Chapter

Inoculant Formulation and Application Determine Nitrogen Availability and Water Use Efficiency in Soybean Production

Canon E.N. Savala, David Chikoye and Stephen Kyei-Boahen

Abstract

Inoculation of suitable rhizobia enhances biological nitrogen fixation in soybean production and are economically viable for use among smallholder farmers due to its low price over inorganic commercial fertilizer blends. In Mozambique, inoculants are available in liquid or solid form (powder/peat or granular). Field studies were conducted in 2017 and 2018 seasons in three agroecologies (Angonia, Nampula and Ruace) in Mozambique to evaluate the performance of inoculants when applied directly to soil and on seed before planting. Data on nodulation, plant growth, nitrogen fixed, ^{13}C isotope discrimination related water use efficiency, yield and yield components were analyzed in Statistical Analysis System® 9.4. Nodulation, yield, and yield components were significant for the different application methods, and solid form tended to be better than liquid form. The nitrogen derived from atmosphere (%Ndfa) were 45.3%, 44.2% and 43.6% with a yield of 2672, 1752 and 2246 kg ha^{-1} for Angonia, Nampula and Ruace, respectively. Overall, inoculants applied on soil or seed increase the amount of biologically fixed nitrogen and has the potential of improving soybean productivity in Mozambique.

Keywords: carbon isotope, nodulation, promiscuous, soybean, rhizobia, water use efficiency, yield

1. Introduction

1.1 Inoculation history in Africa (Mozambique)

Soybean production in Mozambique is gaining pace through land area expansion at the expense of other crops mainly driven by lucrative prices and the unsatisfied market demand particularly the poultry industry [1]. However, climate change effects, low soil fertility and poor crop management keep yield below the world average. Some farmers are seeking solutions to these challenges by adopting region adapted improved varieties, use of soil amendments such as organic manures and inoculant application to improve nitrogen availability. Nitrogen is the most limiting nutrient in soybean production due to its high uptake by plants, vulnerability to leaching, denitrification and removal through crop harvest [2]. Inoculation of rhizobia enhances biological nitrogen fixation (BNF) in soybean production and is economically viable for use among smallholder farmers due to its low price over inorganic commercial fertilizer blends [3, 4]. Likewise, soybean producers have the quest to improve yield which necessitates inoculation with effective rhizobial strains [5–7]. Inoculation improves soybean yield and increases crop resilience to climatic changes effects across Africa such as drought incidences experienced in Mozambique through better water use efficiency (WUE) [8]. Although many African countries currently produce inoculant that is effective for both promiscuous and non-promiscuous soybean varieties and other legumes like beans, cowpea, and groundnuts [9], Mozambique as a country lacks the capacity and facilities for local production. However, production volumes of these inoculants seldom satisfy in-country or regional demand warranting importation of supplementary stocks from as far as south America [10]. Unfortunately, produced inoculants fail to reach smallholder farmers in Africa on time due to logistic constrains linking production and distribution. Development of promiscuous soybean varieties, capable of fixing nitrogen with indigenous rhizobia [11] offer a promising solution to improving BNF. In addition, advancement in research has led to isolation of promising indigenous rhizobia that establish symbiotic association with soybean [12, 13]. The research was based on the notion that African soils have indigenous rhizobia strains capable of colonizing soybean root. Unfortunately, isolated indigenous rhizobia strains are yet to be commercialized despite performing better than or like the well-known USDA 110 strain. Commercial production in solid or liquid form of identified indigenous rhizobia strains is necessary to improve their efficiency since naturally they occur in low populations in the soil coupled with low efficacy as effective nitrogen fixers.

Inoculants can be packaged in liquid, peat, or granular forms. Only the liquid or peat/powder forms of inoculants are found in Mozambique with the latter being more abundant and easier to handle among producers. Both forms of inoculants can be applied on seed or directly on soil before planting. Although both forms of inoculants improve yield, variations in the amount tend to occur due to other factors such as viability, storage and environment especially soil moisture in a specific site [14]. In many cases, seed yield inoculated with liquid formula seldom gives better than the peat inoculants. Liquid inoculants offer limited protection to the rhizobia hence survivability can be a challenge in sub-optimal conditions [15–17] while peat carriers provide more protection to the live cells to a limited extend as it is still important to plant the seed or cover the soil soon after application. Bacterial cells survival on the seed or soil in Mozambique could mainly be affected by desiccation and high temperatures [18]. The most common inoculant application method in Mozambique is on seed although there exists a potential for soil application especially among the large-scale commercial soybean producers who have the capacity to mechanize farm operations.

