effective exotic rhizobia strains as inoculants. When introduced, the inoculant bacteria must adapt to prevailing soil conditions, multiply in the soil and host rhizosphere, and compete with the often ineffective indigenous rhizobia for infection sites [2]. Tapping into the biological potential of legumes is currently constrained by environmental factors including soil pH; temperatures; soil nutrients, mainly nitrate content; soil moisture; and poor nodulation competiveness of the introduced inoculants [3]. A way of improving the success of inoculants would be to use indigenous strains that are effective as well as competitive for nodulation.

In many parts of the world (especially West Africa), soils are experiencing a decline in nitrogen status which is a major determinant of food production [4]. Bationo and Mokyunwe [5] previously identified nutrient deficiencies, mainly nitrogen (N) and phosphorus (P), as the main soil fertility constraints limiting crop yields in West Africa. These authors and Bagayoko *et al.* [6] pointed out that N_2-fixing legume crops such as cowpea can improve the soil fertility of cropping systems, a result of the N supply via biological nitrogen fixation. Cowpea, a legume crop native to Africa is an important annual crop in tropical and subtropical regions worldwide, especially in Sub-Saharan Africa, Asia and Central and South America [7]. The young leaves, pods, and seeds of this plant are a source of dietary proteins, vitamins, and minerals for humans and animals [7]. In rhizobium-cowpea symbiosis, soil fertility status and fertilizer, in the case of external N application, affect both rhizobium and cowpea. Cowpea is also affected when soil N is low and no rhizobium is present. It has been found that high concentration of nitrate (NO_3) in waste effluents and high air temperature can inhibit nodulation and N_2 fixation in symbiotic legumes [8,9]. These findings suggest that while soil low in N can negatively affect the growth of cowpea, soil high in N or amended by N fertilizer, at certain rates, can also have a negative impact on the crop by reducing the N_2 fixation potential. It is therefore important to determine the level of N above which nodulation and nitrogen fixation of rhizobia-inoculated cowpea can be negatively affected. Although some works have been done in this aspect on cowpea, they were mostly limited in assessing the effect of external N on the plant growth [10,11] but not on how the supplied N affect the symbiosis with rhizobia. In parallel, the very high ambient temperatures (correlating to high light intensity) usually promote photosynthetic rates under atmospheric conditions, but drastically increase potential evapotranspiration (PET), thus further decreasing soil moisture needed for root growth and the survival of mutualistic soil microbes such as rhizobia.

Comparatively to soybean, few studies have examined cowpea production under the combination of these various factors, such as inoculation with effective rhizobia, application of mineral nitrogen, and growth under different light intensities. This experiment was designed to investigate the effects of external N application and different light intensities on nodulation, biological nitrogen fixation, growth and production of cowpea inoculated with efficient nitrogen-fixing rhizobia. Such investigation is important to provide information on the level on N at which the benefit of rhizobial inoculation to cowpea growth can be optimized; given that at certain N level, nitrogen fixation, which is reaction consuming more energy, does not occur. Moreover, in farms, cowpea is often planted in sole system or intercropped with cereals such as pearl millet, maize or sorghum. In intercropped systems, the cereal's canopies reduced the amount of radiation reaching the cowpea plant; making the sole system receiving more light intensity. Including the light effect in this study will help determine the best agricultural system that can maximize the dual application of external N and rhizobial inoculants for better cowpea production.

MATERIAL AND METHODS

This work was carried out in two phases, through hydroponic and solid experiments to assess the effect of external nitrogen and light intensity, respectively, on the biological N fixation and growth of inoculated cowpea. All experiments were conducted at Kyushu University, Japan (33° 37'N, 130° 25'E, elevation 3 m a.s.l) from June 2010. Cowpea seeds were from the Laboratory of Plant Nutrition of the Faculty of Agriculture of Kyushu University.

Hydroponic experiment

Seeds of a drought-tolerant cowpea (TVu-11986) were surface sterilized in 1% sodium hypochlorite for 3 min and 99.5% ethanol for 30 sec with 5 replications. They were thoroughly rinsed with distilled water and germinated in autoclaved (121°Cfor 20 min) 15 cm height glass petri dishes with wet filter paperson the bottom and top lids. The seeds were let to pre-germinate in the dark (petri dishes covered with aluminum foil) for 24 hours in a 25°C growth chamber after which the aluminum foil was removed and 10 mL N-free nutrient solution (Saeki *et al.*, 2000) was applied. Germination continued aseptically in the growth chamber for 36 hours before the seedlings were inoculated with rhizobia. The strains used as inoculant were *Bradyrhizobiumyuanmingense*TSB2, TSC7, and TTC9, isolated by Sarr *et al.* [12] from root nodules of cowpea in the South-west part of Japan, and proven to be effective at nodulating the host plant. During a previous pot experiment, these strains showed different symbiotic characteristics and it was important to determine their performance under varied conditions such as different nitrogen levels and light intensity. Prior to inoculation, the strains were grown to the exponential phase in A1E [13] liquid medium and optical densities (OD_{600nm}) of cultures were measured to estimate the number of cells as inocula. Based on their OD_{600nm} values, cultures were diluted to approximately$1x10^7$ cells mL^{-1}[14].

This experiment had a total of 18 treatments that combined six N treatments and three *Bradyrhizobium* strains treatments as a 6 x 3 factorial in three completely randomized blocks. The N treatments consisted of a no N treatment (0 mM) as a control and five N concentrations of 1, 2.5, 5, 7.5, and 10 mM to create conditions of low, medium and high N levels. Pre-germinated uniform seedlings were selected, immersed in 27 mL of the indicated inoculum solution for 5-10 min, and transferred into 7-L hydroponic pots of 30 cm x 25 cm x 13.5 cm dimension (nine plants per pot)filled with N-free nutrient solution [15] first, and N (as $NaNO_3$) concentration modified according to the N treatment. However, because of the huge amount of water needed for preparation, we did not autoclave the nutrient solution. Therefore, a un-inoculated-nitrogen free control was included to verify the effect on non-autoclaving in the symbiosis. White polystyrene

with 9 holes was used to cover the solution inside the pots. The holes where seedlings emerged were fixed with cotton (Figure. 1A). The pots were placed in a glasshouse and covered with aluminum foil for 1 night after which an aeration pump (Rotary compressor 91-22796 Tokico-F, HITACHI) was installed in each pot (Figure. 1B).The pH was adjusted to 6.5 every day by adding 1 M H_2SO_4 or 1 M NaOH as needed and the nutrient solution was renewed 2 weeks after transplanting (WAT), followed by one change a week for the remaining time. Pots were sterilized with 70% alcohol before adding the new solution during each change. This experiment was carried out in hydroponic to mimic the effect of other soil characteristics other than N. The nine plants per pot were harvested on three separate occasions (three plants per time) at 2, 4 and 6 week after transplanting (WAT) corresponding to the growth stages V2, V3, and V5 of cowpea, respectively [16]. The border effect was neglected as the experiment was carried out in a controlled environment.

