DETERMINING MORE PRECISE OPTIMUM NITROGEN RATE FOR EFFECTIVE TESTING OF RHIZOBIA ON GROUNDNUT (*Arachis hypogaea* L.) IN THE GLASSHOUSE

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ABSTRACT

A study was conducted to determine the adequate mineral N requirement of groundnut (Arachis hypogaea L.) for comparison with rhizobia strains in the glasshouse. Ten treatments, six mineral N rates (0, 0.2, 0.5, 1.0, 3.0 and 5.0 mmoles of N twice a week), three indigenous rhizobia (KBU 026, SNN 335 and SNN 345) and a standard rhizobia strain (NC 92) were involved. These were laid down in a Randomized Complete Block Design (RCBD), replicated five times with SAMNUT 24 as a test crop. The plants were harvested at six weeks after sowing. Shoot dry matter, flowers and N uptake of the plants, were determined, and subjected to analysis of variance (ANOVA). The results showed significant differences ($P < 0.01$) among the treatments in influencing all the parameters. The 0.5, 1.0, 5.0, 3.0, 0.2 and SNN 335 gave 88, 86, 85, 39, 23 and 15% higher shoot dry matter relative to 0 mmoles N control, respectively. While increases of 71, 67, 50, 50, 71, 33 and 50% flowers were observed on application of 0.5, 3.0, 5.0 ,1.0, KBU 026, NC 92 and SNN 335, respectively, relative to 0 mmoles N. Therefore, the application of 0.5 mmoles twice week^{-1} gave higher influence on shoot dry matter, flowers and N uptake relative to other N rates. KBU 026 and SNN 335 performed similar to the application of other N rates as shown by the percentages. The study indicates 0.5 mmoles twice a week^{-1} (1.0 mmoles N week^{-1}) as the precise optimum N application rate that could facilitate N economy and accurate effectiveness testing of rhizobia strains on groundnut in the glasshouse.

Key words: groundnut, legumes, mineral nitrogen, Nitrogen fixation, Rhizobia.

INTRODUCTION

Nitrogen (N) is a major nutrient limiting crop production (Date, 2000; Cummings, 2005; Gohari and Niyaki, 2010). It is the main element needed in many important processes in plant metabolism such as synthesis of chlorophyll, proteins and nucleic acids of bacteria and associated plants (Unkovich *et al*., 2008; Gohari and Niyaki, 2010). Legumes generally require large amounts of N to produce high seed yields because of their high protein contents (Singh and Oswalt, 1995; Thilakarathna *et al*., 2019; Kouadio *et al*., 2019). The use of mineral sources for N-fertilization is costly, and has a tendency to contaminate the environment through inefficient utilisation and leaching (Bohlool *et al*., 1992; Sanginga, 2003; Yakubu *et al*., 2010). On the other hand, symbiotic N_2 fixation between legumes and root nodule bacteria (rhizobia) is inexpensive, convenient and an environmentally sound option to provide N for many farming systems (Nelson, 2004; Yakubu *et al*., 2010). Hence, inoculation of legumes with effective N_2 fixing strains of rhizobia is a well-established farming practice (Manyong *et al*., 2001; Rivas, 2009; Singh *et al*., 2011; Peix *et al*., 2015). Regular failure of rhizobia inoculants, meant to facilitate N_2 fixation, when introduced to new environments calls for selection of effective

indigenous strains adapted to those environments (Brockwell and Bottomly, 1995; Deaker *et al*., 2004; Sajjad *et al*. 2008; Weilbo, 2012). Hence, there is a need to select for effectiveness among inoculant strains in the glasshouse using appropriate techniques. These, involve comparison with mineral N application and control of the operating environment, before testing the selected strains in the field (Yates *et al*., 2016).