1.2 Plant nitrogen uptake

Soybeans acquire N from either BNF or soil and sometimes inorganic N fertilizer if applied. Maximum N demand in soybean occurs between the R3 and R5 stages of development [19]. Proportions of N absorbed from these sources differ with the cropping system and management. Since BNF is an energy consuming process, soybean will not invest in it where either the soil or fertilizer N is adequate. On the other hand, unavailability of N from any of the sources during plant growth will result in N translocation from other parts of the plant such as leaves to the grain, which diminishes the photosynthesis thus reducing yield potential [20]. Soybean plant N derived from BNF leads to improved productivity. Nitrogen availability in soybean production can be enhanced through inoculation. Inoculating soybean with liquid or peat based effective rhizobia strains promotes nodulation and plant growth that contribute to increased yield. Through BNF, soybean can satisfy between 50% and 60% of its nitrogen requirement [21]. Farmers in Mozambique rarely apply external inorganic fertilizer on soybean. Therefore, the N sources of soybean production is either soil or BNF where inoculants are applied, or effective indigenous rhizobia strains exist in the soil. More so, where inoculants are applied, there exists no means to quantify the amount of N fixed in the fields other than the yield obtained. Benefits of BNF are higher when phosphorus fertilizer is applied in addition to rhizobia inoculation on soybean [5] or cowpea [3] in Mozambique.

1.3 Carbon isotope discrimination, water use efficiency and yield

Carbon is released from the plant through the leaves as CO_2 during transpiration. Likewise, water is lost from the plant by the same process through the stomata. Transpiration is important in plants as it facilitates mass-flow movement of nutrients from the roots to the above ground parts. This process is inversely correlated to availability of soil moisture content hence affecting plant WUE [22]. WUE is the ratio of plant dry matter production against the water used over a period. It can also be defined at a point in time as the ratio between the rate of carbon fixation and the rate of transpiration. ^{13}C isotope discrimination is used to determine a fraction of carbon isotope during CO_2 uptake and fixation and related to WUE that is an important physiological character as an indicator of plant adaptability to drought conditions through the functioning of the stomata [23]. It is strongly linked to the ratio of the intercellular and atmospheric concentration of CO_2 (C_i/C_a) associated with stomatal conductance and chloroplast affinity for CO_2 [24]. Therefore, the intercellular and atmospheric CO_2 ratio theoretically links WUE to ^{13}C isotope discrimination. These relationship is useful in breeding for selection of high transpiration efficiency, and increased and grain yield in soybean as demonstrated with wheat [25]. Kumar et al. [26] demonstrated a positive relationship between grain yield and ^{13}C isotope discrimination and a negative one to transpiration efficiency. Since transpiration is inverse to WUE the increase in ^{13}C isotope discrimination and WUE lead to increase in grain yield. In essence, ^{13}C isotope discrimination offer a promise to selection of criterion for high yielding drought adapted varieties. Therefore, in our study, we sort to understand how liquid or solid inoculant affect soybean WUE and yield. Earlier studies have reported that inoculation improves yield as it leads to more available N from the BNF process. However, the yield increase varies with soybean varieties and type of inoculant especially nitrogen availability even if similar strains are used [8]. The objective of this study was to evaluate soybean WUE and yield response to liquid or solid inoculants applied to soil and on seed before planting.

2. Materials and methods

2.1 Site selection and description

Field studies using soybean variety '*Safari*' (SeedCo. material) were conducted in 2017 and 2018 growing seasons at three locations, Nampula 15.2741° S, 39.3150° E, 365 m above sea level (m a.s.l.), Angonia 14.5473° S, 34.1873° E, 1224 m a.s.l. and Ruace 15.2345° S, 36.6887° E, 772 m a.s.l. in Mozambique. New fields previously under maize for two growing periods were used for each season. According to the Soils Atlas of Africa, the predominant soil type at the sites in Nampula is Haplic Lixisols while in Angonia and Ruace are Chromic Luvisols [27]. Ten soil samples were taken from 0 to 30 cm soil layer using a soil auger in a W pattern across the field for the trial before plowing or harrowing. Soils from each site were combined into a composite sample and four subsamples drawn for chemical and particle-size analysis (**Table 1**). The pH was determined using a high impedance voltmeter on 1:2 soil–water suspension. Total N was determined using The Kjeldahl method, P by Olsen's method, and K plus other bases by ICP-OES after extraction with Mehlich 3.