Solid experiment

The experiment had a total of 12 treatments, a combination of four light intensity treatments and three *Bradyrhizobium* strains treatments, and was conducted as a completely randomized 4 x 3 factorial design with 3 replications. The light intensity treatments were 25%, 50%, 75% of the natural light (artificially produced in the glasshouse using white and/or black cheesecloth), and 100% that corresponded to the natural light inside the glasshouse. These intensities were used to represent conditions of light in sole cropping systems and in intercropping systems where the intensity of the light reaching the soil surface decreases based on the tillage of the selected crop for intercropping. Studying the light effect is important as cowpea is generally grown in tropical regions that have strong light intensities during the growing seasons. The three *Bradyrhizobium* strains treatments were TSC7, DTC8, and TTC9 from Sarr *et al.* [12] collection. This experiment was carried out in 1/5000 Wagner pots (3 L) filled with a mixture of autoclaved sand and vermiculite (ratio by weight 15:7) and 1.8 L N-free nutrient solution (similar to the one used in the hydroponic experiment). Three sterilized seeds (as described above) of the cowpea variety TVu-11986were sown per pot. Thereafter, 3 mL of $1x10^7$ cells mL^{-1}bradyrhizobial culture were spread above each seed. A un-inoculated-nitrogen free control pot was included to assess the effect of not autoclaving the nutrient solution during the experiment. Pots were covered with aluminum foil (AF) after inoculation and transferred within the glasshouse at the corresponding light intensities. AF was removed after germination and the nutrient solution was supplied weekly for up to 3 weeks, by adding 2 L at the top of the pot which leached slowing from an open hole at the bottom of the pot. At 3 weeks after sowing (WAS), 0.5 mM N as$NaNO_3$ was added to the nutrient solution to enhance cowpea growth. Plants were watered regularly for the remaining period and the experiment lasted from June 23 to October 20 (4 months). A Model LI-1400 data logger was installed in the glasshouse for continuous (daily) measurements of the photosynthetic photon flux density (PPFD) at 70 cm and 120 cm aboveground surface(corresponding to the minimum and maximum heights of the plant at maturity) for each of the four light intensities (100%, 75%, 50%, and 25%). The red (R, 660 nm) and far-red (FR, 730 nm) light ratios were measured and the R/RF ratio calculated. Data were collected at 6, 10 and 16 WAS. 6 WAS corresponds to the vegetative growth stage V5, while 10 WAS and 16 WAS correspond to the reproductive growth stage R3.5 and the physiological maturity RH, respectively[16].

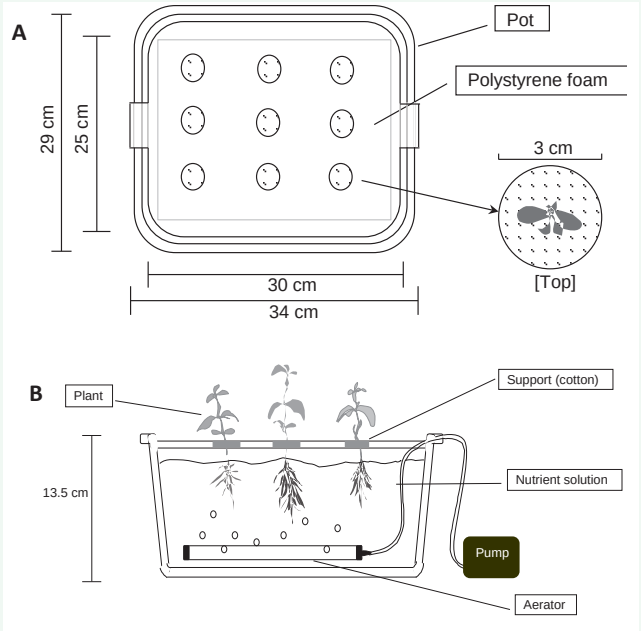

Figure 1 Schematic diagram of the hydroponic culture.

Data collection and plant analysis

In the hydroponic experiment, three plants (three replicates) per treatment were individually removed from the pot at each of the three harvest periods. After harvesting, the plant samples (experimental units) were washed in running tap water and their nitrogen fixation potential was assessed using the Acetylene Reduction Activity (ARA) method. Following ARA assessment, the nodules from each root system were detached and counted, and the plant dry weight was obtained after drying the samples (aerial parts + roots) for 72 h at 70°C.

In the solid experiment, the three plants grown in a pot (treatment) were collected separately at each harvest period by carefully removing them from the substrate (sand + vermiculate). There were 3 replicated pots for the 3 harvest periods and each pot contained 3 plants that corresponded to the 3 replicates plants per harvest. After ARA assessment at each harvest period, the nodule number was recorded and the nodule mass calculated after nodules were oven dried. At 16 WAS corresponding to the maturity period, the nodule (fresh and decayed) number, the dry weight, the N concentration, and the distribution of N in the different plant components (root, nodule, shoot, leaf, pod + seed) were recorded. Fresh nodules had pink-red color due to the presence of leghemoglobine while decayed nodules gray colored. Dry weight was obtained after drying the plant component samples for 72 h at 70°C. N concentration was determined by using salicylic-sulfuric acid (H_2SO_4)-hydrogen peroxide (H_2O_2) digestion [17] followed by the indophenol method [18]. N distribution (%) was calculated using the formula: N distribution (%) = (Component N/Total plant N) * 100:

In both hydroponic and solid experiments, ARA was determined as described by Sarr *et al.* [14]. Plant roots (per replicate) with intact nodules were severed at the cotyledonary

node and placed in 100 ml conical flasks. Flasks were sealed with a serum stopper and 10% air gas was replaced with acetylene (C_2H_2) gas. The nodulated roots were incubated at room temperature and 1 ml subsamples were analyzed for ethylene (C_2H_4) productionat 5 and 65 min after incubation using flame ionization and gas chromatograph (Shimadzu GC-14A Kyoto, Japan). Column and injector temperatures were 35, 45°C, respectively. Carrier gas was N_2 (flow rate: 30 ml min^{-1}). ARA was calculated as µmol of ethylene produced per hour per plant.