Glasshouse experiments are often required to screen isolates of root nodule bacteria obtained from indigenous populations in centres of origin of the host legumes. They must then be screened for effectiveness in N_2 fixation with the target and non-target hosts (Howieson *et al*., 2016). Obtaining the optimum rate of mineral N for the legume in the N-fed control comparable with N_2 fixed by the rhizobia is key to their successful screening (Waswa *et al*., 2014). Yet, no standard optimum rate of N is universally recommended for all crops. The N applied must be sufficient for maximum growth and development of the crop, but neither deficient nor in excess, both of which hinder the crop growth (Yates *et al*., 2016). Growth of the crop due to optimum N application is then compared with N_2 fixation through symbiosis between legumes and the rhizobia strains. Similarly, the negative N or uninoculated control simulates the worst performance of the crop on deprivation of N (Waswa *et al*., 2014).

Abdullahi (2018) conducted a study using three N rates that were relatively wide apart, which indicated 1.0 mmole N as the optimum rate for groundnut in the glasshouse. Therefore, there is a need to use more varied concentrations, which are close to each other to confirm or come up with a more precise optimum rate of mineral N application for more efficient symbiotic effectiveness of rhizobia strains on groundnut in the glasshouse.

MATERIALS AND METHODS

Treatments and experimental design

The study was conducted under axenic N-free conditions in the glasshouse to confirm a more precise N rate for N fixation by groundnut, based on finding of Abdullahi (2018) that 1.0 mmole of N weekly as the optimum application rate of N for groundnut in the glasshouse using potassium nitrate as N source. Ten treatments were evaluated including six N levels (0, 0.2, 0.5, 1.0, 3.0 and 5.0 mmol of N) applied twice weekly using potassium nitrate (KNO_3) as N source and three indigenous rhizobia isolates (SNN 382, SNN 335 and KBU 026) for comparison as well as a standard commercial groundnut rhizobia strain (NC 92), obtained from the Centre for Rhizobium Studies (CRS), Murdoch University, Western Australia. The treatments were arranged in a Randomized Complete Block Design (RCBD) so as to capture the gradient in solar radiation in the glasshouse, replicated five times with SAMNUT 24 as a test crop. It is an extra early maturing (75 – 90 days), commercial groundnut genotype, released in 2011 by the Institute for Agricultural Research, Ahmadu Bello University, Zaria, Nigeria.

Surface sterilization, pre-germination of the seeds, sowing and thinning

The seeds were surface sterilized by first immersing for 10 seconds in 70% (v/v) ethanol, followed by 3 minutes in 3% (v/v) hydrogen peroxide, then 6 rinses of sterile deionised (DI) water. This was followed by pre-germination in moist paper towel placed in a lunch box under sterile conditions at 28^{o}C for 3 days. Four of the pre-germinated seeds were then sown per pot and later thinned to two at 2 weeks after sowing (WAS).

Inoculant source, preparation and application

The three indigenous rhizobia isolates (SNN 335, KBU 026 and SNN 345) used for comparison and NC 92 were grown on Yeast Mannitol Agar (YMA) plates (Vincent, 1970) for 7 days and colonies were ensured to be uniform, using a light microscope. The inoculant for each was prepared by carefully wiping primary cultures with sterile wooden applicator sticks into 10 ml of 1% w/v sucrose solution. The optical density (OD) of the inoculants was measured at 600 nm using spectrophotometer and adjusted to ensure uniformity of OD 1 (estimated as10^6 cells). A millilitre of the inoculant was used to drench each seed in the inoculated pots at sowing according to the treatments.

Plant growth conditions

The plants were grown in 10 kg capacity pots lined at the bottom with absorbent paper, containing a 1:1 ratio of a mixture of steam sterilized, N-free coarse lawn and yellow sands. The pots were pre-sterilized by steaming at 99^{o}C for 3 hours, flushed twice with hot, sterile, deionised (DI) water to leach out any trace of inorganic N. Sterile polyvinyl chloride tubes (25 cm in diameter) were inserted into the sand mixture for supply of sterile DI water and nutrients. The surface of the soils in the pots were covered with alkathene beads after application of the treatments (Yates *et al.,* 2016). The nutrient solution composition and microbiological procedures to avoid cross-contamination, including CRS N-free nutrient solution were as described by Howieson *et al.* (1995). The plants were grown in the glasshouse at 28/20^{o}C day/night temperatures for 6 weeks. Uniform amount of sterile DI water was applied to each pot daily, based on the plant's growth requirement, while the CRS N-free nutrient solution was also uniformly applied to the plants based on their requirement, on weekly basis.