2.2 Inoculant sources and preparation

Two inoculants were sourced from Novozymes BioAg (Cell-Tech® liquid and Cell-Tech® peat) in Saskatoon, SK Canada and Soygro (Soyflo-liquid and Soycap-powder) in Potchefstroom South Africa. According to the manufacturers' specifications, the inoculants contained 2×10^9 cells/ml or cells/g of *Bradyrhizobium diazoefficiens* formerly known as *Bradyrhizobium japonicum* [28] USDA110 strain for Cell-Tech® and USDA122 strain for Soygro. The Cell-Tech® liquid inoculant was applied at the rate of 1900 ml/ha (3.8 ml/20 m^2 plot) while the Cell-Tech® peat was 2.32 kg/681 kg seed (170.5 g/50 kg seed/ha). On the other hand, the Soyflo-liquid and Soycap-powder were applied at 2000 ml/ha (4.0 ml/20 m^2 plot) and (250 g/50 kg seed/ha) respectively.

2.2.1 Seed application

Liquid inoculants required for 2 kg soybean seed were weighed and diluted with 100 ml of distilled water before applying on seed in a plastic bag. The seeds were then mixed well for the surfaces to be fully coated with the inoculant. For the solid-based

Location	Angonia	Gurue	Nampula
pH	6.4	6.2	6.6
P (ppm)	25.0	44.8	0.3
K (ppm)	122.8	421.0	90.4
Ca (ppm)	772.8	1755.0	800.5
Mg (ppm)	165.5	301.8	113.0
Na (ppm)	29.4	17.9	29.3
EC (dS/cm)	0.059	0.057	0.050
CEC ($cmol_c$/kg)	6.6	15.0	6.0
N (%)	0.09	0.15	0.13
Sand (%)	64.0	56.8	63.2
Silt (%)	6.6	12.1	2.0
Clay (%)	29.4	31.1	34.8

Table 1.
Soil characteristics at the experimental sites' soils.

inoculants, the seeds were weighed into a plastic bag then moist with water for Cell-Tech® peat or Mollyflo for the Soycap-powder. Seeds were then mixed well in the plastic bag until all the surfaces were coated with a film of water or Mollyflo. Then respective quantities of solid-based inoculants added and mixed well to cover the surfaces of all the seeds. All the preparations were done under shade and the seeds planted within 2 h of mixing with the inoculant.

2.2.2 Soil application

Volumes of inoculants to be applied on soil per plot were measured using a syringe into 2 l hand sprayers before adding 1 l of distilled water. The mixture was then agitated gently to equally distribute the inoculant cells in the water. Later the mixture was sprayed into open seed furrows followed immediately with seed placement and covering with soil. To apply the solid-based inoculants onto soil, quantities of respective plot inoculants were weight and mixed with 100 g moist fine sieved (1 mm sieve) soil in a wide mouth plastic container with a lid. Then soil and inoculant were mixed thoroughly by shaking. The lid was then perforated using a hot nail to open many holes like a saltshaker. This mixture was then applied in open furrow followed by immediate planting of seeds and covering with soil. To avoid scorching of the rhizobia strains to death in the sun, immediately planting the seeds and covering with soil is recommended.

2.3 Experimental layout

A disc plow was used for land preparation followed by two passes of harrowing. Both seasons' experiments were planted between 16 and 24 December depending on the onset of rains in each site. Experimental treatments were formulated by combining the two inoculants, their formula (liquid or solid) and place of application (seed or soil) plus a control (no amendment). These resulted in nine treatments that were layered out in a Randomized Complete Block Design (RCBD). A non-promiscuous soybean variety Safari was planted in plots of 20 m^2 in four replications. Plots consisted of seven rows of 8 m in length, 0.50 m row-spacing and 0.1 m between plants within rows. During establishment of the trials, similar treatments were planted by one person for all the four replicates to avoid contamination. Planting and weeding (twice) were done by hand at site-specific scheduling. The experiment was conducted under rainfed conditions for both seasons with no external water supply through irrigation. Pests were controlled once at beginning of flowering using 100 ml of Cypermethrin (200 g active ingredient/l) and 50 ml of Lambda Cyhalothrin (50 g active ingredient/l) applied using 15 l knapsack sprayer.