Statistical analysis

In both experiments, all recorded data were subjected to ANOVA ($P \leq 0.05$), using CropStat 7.2 developed by the International Rice Research Institute (Manila, Philippines). First, two-way ANOVA was applied to data of each harvest period, to analyze the interaction between external N and bradyrizobial strains (hydroponic experiment), and between light intensity and bradyrhizobial strains (solid experiment). When the interaction was significant, one-way ANOVA was carried out using data obtained from each strain. The Least Significant Difference (Tukey's test) was used to compare mean data when the probability level was significant.

RESULTS

Hydroponic experiment

The possibility of contamination with non-relevant cowpea-nodulating bacteria was excluded by the confirmation of the absence of nodules in the un-inoculated negative control culture (data not shown).The (Table 1) shows that the interaction between N concentration and inoculant was not significant for nodule number at 2 WAT, for ethylene production at 4 WAT, and for plant dry weight at 2 and 4 WAT. Therefore, the main effects of inoculants and N concentration were evaluated and data are shown (non-bold) in the table and are discussed. However, significant interactions were observed between these two factors for nodule number at 4 and 6 WAT, for produced ethylene at 2 and 6 WAT, and for plant dry weight at 6 WAT. Where interaction was significant, one-way ANOVA was separately applied to each inoculant for discussion, and mean effects (not discussed) are reported in bold in (Table 1). Results of the one-way ANOVA are shown in (Figure 2). At 2 WAT, no difference was observed in the main effect of inoculants for nodule number, and the main effect of the N showed that N ≥ 2.5 mM significantly repressed the nodulation (Table 1). At 4 and 6 WAT, the effect of N on cowpea nodulation varied within the three inoculants (Figure 2a,2b). The statistical analysis was not significant for TSB2-inouclated cowpea. At 4 WAT, N ≥1mMsignificantly repressed the nodulation of TTC9-inoculated cowpea, while the repression was observed at N ≥ 5 mM for TSC7-inoculated cowpea. At 6 WAT, significant nodulation repressions were observed at N ≥ 5 mM for TTC9- and N ≥7.5 mM for TSC7-inoculated cowpeas in comparison to N = 0 mM. Interestingly, N ≥2.5 mM increased the nodule number at 4 and 6 WAT.

Figure 2c shows the interaction between ethylene production and N concentration for the 3 inoculants at 2 WAT (one-way ANOVA). While N ≥1mM enhanced the nitrogen fixation for TSB2 and TTC9, it significantly reduced it for TTC9. All N concentrations ≥ 5 mM significantly decreased the ethylene production for the 3 strains at this stage. At 4 WAT, the main N effect following the two-way ANOVA (Table 1) also shows that N ≥ 2.5mM significantly reduce the ethylene production. At this stage, no significant difference was observed in the main effect of inoculants. (Figure 2d) (one-way ANOVA) shows that the statistical analysis was not significant for TSB2-inoculated cowpea at 6WAT. In TTC9-inoculated cowpea, N ≥1mM significantly reduced the amount of produced ethylene, while this N concentration positively enhanced the ethylene production in TSC7-inoculated cowpea. Higher N concentration (≥2.5mM) did not negatively affect the ethylene production of TSC7-inoculated cowpea compared to N = 0 mM.

Considering the total plant dry weight (Table 1), the main inoculant effect shows no significant difference within strains at 2 WAT. But at 4 WAT, the effect of TTC9 was significantly higher than that of TSC7 and TSB2. The main N effect shows that N ≥1mM significantly increase plant dry weight at 2 WAT. The effect of different N concentrations did not significantly differ within them, except N = 7.5 mM which led to significant higher plant dry weight compared to N = 1 mM.At4 WAT, N ≥5mM significantly increased plant dry weight compared to the N = 0 mM, and no significant difference was observed within the five N concentrations. At 6 WAT, one-way ANOVA was applied (Figure 2e). Results show that N ≥1mM, significantly increase plant dry weight compared to the N = 0 mM for TSC7-inoculated cowpea. For TTC9-inoculated cowpea, the significant plant dry weight increase compared to the N = 0 mM was observed at N ≥5mM. Application of N ≥2.5mM enhanced plant dry weight of TSB2-inoculated cowpea, except that significant decrease was observed at N = 5 mM.

Solid experiment

Similarly to the hydroponic experiment, the possibility of contamination with non-relevant cowpea-nodulating bacteria was excluded in this experiment by the confirmation of the absence of nodules in the un-inoculated negative control (data not shown). The results in (Table 2) show a higher PPFD at 100% light intensity which decreases with the decrease of light intensity. We also observed differences in R and FR among the different light intensities inside the glasshouse, but the R/FR ratios did not show much variation. Considering the results of the hydroponic experiment where high N concentrations limited the nodulation of cowpea, a low N concentration of 0.5 mM was applied in this second experiment which investigated the dual effect light intensities and rhizobial inoculation on cowpea growth characteristics.

Table 3 shows that the interaction between light and inoculant was not significant at $P \leq 0.05$ for ethylene production and nodule mass at 6 and 16 WAS, and for nodule number at 16 WAS. The main effects of inoculants and light intensities were therefore considered and results are reported (non-bold) in the table and discussed. The results indicated no significant differences within light intensities and within inoculants for the analyzed parameters at the indicated periods; except for the nodule number at 16 WAS where TTC9 showed significant less decayed nodules compared to DTC8.Fresh nodules were mainly (92-100%) located at the upper part of the lateral roots (data not shown). At 10 WAS, the interaction between the two factors was significant for ethylene production and nodule mass; indicating a different pattern of the effect of light intensities when cowpea

Table 1: "N x inoculant" interaction on cowpea characteristics (2-way ANOVA).