Data collection and statistical analyses

The root nodules induced by SNN 335, KBU 026 and SNN 345 indigenous isolates and NC 92 were scored using a scoring system developed by Abdullahi *et al*. (2018) based on size, distribution and number of nodules per plant in each of the inoculated treatments (Table 1) and the number of flowers pot$^{-1.}$ The shoots were harvested at the end of the experiment (6 WAS), oven dried at 60^{o}C for 48 hours and weighed. The N concentrations in the plant shoots was determined using LECO TruMac N combustion N determiner, LECO, FP 528 (LECO, 2010) and subsequently uptake by the plants were determined. The data were subjected to Analysis of Variance (ANOVA) at 5 % level of significance, using IBM SPSS Statistics 20 and where F values were significant, the means were separated using Tukey HSD.

TABLE 1. THE NODULE SCORING SYSTEM

Score	Description
0	No nodules
1	0 - 10 small nodules on the tap root and few to many minute/small nodules on the lateral roots
2	> 10 small nodules on the tap root and many minute nodules on the lateral roots
3	> 10 medium nodules on the tap root and many minute/small nodules on the lateral roots
4	20 -100 medium nodules on the tap root and 20 - 150 medium/large nodules on lateral roots
5	>100 large nodules on the tap root and >150 large nodules on the lateral roots