2.4 Data collection

Data on nodulation, plant growth, nitrogen fixed, ^{13}C related WUE, yield and yield components were collected. At R3 (flowering to podding) growth stage when pods had reached 10–12 mm long at one of the four uppermost nodes on main stem, five randomly selected soybean plants were excavated using a hoe and spade from each plot ensuring that all the roots were recovered. All the soil was washed out of the roots and all nodules plucked out carefully by hand. The nodules were counted and later placed in envelopes before drying in an oven at 60°C for 48 h to determine nodule dry weight. Plant biomass were also dried in an oven at 60°C until constant dry weight was achieved. Later the biomass was ground to pass through a 2-mm mesh sieve for plant tissue N analysis stable light isotope ratio mass spectrometer. At maturity, 10 plants were randomly selected and harvested for determination of pod density and seed weight. Pods from each plot were threshed manually and grain yield was determined. The moisture content of grain samples from each plot was measured using Farmex MT-16 grain moisture Tester (AgraTronix LLC, Streetsboro, Ohio, USA) and grain yield in kg ha^{-1} was adjusted to 13% moisture content. Above-ground plant biomass from whole plots were sun-dried to 10% moisture content for 10 days to determined harvest biomass weight.

2.4.1 Measurement of shoot N and C isotopes

The isotopic analyses of ^{15}N and ^{13}C were performed at the Mammal Research Institute, University of Pretoria, Pretoria, South Africa using a Stable Light Isotope Laboratory on a Flash EA 1112 Series coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a ConFlo IV system (Thermo Fischer, Bremen, Germany). Aliquots of 1.2 mg were weighed into toluene pre-cleaned tin capsules. During the analysis, a standard (Merck Gel: $\delta^{13}C = -20.57‰$, $\delta^{15}N = 6.8‰$, C% = 43.83, N % = 14.64) and a blank sample were run after every 12 samples. The air nitrogen was used as the reference isotope values for nitrogen. The ^{15}N natural abundance expressed as the δ (delta) notation is the ‰ deviation of the ^{15}N natural abundance of the sample from atmospheric N_2 (0.36637 atom % ^{15}N) was calculated [29] with the analytical precision values used being $<0.2‰$ for $\delta^{13}C$ and $< 0.2‰$ for $\delta^{15}N$.

$$\delta^{15}N = \left[\left(\left({}^{15}N/{}^{14}N\right)_{plant} - \left({}^{15}N/{}^{14}N\right)_{atm}\right)/\left({}^{15}N/{}^{14}N\right)_{atm}\right] \times 1000 \qquad (1)$$

The percentage *N* derived from the legume (%Ndfa) was determined using [30]:

$$\%Ndfa = \left(\left(\delta^{15}N_{ref} - \delta^{15}N_{plant}\right)/\left(\delta^{15}N_{ref} - B_{value}\right)\right) \times 100 \qquad (2)$$

Where, $\delta^{15}N_{ref}$ is the mean ^{15}N natural abundance of the collected reference plants (maize), $^{15}N_{leg}$ is the ^{15}N natural abundance of soybean, and the *B* value is the ^{15}N natural abundance of the test legume wholly dependent on N_2 fixation for its N nutrition. The B_{value} replaces atmospheric N_2 as it incorporates the isotopic fractionation associated with N_2 fixation. The *B* value used for estimating %Ndfa in this study was $-0.72‰$ [29, 31, 32]. The amount of N-fixed was calculated based on the method established by [33].

$$N - \text{fixed} = (\%Ndfa/100) \times \text{legume biomass N} \qquad (3)$$

Where legume biomass N refers to the N content of plants shoots.

2.4.2 Carbon assimilation and water use efficiency

To perform the $^{13}C/^{12}C$ isotopic analysis, the plants shoots were weighed (subsampled) into tin capsules and analyzed on a mass spectrometer as described for the $^{15}N/^{14}N$ isotopic analysis. Shoot C content was calculated by relating plant %*C* to the biomass of the plant.

$$\text{Shoot C content} = \%C \times \text{shoot biomass per plant} \qquad (4)$$

Reference carbon isotope values were the Vienna Pee-Dee Belemnite (PDB). Change in $_{13}C$ (Δ13C) was calculated as follows

$$\Delta^{13}\mathrm{C} = \left(\delta^{13}\mathrm{C}_{\mathrm{atm}} - \delta^{13}\mathrm{C}_{\mathrm{plant}}\right)/\left(1 + \delta^{13}\mathrm{C}_{\mathrm{plant}}\right). \tag{5}$$

Where $\delta^{13}C_{atm}$ is ^{13}C change in atmospheric CO_2 (−8) and $\delta^{13}C_{plant}$ in plant material.