Treatments	Nodule number			C_2H_4 µmol h^{-1} $plant^{-1}$			Plant dry weight (g)		
	2 WAT	4 WAT[a]	6 WAT[a]	2 WAT[a]	4 WAT	6 WAT[a]	2 WAT	4 WAT	6 WAT[a]
Inoculant x N Effect	ns	**	***	*	ns	***	ns	ns	**
Inoculants									
TSC7	11.28	24.28	27.33	0.535	1.376	1.392	1.06	4.14 a	10.57
TSB2	9.33	10.39	9.94	0.37	0.929	0.329	1.17	4.50 a	8.46
TTC9	8.94	18.39	24.19	0.986	1.051	4.391	1.28	5.37 b	11.82
Inoculant Effect	ns				ns		ns	*	
N (mM)									
0	20.67 b	45.67	40.83	1.275	4.108 b	7.631	0.59 a	3.06 a	4.71
1	10.56 ab	23.89	21.11	2.071	2.136 ab	2.071	1.06 b	4.44 ab	8.18
2.5	09.44 a	26	38.67	0.28	0.363 a	1.516	1.36 bc	4.21 ab	10.51
5	06.56 a	7.44	19.89	0.068	0.103 a	0.881	1.23 bc	4.88 b	10.67
7.5	05.44 a	1.33	2.44	0.047	0.002 a	0.117	1.55 c	5.92 b	13.05
10	06.44 a	1.78	0	0.039	0.0003 a	0.006	1.23 bc	5.50 b	14.6
N Effect	*				*		**	*	

Abbreviations: WAT: Week After Transplanting; ns: not significant (P>0.05)
*significant level: P≤0.05, **significant level: P≤0.01, ***significant level: P≤0.001. TSC7, TSB2, and TTC9 are strains of Bradyrhizobiumyuanmingense. Two-way ANOVA (inoculant x N concentration interaction) was performed using CropStat ver. 7.2. When the interaction was not significant (ns), mean data for inoculants and N concentration (mM) were taken into consideration (non-bold). When the interaction was significant (a), inoculants performed differently across the different N concentrations; and therefore 1-way ANOVA was performed separately for each inoculant (see figures) for discussion. However, mean data (not discussed) are reported in bold in the table. For inoculants, values are the means of 3 replicates for 6 N concentrations giving a total of 18 observations per inoculant at each period. For N concentrations, values are the means of 3 replicates for 3 inoculants giving a total of 9 observations per N concentration at each period. Means with the same letter in a column are not significantly different at the indicated level of significance (Tukey's test).

Table 2: Photon flux density (PFD) inside the different cowpea growing units of the glasshouse and the amount of red (R) and far-red (FR) light intensity ratios.

Height	Light	PFD (µmolm^{-2} sec^{-1})			R/FR
(cm)	Intensity (%)	PPFD	R (660 nm)	FR (730 nm)	
70	100	705.2	61.05	59.31	1.029
	75	535	46.87	45.45	1.031
	50	302.7	30.4	29.57	1.028
	25	154.2	13.37	12.01	1.113
120	100	726.2	63.17	61.47	1.028
	75	537	48.57	47.36	1.026
	50	310.6	31.46	30.06	1.047
	25	185.2	19.37	18.01	1.076

Abbreviations: PPFD: Photosynthetic photon flux density.
Results are the average of periodical measurements during the experimental period

was inoculated by the three strains. Because of the significance of the interaction and the difference within the three strains that it indicates, the mean values (from the three strains) reported in the table aren't discussed. One-way ANOVA was therefore applied to each strain and results shown in (Figure 3) are discussed. No significant difference in nodule mass within the four light intensities was observed for each of the three strains (inoculants), although TSC7-inoculated cowpea had higher nodule mass at higher light intensities (Figure 3a,3b) shows that there is no significant difference in ethylene production within the four light intensities for DTC8-inoculated cowpea. When light intensity was decreased to 75% of the natural light, the ethylene production was significantly reduced for TSC7-inoculated cowpea. However, the 75% light intensity enhanced the ethylene production for TTC9-inoculated cowpea.

Table 4 shows the effects of the two factors (light, inoculant) on some cowpea characteristics at maturity (16 WAS). The interaction between the two factors was not significant for the dry weights of shoot, leaf, and pod + seed; for the N concentration