Minute: < 1 mm, small: 1-2 mm, medium: 3 - 4 mm and large: > 4 mm in diameter

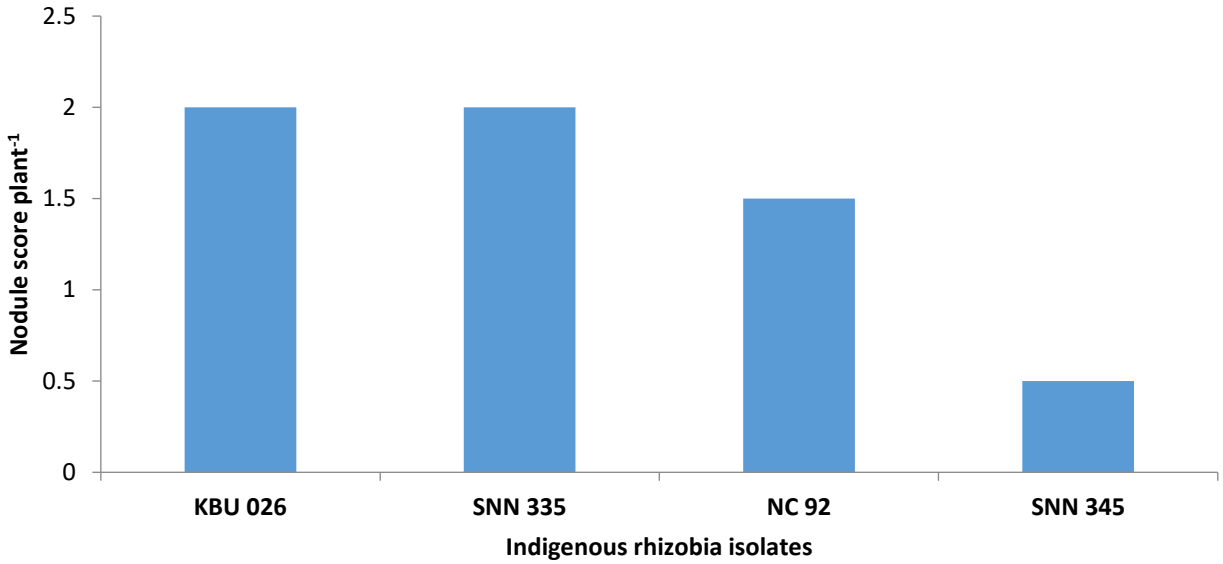
RESULTS

Plants inoculated with KBU 026 and SNN 335 had 25% higher nodule scores than those inoculated with NC 92, while those inoculated with SNN 345 had the least nodule score (75% lower than those induced by KBU 026 and SNN 335) (Fig. 1). This indicates the compatibility of the rhizobia isolates with SAMNUT 24. The hygiene of the experiments was ensured by the absence of nodules on the plant roots of all the control (0 mmoles N twice week^{-1} and uninoculated) as well as the N-fed plants throughout the experiment.

The influence of the treatments on shoot dry matter of SAMNUT 24 is shown in Fig. 2. Significant differences ($P < 0.01$) were observed among the treatments in influencing the shoot dry matter of SAMNUT 24. The highest shoot dry matter was observed on application of 0.5 and 1.0 mmole N. Even though, the application of 3.0 and 5.0 mmoles N gave lower shoot dry matter than the application of 0.5 mmoles N, they were statistically similar to the application of 1.0 mmoles N and in turn similar to the application of 0.2 mmoles N and inoculation with SNN 335. The application of 0.2 mmoles N and inoculation with KBU 026, SNN 335 and SNN 345 were also similar in influencing the shoot dry matter. Inoculation with SNN 335, SNN 345, NC 92 and the application of 0 mmoles N were also similar. The 0.5, 1.0, 5.0, 3.0, 0.2 mmoles and SNN 335 gave 88, 86, 85, 39, 23 and 15% higher shoot dry matter relative to the 0 mmole N control, respectively.

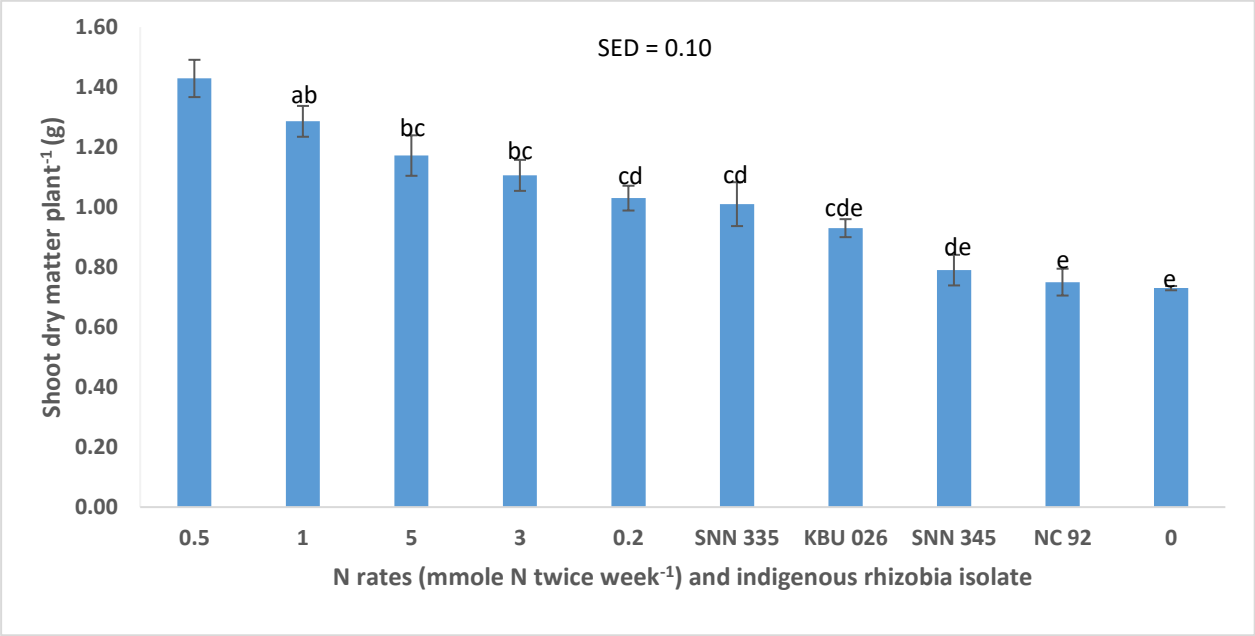
Fig. 3 shows the effect of the treatments on the number of flowers of SAMNUT 24. There was also significant difference ($P < 0.01$) among the treatments in terms of the number of flowers of the plants. The highest numbers of flowers were observed on application of 0.5, 3.0, 5.0 and 1.0 mmoles N and inoculation with KBU 026. While the application of 5.0, 1.0 mmoles N and inoculation with KBU 026 gave lower number of flowers than 0.5 and 3.0 mmoles N, and were similar to all other treatments (NC 92, SNN 335, SNN 345 and the application of 0.2 and 0 mmoles N), inoculation with NC 92, SNN 335 and SNN 345 and application of 0.2 and 0 mmoles N were similar. Increases of 71, 67, 50, 50, 71, 33 and 50% flowers were observed on application of 0.5, 3.0, 5.0, 1.0, and inoculation with KBU 026, NC 92 and SNN 335, higher than the 0 mmoles N treated plants, respectively.