The relationship between carbon fixation and stomatal conductance in soybean at R3 stage was determined based on the model linking the isotope discrimination ($\Delta^{13}C$) to plant and atmospheric ^{13}C [34]. A linear relationship was used to relate the isotope discrimination to plant physiological properties.

$$\Delta^{13}\mathrm{C} = a + (b\text{–}a)/(C_{\mathrm{i}}/C_{\mathrm{a}}). \tag{6}$$

Where a is the discrimination against $^{13}CO_2$ during CO_2 diffusion through the stomata (a = 4·4‰), b is the discrimination associated with carboxylation (b = 27‰), and C_i and C_a are the intercellular and atmospheric ambient CO_2 concentrations respectively. According to Fick's law (1855) that states 'the rate of diffusion of a substance across unit area (such as a surface or membrane) is proportional to the concentration gradient'. Then Movement of CO_2 can be expressed as;

$$A = g_{\mathrm{CO2}}(C_{\mathrm{i}}/C_{\mathrm{a}}) \tag{7}$$

Since the ratio of leaf conductance to water vapor is 1.6 g CO_2, and therefor change in ^{13}C can be related to the A/gH_2O ratio as follows:

$$\Delta^{13}\mathrm{C} = a + (b\text{–}a)\left(1 - 1.6\left(A/C_{\mathrm{a}}\ \mathrm{g}\ \mathrm{gH_2O}\right)\right. \tag{8}$$

WUE defined as the ratio of the fluxes of net photosynthesis and conductance for water vapor (*A/E*) which indicates carbon assimilated per unit of water umol mol^{-1}) [35]. Therefore, water-use efficiency at growth level (WUE_g) − biomass accumulated over water transpired (g C kgH_2O^{-1}) was calculated as:

$$\mathrm{WUE_g} = C_{\mathrm{a}}\left[\left(b - \Delta^{13}\mathrm{C}\right)/1.6(b\text{–}a)\right] \tag{9}$$

2.5 Data analysis

Analyses of variance (ANOVAs) were performed using PROC GLM in Statistical Analysis System (SAS)® 9.4 [36]. First a combined analysis across locations and cropping seasons was performed. Since location and season effects were dominant, the two variables were combined to form environment. Secondly, a factorial ANOVA was performed, to evaluate the effects of environment, treatment, and their interactions. Environments effects were considered random and were significant for all the variables [37] while the treatments factors were fixed effects for each environment. Means were determined for treatments, and comparisons done using Tukey adjustment at $p \leq 0.05$ significance level based on the standard error of means (SEM) [36].

3. Results

3.1 Nodulation

Formation of nodules is an indicator of BNF through the symbiotic relationship of soybean plant and the inoculant strains. Data on nodule count and dry weight per plant were collected for both crown and lateral nodules. There were no significant differences ($p \leq 0.05$) in the nodule count and dry weight between treatments, sites and their interactions for both crown and lateral nodules. It was however evident that crown nodules of inoculated soybean averaging at 20.4 nodules plant^{-1} were more than lateral nodules at 18.6 nodules plant^{-1} against the check of 3.4 nodules plant^{-1} and 3.2 nodules plant^{-1} respectively. Total nodule counts, and weight combined both crown and lateral nodules were significant between treatments at Angonia in 2017, Ruace in 2017 and Ruace in 2018 (**Tables 2** and **3**). Angonia and Ruace sites are in well suited high potential soybean production agroecologies while Nampula site is in a low to marginal production region.

In Angonia and Ruace in 2017, nodule counts were lowest for the uninoculated soybean and the nodule count per plant was observed to be the highest from seed inoculated soybean with Soycap-powder (**Table 2**). Comparable nodules were formed for inoculated soybean at Ruace in 2018 except for Soycap-powder soil application. A common trend was observed between manufacturers/source liquid and solid inoculants regardless of the application on soil or seed. The liquid inoculants had numerically lower nodules formed than the solid (peat or powder) based. Generally, liquid based inoculants averaged at 36.5, 37.5 and 41.2 versus 56.3, 56.2 and 45.8 nodules plant^{-1} for Angonia 2017, Ruace 2017 and Ruace 2018 respectively. Except for Ruace 2018 with 50.2 and 36.8 nodules plant^{-1} for seed and soil inoculant application, mean number of nodules formed between the two inoculation methods were not different for the other environments. The total number of nodules formed per plant were significantly higher ($p \leq 0.05$) for the inoculated soybean in all the sites at 46.4, 46.9 and 43.5 than the uninoculated plants at 9.0, 8.5 and 11.1 nodules plant^{-1} (**Table 2**).