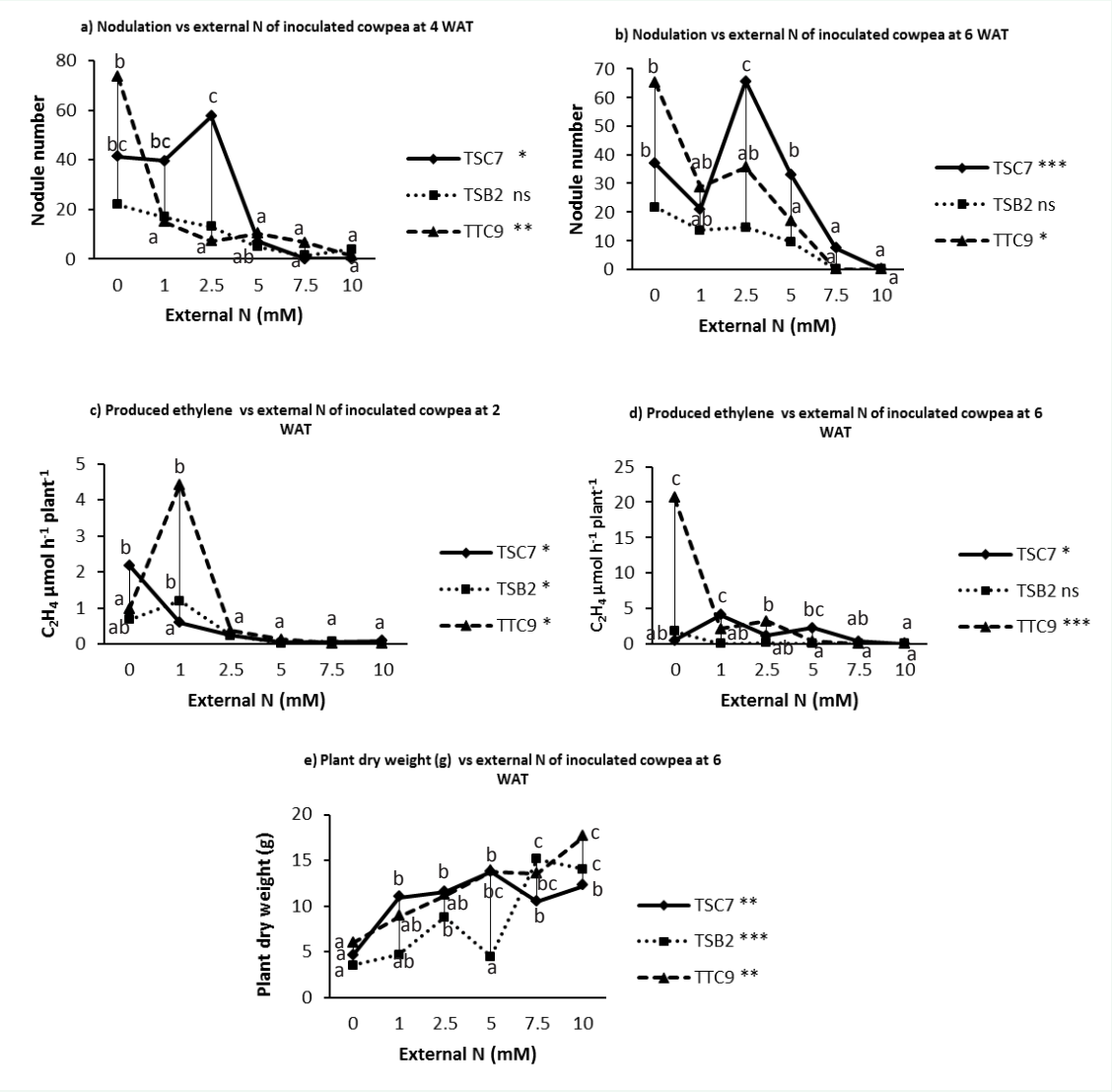

Figure 2 Effect of the application of different N rates (mM) on nodulation (a, b), ethylene production (c, d) and growth of cowpea (e) at different stages after inoculation with three *Bradyrhizobiumyuanmingense* strains (TSC7, TSB2, TTC9)
Abbreviations: WAT: week after transplanting, ns: not significant at $P>0.05$,
*: significant at $P\leq0.05$, **: significant at $P\leq0.01$, ***: significant at $P\leq0.001$.
One-way ANOVA (CropStat ver. 7.2) was applied to each inoculant to determine the level of significance among different N rates, for each analyzed component. In an inoculant line, means followed by the same letter are not significantly different at the indicated level of significance. In c), "a" allocated to the means from N 2.5 to 10 mM represents the three inoculants.

in root, nodule, lead, and pod + seed; and for the N distribution in root, leaf, and pod + seed. The main effects of inoculants and light intensities were therefore considered and results (non-bold) are reported in the table. The statistical analysis was not significant within inoculants for the indicated parameters. The main effect of light intensities shows a significance of the analysis only for dry weights of leaf and pod + seed, N concentration in roots, and N distribution in leaf and pod + seed. When the light intensity decreased to 50% and 25% of the natural light (100%), dry weight of leaves was significantly increased. Although, leaf dry weight in the 75% light intensity was not significantly different with that of the natural light, an important decrease was observed at this light intensity. In contrast to the dry weight of leaves, the dry weight of pod + seed was significantly higher at the 75% light intensity. When the light intensity decreased to 25% of the natural light, the dry weight of pod + seed was significantly reduced compared to the other lights. Results of the one-way ANOVA (Figure 3c) following the significance of the "light x inoculant" interaction show a significance of the analysis only for TSC7-inoculated cowpea. It indicates that light intensities lower than the natural light (100%), significantly reduce the root dry weight. For nodule dry weight, significant differences were observed at TTC9-inouclated cowpea only; with the 75% light intensity enhancing the nodule dry weight (Figure 3d). Interestingly, the 75% light intensity which lowered the leaf production, led to significant higher pod + seed production and root dry weight (TTC9), has significant higher N concentration in roots in comparison to the other light intensities (Table 4). Furthermore, the one-

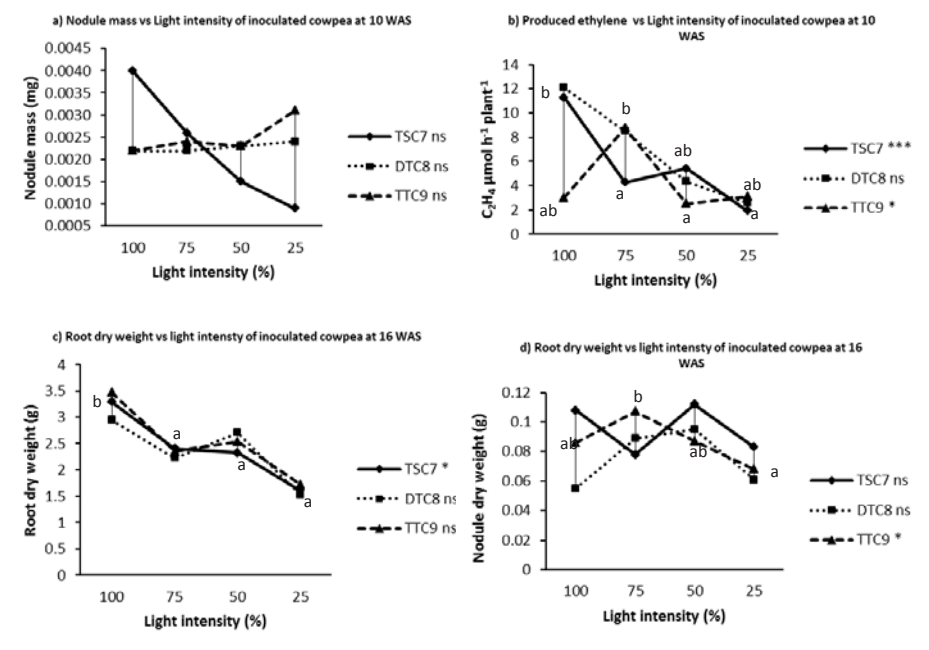

Figure 3 Effect of light intensity on nodule mass (a) and ethylene production (b) at 10 week after sowing (WAS), and on root (c) and nodule (d) dry weight at 16 WAS after inoculation with three *Bradyrhizobiumyuanmingense* strains (TSC7, DTC8, TTC9).
ns: not significant at $P>0.05$,
*: significant at $P\leq0.05$, ***: significant at $P\leq0.001$. One-way ANOVA (CropStat ver. 7.2) was applied to each inoculant to determine the level of significance among different light intensities for each analyzed component. In an inoculant line, means followed by the same letter are not significantly different at the indicated level of significance.