The effect of the treatments on N uptake by SAMNUT 24 is shown in Fig. 4. The applications of 0.5, 1.0, 3.0 and 5 mmoles N and inoculation with KBU 026 had higher influence on N uptake of the plants than the rest of the treatments (application of 0.2 and 0 mmoles N and inoculation with NC 92, SNN 345 and SNN 335). The applications of 0.5, 1.0, 3.0 and 5 mmoles N and inoculation with KBU 026 gave 63, 74, 75, 78 and 68% higher N uptake by the plants over the 0 mmoles N treated plants.



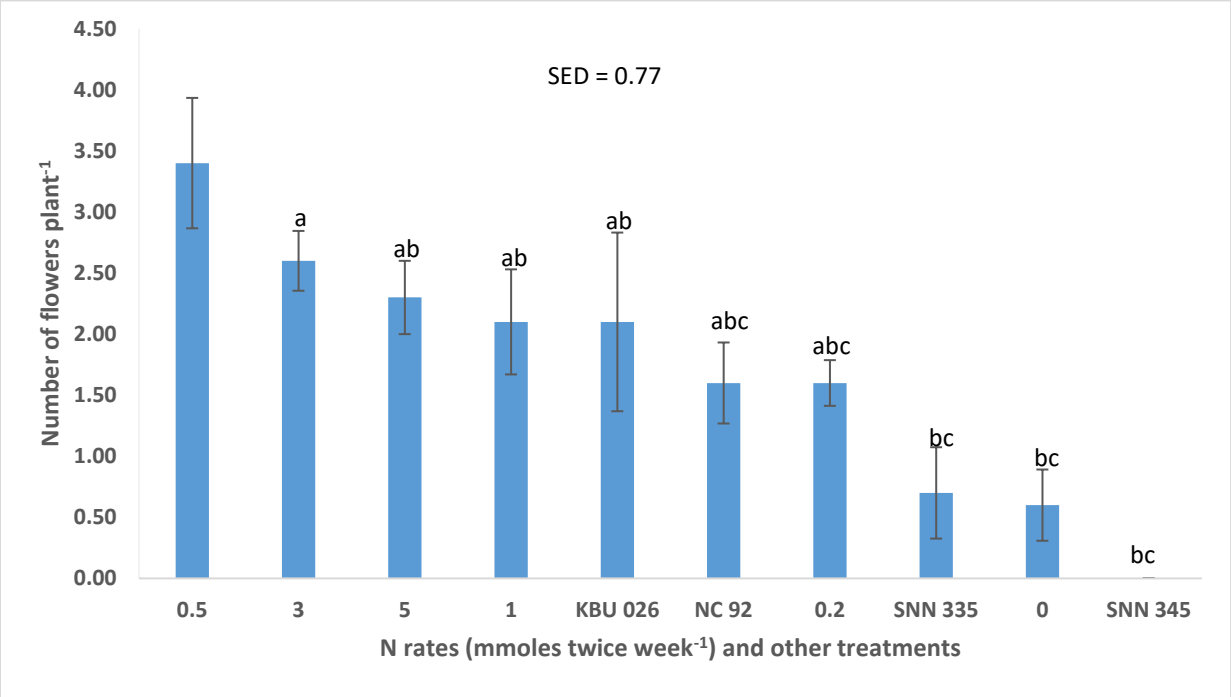
KBU 026, SNN 335 and NC 92 are indigenous rhizobia isolated from the Nigerian Savanna, while NC 92 is an international commercial rhizobia strain for groundnut.