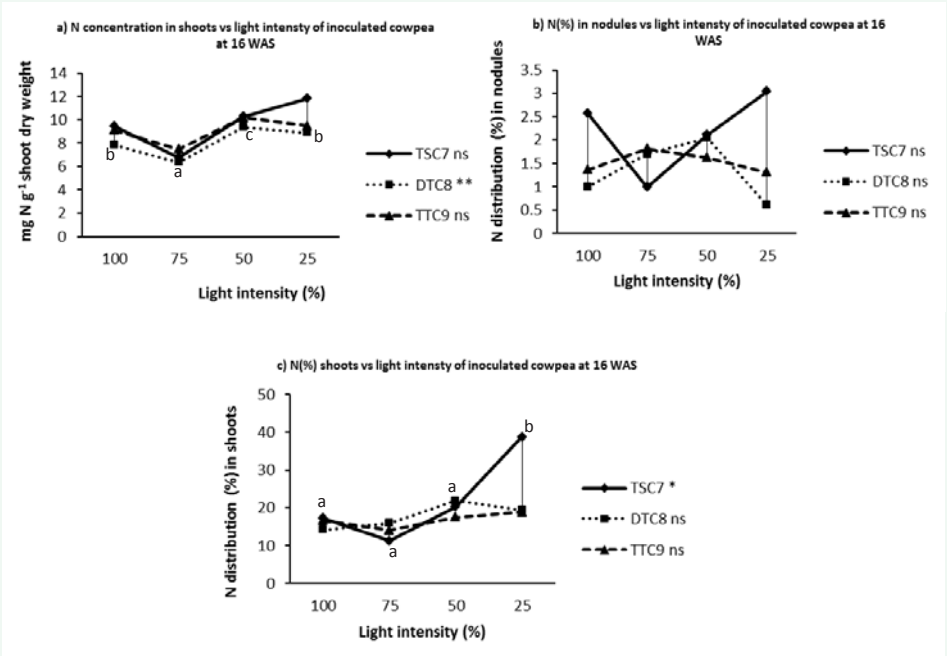

Figure 4 Effect of light intensity on N concentration in shoots (a) and N distribution in nodules (b) and shoots (c) at 16 week after sowing (WAS) and inoculation with three *Bradyrhizobiumyuanmingense*strains (TSC7, DTC8, TTC9).
ns: not significant at $P>0.05$
*:significant at $P\leq0.05$, **: significant at $P\leq0.01$. One-way ANOVA (CropStat ver. 7.2) was applied to each inoculant to determine the level of significance among different light intensities for each analyzed component. In an inoculant line, means followed by the same letter are not significantly different at the indicated level of significance. N distribution corresponds to the percentage of N out of the total N of the plant that is found in a component (root, shoot, leaf, nodule, pod + seed).

way ANOVA revealed that the 75% light intensity significantly decreased the N concentration in shoots, while the 50% light intensity increased it (Figure 4a). Considering the N distribution, only 3.55% of the total plant N was distributed to leaves at 75% light intensity when we consider the main light effect (Table 4). This value is significantly lower than that of N distribution in leaves when the plant is grown at 50% and 25% light intensities. In parallel, the N distribution in pod + seed (68.09%) was significantly higher at 75% light intensity compared to 50% and 25% light intensities. For both N distributions in leaf and pod + seed, no significance difference was observed between the 75% and the 100% light intensities. The N distribution in nodules as revealed by the one-way ANOVA (Figure 4b) was not significant for either inoculant. Considering the N distribution in shoots, the statistical analysis was only significant for TSC7-inoculated cowpea (Figure 4c). Results show that when the light intensity is reduced to 25% of the natural light, the N distribution in shoot is significantly enhanced. No significant difference was observed within the three remaining light intensities.

DISCUSSION

Several studies have been carried out to evaluate the effect of N fertilizers on cowpea growth. However, cowpea N requirement is not well documented. As its seeds contain a less proportion of protein of about 25% [19]; it may require less amount of nitrogen compared to soybean. This author reported that cowpea can fix about 40 kgN/ha in the presence of right rhizobia, which can satisfy the crop nitrogen requirements. Krasova-Wade *et al.* [20] reported that stronger N_2 fixing-potential is obtained from more adapted strains due to host specificity. In the present study, the results of the inoculation test with 4 rhizobial strains are in accordance with Sarr *et al.* [12] who reported slight differences in the fixation patterns of a set of 57 isolates from which the used strains were chosen. Our results show better performances of the strains TTC9 and TSC7, with generally higher N_2 fixing-potential.

Hydroponic experiment

In this experiment, the increase in ethyleneproduction following the supply of 1 mM N at 2 WAT for TTC9- and TSC7-inoculated cowpea indicated the importance of little amounts of initial N for efficient N_2 fixation of cowpea. At later stages (4 and 6 WAT), this N concentration does not significantly inhibit the nitrogen fixation compared to the 0 mM N, and even could significantly boost the plant dry weight production (strain-dependent). This positive effect of the N =1 mM concentration on the nitrogen fixation of cowpea could be related to the non-inhibition of the nodulation. The nodule number was significantly increased at 4 and 6 WAT for TSC7-inoculated cowpea with the supply of N = 2.5 mM. However, this increase in nodule number was not directly translated to significant increases in nitrogen fixation. A possible cause could be nodule's size. Matured bigger nodules with stronger hemoglobin activity, rather han the nodule number, are better drivers for increased N_2 fixation in legume crops. Gil-Quintana *et al.*[21], and King and Purcell [22] suggested an N-feedback inhibition of nitrogen fixation as the possible cause for the decline of nitrogenase activity. The inhibition of nodulation by higher N concentrations in our study supports the already well known observation that heavy supply of nitrogen fertilizers often causes the inhibition of nodulation and nitrogen fixation. Studies on soybean show that when it is only dependent on nitrogen fixation, it has poor growth and low seed yield, because of the early decline in photosynthesis that results from a decreased supply of nitrogen during the vegetative stage. Our results agree with this statement based on the good maintenance of nodulation during the vegetative growth of cowpea in the hydroponic culture after the application of little

Table 3: "Light x inoculant" interaction on cowpea characteristics (2-way ANOVA).