FIGURE 1. NODULE SCORES OF SAMNUT 24 PLANTS ON INOCULATION WITH NIGERIAN INDIGENOUS RHIZOBIA ISOLATES AND NC 92.



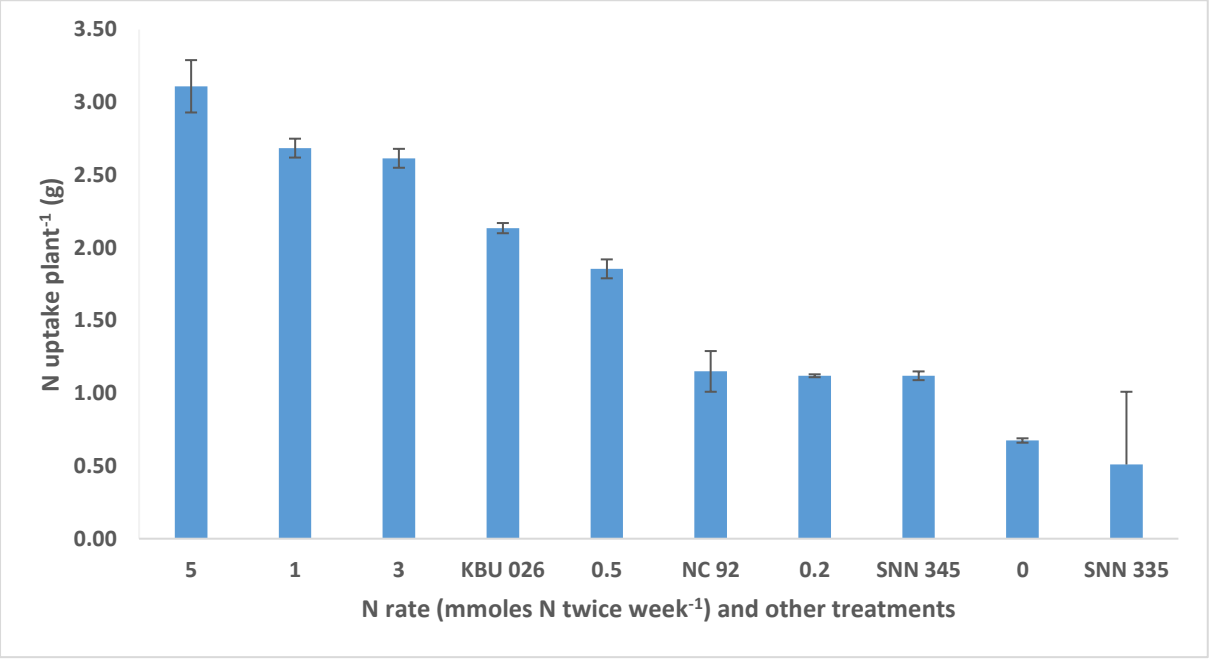
KBU 026, SNN 335 and NC 92 are indigenous rhizobia isolated from the Nigerian Savanna, while NC 92 is an international commercial rhizobia strain for groundnut.

FIGURE 2. SHOOT DRY MATTER OF THE SAMNUT 24 IN RESPONSE TO N APPLICATION AND INOCULATION INDIGENOUS RHIZOBIA ISOLATES. VERTICAL BARS REPRESENT STANDARD ERROR (±).



KBU 026, SNN 335 and NC 92 are indigenous rhizobia isolated from the Nigerian Savanna, while NC 92 is an international commercial rhizobia strain for groundnut.

FIGURE 3. NUMBER OF FLOWERS OF SAMNUT 24 AS INFLUENCED BY N APPLICATION AND INOCULATION WITH INDIGENOUS RHIZOBIA ISOLATES. VERTICAL BARS REPRESENT STANDARD ERROR (±).



KBU 026, SNN 335 and NC 92 are indigenous rhizobia isolated from the Nigerian Savanna, while NC 92 is an international commercial rhizobia strain for groundnut.

FIGURE 4. N UPTAKE OF SAMNUT 24 PLANTS IN RESPONSE TO N APPLICATION AND INOCULATION WITH INDIGENOUS RHIZOBIA ISOLATES. VERTICAL BARS REPRESENT STANDARD ERROR (±).

The response curves of shoot dry matter and N uptake of SAMNUT 24 to the treatments is shown in Fig. 5. The shoot dry matter yield reached its peak at the application of 0.5 mmole of N, indicating it to be the critical N application rate. The shoot dry matter eventually declined afterwards, even though the N uptake of the plants continued to increase with N application, indicating the higher rates supplied excessive N to the plants that retarded their growth.

DISCUSSION

Nodulation of the inoculated plants with the rhizobia isolates indicates compatibility of the isolates' nod genes with the flavonoids released by SAMNUT 24 plants, an indication of symbiotic relationship for N_2 fixation and a favourable environment for the process (Lira Jr *et al.*, 2015; Andrews and Andrews, 2017). Lack of nodulation of the N treated and the control (0 mmoles N) plants was a clear indication of lack of cross-contamination in the system, an assurance on the quality of the results that enable selection of (Waswa *et al.*, 2014; Yates *et al.*, 2016). The application of 0.5 mmoles of N twice week^{-1} indicated optimum influence on plant growth and reproductive parameters; shoot dry matter and flowers relative to all other rates of N application (Fig. 2 and 3). This was clearly confirmed by the N calibration curve of the relationship between shoot dry matter and N uptake of the plants (Fig. 5). The result was in conformity with earlier finding of the critical rate of N application to the plants as 1.0 mmoles N week^{-1} in an experiment using four N rates (0, 1.0, 5.0 and 10.0 mmoles N week^{-1}) and NC 92 by Abdullahi (2018). Application of 0.5 mmoles twice week^{-1} supplied exactly the same amount of N. Therefore, no additional growth and development in shoot was derived from the additional N application, even though it led to higher N uptake in the plant tissue. This indicated that the additional N absorbed by the plants has not been assimilated by the plants for the formation of chlorophyll, amino acids, and nucleic acids as found by Karanja *et al.* (2011) and Sprent *et al.* (2013). The split application of N under glasshouse conditions was, therefore, unnecessary since there was no additional advantage derived from the practice. The application of the lowest rate of 0.2 mmoles of N twice week^{-1} and the higher rates of N application; 1.0, 3.0 and 5.0 mmoles of N twice a week^{-1} only ensured a precise N response curve from deficiency through sufficiency to excessive amounts of N supply to the plants (Fig. 5).