Treatments	C_2H_4 µmol h^{-1} $plant^{-1}$			Nodule mass (mg)			Nodule number (16 WAS)	
	6 WAS	10 WAS[a]	16 WAS	6 WAS	10 WAS[a]	16 WAS	Fresh	Decayed
Inoc. x Light Effect	ns	*	ns	ns	***	ns	ns	ns
Inoculants								
TSC7	5.519	5.715	1.047	2.04	2.25	1.12	85.67	50.58 ab
DTC8	3.964	6.779	0.242	2.28	1.73	0.91	89.09	59.92 b
TTC9	3.483	4.344	0.74	2.31	2.51	1	89.68	38.17 a
Inoculant Effect	ns		ns	ns		ns	ns	*
Light intensity (%)								
100	4.006	8.641	0.342	2.06	2.94	1.01	89.25	51.78
75	4.224	7.181	0.012	2.17	2.29	1.07	86.67	49.22
50	5.274	4.096	0.666	2.35	1.77	1.04	96.22	51.78
25	3.782	2.532	1.686	2.25	1.64	0.92	80.44	45.44
Light Effect	ns		ns	ns		ns	ns	ns

Abbreviations: WAS: week after sowing, ns: not significant (P>0.05),
*significant level: P≤0.05, ***significant level: P≤0.001. TSC7, DTC8, and TTC9 are strains of Bradyrhizobiumyuanmingense, Inoc. : Inoculant
Two-way ANOVA (inoculant x Light intensity interaction) was performed using CropStat ver. 7.2. When the interaction was not significant (ns), mean data for inoculants and light intensities (%) were taken into consideration. When the interaction was significant (a), inoculants performed differently across the different light intensities, and therefore 1-way ANOVA was performed separately for each inoculant (see figures) for discussion. However, mean data (not discussed) are reported in bold in the table. For inoculants, values are the means of 3 replicates for 4 light intensities giving a total of 12 observations per inoculant at each period. For light intensities, values are the means of 3 replicates for 3 inoculants giving a total of 9 observations per light intensity at each period. Means with the same letter in a column are not significantly different at the indicated level of significance (Tukey's test).

Table 4: "Light x inoculant" interaction on cowpea characteristics at 16 WAS (2-way ANOVA).

Treatments	Dry weight (g)					N concentration (mg N g^{-1} dry weight)					N distribution (%)				
	Root[a]	Nodule[a]	Shoot	Leaf	Pod + seed	Root	Nodule	Shoot[a]	Leaf	Pod + seed	Root	Nodule[a]	Shoot[a]	Leaf	Pod + seed
Inoc. x L Effect	***	*	ns	ns	ns	ns	ns	*	ns	ns	ns	*	*	ns	ns
Inoculants															
TSC7	2.4	0.095	6.17	1.87	5.85	16.41	63.43	9.58	19.44	29.04	13.36	2.18	21.89	11.46	51.11
DTC8	2.35	0.075	5.63	1.35	9.91	17.29	56.01	8.11	22.39	28.39	13.69	1.33	17.87	16.11	51.27
TTC9	2.52	0.087	5.85	1.54	6.8	17.65	58.65	9.05	22.38	29.85	12.98	1.56	17.14	11.22	57.11
Inoc. Effect			ns	ns	ns	ns	ns		ns	ns	ns			ns	ns
Light intensity (%)															
100	3.32	0.083	5.54	1.52 ab	6.54 b	15.51 a	66.39	8.75	21.73	27.71	15.67	1.7	16.07	11.92 ab	54.64 ab
75	2.32	0.091	5.91	0.41 a	8.86 c	20.91 b	58.8	6.88	21.07	28.86	14.22	1.58	12.94	3.55 a	68.09 b
50	2.51	0.098	6.4	2.29 b	6.72 b	14.27 a	63.57	9.94	24.03	29.62	11.35	1.93	19.89	16.85 b	49.99 a
25	1.62	0.071	5.64	2.12 b	3.74 a	17.54 ab	49.56	10.06	18.71	29.95	12.48	1.65	25.67	16.15 b	44.05 a
Light Effect			ns	**	*	*	ns		ns	ns	ns			*	*

Abbreviations: WAS: week after sowing, ns: not significant (P>0.05)
* significant level: P≤0.05, *** significant level: P≤0.001. TSC7, DTC8, and TTC9 are strains of Bradyrhizobiumyuanmingense, Inoc. : Inoculant
Two-way ANOVA (inoculant x Light intensity interaction) was performed using CropStat ver. 7.2. When the interaction was not significant (ns), mean data for inoculants and light intensities (%) were taken into consideration. When the interaction was significant (a), inoculants performed differently across the different light intensities, and therefore 1-way ANOVA was performed separately for each inoculant (see figures) for discussion. However, mean data (not discussed) are reported in bold in the table. For inoculants, values are the means of 3 replicates for 4 light intensities giving a total of 12 observations per inoculant at each period. For light intensities, values are the means of 3 replicates for 3 inoculants giving a total of 9 observations per light intensity at each period. Means with the same letter in a column are not significantly different at the indicated level of significance (Tukey's test).

amounts of N (1 mM). In other words, when the inoculation of cowpea with adequate bradyrhizobialstrains is combined with an appropriate N supply, better nitrogen fixation and plant growth can be obtained. It has been recognized and demonstrated that application of small quantity of nitrogen fertilizer enhances early vegetative growth [23]. Earlier, Burries [24] stated that nitrogen has a stimulating effect on root activity and rooting pattern of cowpea, and allows seedlings to make a good start before nitrogen fixation has a chance to occur. In the case of soybean, Harper [25] reported that both soil N and symbiotic N are required for optimum production. Later, when investigating the effect of external nitrate application on soybean, Streeter [8] found that external N concentrations of 1-2 mM enhanced soybean nodulation, while higher concentrations above 5 mM inhibited the nodulation phenotype and N_2 fixation activity. The inhibition of the nodulation and N_2 fixation intervenes when N is supplied at the nodule-forming zone, which happens to be the upper lateral root area for cowpea as shown in this study, and soybean. Many hypotheses are proposed for the cause of nitrate inhibition of nodulation and nitrogen fixation, i.e. carbohydrate deprivation in nodules [26], feedback inhibition by a product of nitrate metabolism such as glutamine [27], as paragine [28], an indirect effect of nitrates via an increase in the resistance to oxygen diffusion across the nodule cortex resulting in a reduced availability of oxygen to the bacteroids which restricts their respiration [29,30]. In an attempt to reconcile these hypotheses, Minchin *et al.* [31] proposed that nitrate inhibition of nitrogen fixation occurs in two stages: (i) an initial increase in oxygen diffusion resistance, followed by (ii) the entry of nitrate into the bacteriod region with inhibition and/or damage resulting from its metabolism. In our study, the hydroponic culture facilitated the homogeneous distribution of the supplied N to the whole root system, leading to an easier inhibition of the nodulation at higher N concentrations. Fujikake *et al.* [32] studied the effect of various combinations of culture solution with 0 mM or 5 mM nitrate on nodulated soybean during three successive weeks. They found that the number of the already existing soybean nodules was not significantly affected in contrast to the nodule weight which was significantly affected by the period of nitrate application. Similarly, significant repression of acetylene reduction activity per plant was observed following the addition of nitrate, while the withdrawal of the nitrate and its replacement by nitrate-free solution led to higher ARA compared to the control (no nitrate). This indicates that when nitrates are supplied at later stages (3-4 WAS) to legume crops, after the establishment of the nodulation, the double advantage of the N_2 fixation and potential of the nitrates could be obtained.

Solid experiment

In the second study, the low concentration of 0.5 mM N was purposely supplied to plants at 3 WAT, once the nodulation was already established, owed that there was no risk of nodulation inhibition. N_2 fixation depends on photosynthesis to provide ATP for energy and carbon compounds as electron donors [33]. This suggests that appropriate light is important for optimal biological nitrogen fixation when other factors such as effective rhizobial strains, moisture, carbon balance, temperature, and supply of assimilates are satisfied. Studies on the effect of light intensity on the growth of legume crops have been mostly carried out on soybean. The reduction of light reaching the legume canopy when intercropped with maize was about 30% - 50% of the total incoming radiation and starts around 30- 35 days after seeding [34]. In this condition of low light intensity, decrease of soybean grain yield has been reported [35]. Polthanee *et al.* [36] studied

the influence of low light intensity on the growth and yield of four soybean cultivar. They found that while low intensity of about 30% of normal light negatively affected the yield of three cultivars, it did not reduce the yield of the fourth; indicating that the influence of light on legume growth is variety-dependent. Pursuing the research of the effect of light intensity on seed quality of soybean, Bellaloui *et al.* [37] reported that a 50% reduction of normal light intensity resulted in lower protein, lower nitrogen assimilation, and lower chlorophyll concentration. The significant lower N distribution (%) in pod + seed at 50% and 25% light intensities observed in our study after inoculation with appropriate bradyrhizobial strains, are in agreement with the above results. Although shading affects growth and yield, only a few studies have been made on the effect of shading on N_2 fixation. Shading reduces nodule number, nodule size and N_2 fixation on cowpea [38], lupin [39], soybean [40]. Studying the effect of solar radiation regimes on growth and N_2 fixation of soybean, cowpea, and bushbean, Eriksen and Whitney [41] reported that a 27% of normal light intensity highly reduced the crops yields. However among the three plants, cowpea was the least shade tolerant, and produced more dry matter at full sun than either soybean or bushbean. Results in the present study where the normal light (100%) and the 75% normal light intensities produced the higher ARA values at 10 WAS, and the higher pods + seeds yields at 16 WAS, are in agreement with these previous studies, and indicate that cowpea prefers high to slightly high light intensities for optimal growth. Below these light intensities (50% and 25%), the leaf production was promoted while the pod + seed yield was significantly reduced, presumably due to the reduced availability of photosynthetic products to support N_2 fixation [42]. An important outcome demonstrated in this study is the significant lower leaf production in the 75% light intensity and the highest cowpea pod + seed production this light intensity provoked. As a consequence, the N distribution was significantly higher in pods + seeds. The loss of leaves observed at the 75% light intensity during the late growth stages could be a good strategy developed by cowpea, enabling the redistribution (translocation) of leaves' N to pods; and contributing to enhance the protein content in seeds. This attribute could be particularly important for humans when cowpea production is rather oriented to human consumption (seeds) than livestock feeding (aerial biomass). However, the physiological mechanisms involved in this loss of leaves are not clearly identified and may require further investigations. Therefore, reducing the natural light (above 700 µ $molm^{-2}$ sec^{-1}PPFD) to about 535 µ $molm^{-2}$ sec^{-1} PPFD, contributes to maximize nitrogenase activity (NA) and cowpea production. This value of PPFD could be considered as optimum for cowpea growth, and cowpea seed quality (N content) could be improved if appropriate lightning can be developed in cowpea producing areas. However, the optimum photosynthetic photon flux density level varies according to species. In many studies, *Nostoc* showed an increase in NA up to 100-150 µ $molm^{-2}$ sec^{-1} PPFD, after which maximal NA rates are maintained [43,44]. Rychert *et al.* [45] found that a *Microcoleus-Scytonema-Nostoc-Collema* crust reached optimal NA rates at 200 µ$molm^{-2}$ sec^{-1}PPFD.

CONCLUSION

This study reveals that cowpea N_2 fixation and growth can be enhance or maintained by external N supply at low rates (up to1mM), following inoculations with efficient rhizobia. When efficient rhizobial inoculations and appropriate supply of N are carried out, N_2 fixation and cowpea production can be improved when the light intensity of the cultivated area has optimal photosynthetic photon flux density of 535 µ$molm^{-2}$ sec^{-1}(75%of normal light). At this level, a higher grain production and seed N concentration are reported at maturity. The results from the light intensity trial indicate that cowpea fits well with mix cropping systems. The study reveals that cowpea symbiosis is dependent to the combination of various factors, that if set up at optimum levels, can greatly enhance its production. This work provides additional information on the nodulation, N_2 fixation, and growth of cowpea, under different nitrogen concentrations and solar radiation regimes.

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