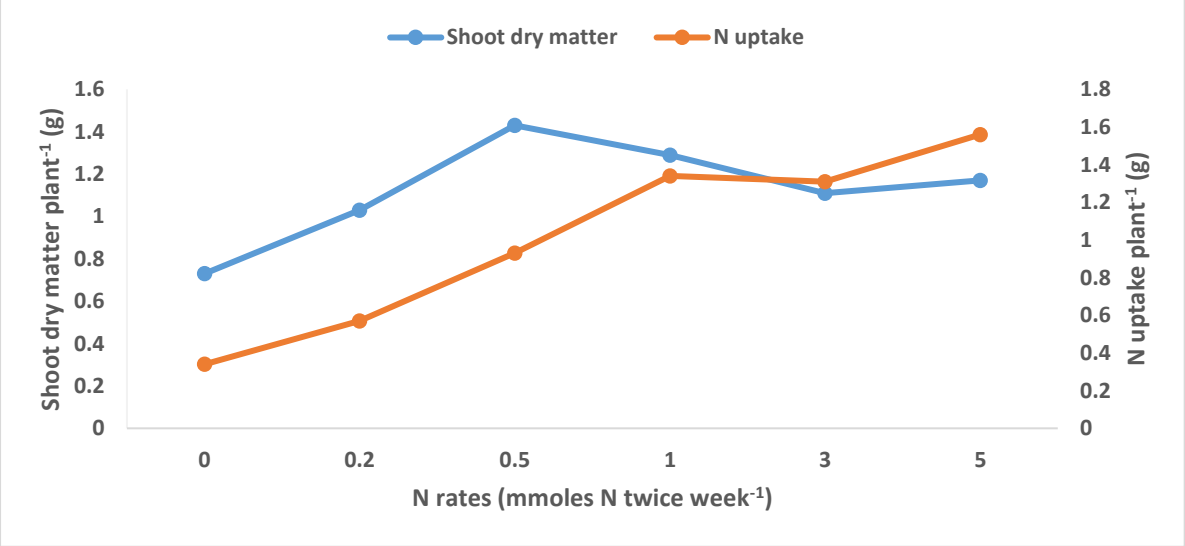


FIGURE 5. RESPONSE CURVE CURVES OF SHOOT DRY MATTER AND N UPTAKE OF SAMNUT 24 DUE TO N APPLICATION.

This confirms the Liebig's law of "the minimum" that growth is controlled by the factor in lowest proportion relative to its requirement (Browne *et al*., 1942). Hence, further application of N, beyond the critical level could not influence further growth and development of the plants as observed by Yates *et al*. (2016). The findings also corroborate earlier ones that groundnut growth and yield response to applied N, varied from nil to an increase, directly related to fertilization (Cummings, 2005; Smith *et al*., 1993) and high rates of N fertilization above optimum requirement of the plants reduces growth (Smith *et al*., 1993; Yates *et al*., 2016). The findings, however, contradict report of Giller *et al*. (1987) of low response of groundnut to added fertilizer, since this study clearly shows high response of the crop to mineral N application. The observations of these authors could be due to variations in N sources used as groundnut prefers nitrate (NO_3^-) to ammonium (NH_4^+) form of N for its growth and nutrition (Ribeiro *et al.,* 2012). It may also be due other environmental, crop or soil factor, as residual fertilizer N that may have hindered or obstructed the N response as observed by Ajeigbe *et al.* (2014), especially under field conditions.

On the other hand, the decline in growth of the plants caused by the treatments above 0.5 mmoles twice week^{-1} was an indication of excessive N supply to the plants that reduced yield due to toxicity (Bennet, 1993; Marshner, 1995; Yates *et al*., 2016). This was clearly shown by the performance of the plants on application of 1.0, 3.0 and 5.0 mmoles twice week^{-1}.

The results also indicated the indigenous rhizobia isolates included in the study, especially SNN 335 and KBU 026 had moderate rate of N_2 fixation. They also had influence on plant growth similar to those of the applications of 1.0, 3.0, 5.0 mmoles of N twice week^{-1} and higher than the standard inoculant strain (NC 92). However, SNN 345 showed less effectiveness in N_2 fixation through lower influence on the shoot dry matter, number of flowers and N uptake of the plants. This indicates that selection of rhizobia for effectiveness is possible with similar the system.

CONCLUSION

The study indicated 0.5 mmoles N twice week^{-1} (a total of 1.0 mmole week^{-1}) as the precise optimum rate of mineral N application to groundnut for efficient effectiveness testing of rhizobia isolates in the glasshouse. An assessment of indigenous rhizobia isolates in the study also indicated SNN 335 and KBU 026 as relatively effective rhizobia isolates.

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