Similar trends of nodules plant^{-1} were also observed for the nodule dry weight (mg plant^{-1}). Inoculated soybean had heavier nodules than the uninoculated ones averaging at 206.9, 218.8 and 249.7 mg plant^{-1} versus 33.5, 36.6 and 69.9 mg plant^{-1} for Angonia 2017, Ruace 2017 and Ruace 2018 respectively (**Table 3**). It was also noted that the dry weight per nodule at Ruace in 2018 was higher than at Angonia and

Treatment (inoculant application)	**Angonia 2017**	**Ruace 2017**	**Ruace 2018**
Control	9.0^{d}	8.5^{b}	11.1^{c}
Seed Cell-Tech liquid	36.6^{bc}	42.6^{a}	48.1^{ab}
Seed Cell-Tech peat	52.6^{abc}	57.5^{a}	60.5^{a}
Seed Soyflo-liquid	38.8^{bc}	38.3^{a}	36.0^{ab}
Seed Soycap-powder	63.9^{a}	58.6^{a}	56.1^{ab}
Soil Cell-Tech liquid	37.9^{bc}	34.9^{a}	42.9^{ab}
Soil Cell-Tech peat	53.4^{abc}	54.4^{a}	37.8^{ab}
Soil Soyflo-liquid	32.8^{c}	34.2^{a}	37.6^{ab}
Soil Soycap-powder	55.4^{ab}	54.4^{a}	28.7^{bc}
%CV	10.3	13.2	14.7
p-Value	<0.0001	<0.0001	0.0001

The subscripts signify statistical differences at p < 0.05. Same letters indicate no differences while different letters show significance in the treatments within the season.

Table 2.
Nodule count per plant of inoculated soybean.

Treatment (inoculant application)	Angonia 2017	Ruace 2017	Ruace 2018
Control	33.5^{d}	36.6^{b}	69.0^{b}
Seed Cell-Tech liquid	134.3^{cd}	174.1^{a}	247.0^{ab}
Seed Cell-Tech peat	259.4^{ab}	247.3_{a}	275.1^{ab}
Seed Soyflo-liquid	155.4^{bc}	176.6^{a}	228.0^{ab}
Seed Soycap-powder	294.3^{a}	295.7^{a}	310.7^{a}
Soil Cell-Tech liquid	147.0^{bc}	165.1^{a}	249.5^{a}
Soil Cell-Tech peat	238.9^{abc}	255.3^{a}	228.3^{ab}
Soil Soyflo-liquid	169.6^{bc}	180.1^{a}	239.0^{a}
Soil Soycap-powder	256.7^{ab}	256.7^{a}	220.5^{ab}
%CV	28.8	26.9	35.6
p-Value	<0.0001	<0.0001	0.0127

The subscripts signify statistical differences at p < 0.05. Same letters indicate no differences while different letters show significance in the treatments within the season.

Table 3.
Nodule weight (mg) per plant of inoculated soybean.

Ruace 2017 for all the treatments. The average weight per nodule was Angonia 2017 (4.3 mg nodule^{-1}), Ruace 2017 (4.6 mg nodule^{-1}) and Ruace 2018 (6.0 mg nodule^{-1}). The heaviest weight per nodule was from soybean that were inoculated with Soycap powder applied on the soil at 7.7 mg nodule^{-1} in Ruace 2018. As observed for the nodule counts, significantly heavier nodules ($p \leq 0.05$) were obtained when Soycap-powder inoculant was applied on seed which gave 294.3, 295.7 and 310.7 mg plant^{-1} of dry nodule weight at Angonia 2017, Ruace 2017 and Ruace 2018 respectively (**Table 3**). Application of the inoculants in liquid form had lighter nodules for all the sites at 151.5, 174.0 and 240.9 mg plant^{-1} against using inoculants in solid form with 262.3, 263.7, and 258.6 correspondingly. From the contrast analysis, nodule dry weight had a likelihood of increasing over the uninoculated by 173.4 mg plant^{-1} in Angonia 2017, 181.9 mg plant^{-1} in Ruace 2017 and 180.4 mg plant^{-1} in Ruace 2018 when using inoculant either as liquid or in solid form. There was a strong correlation between number of nodules and dry weight in all the environments with the coefficients ranging between 0.92 and 0.96 (**Table 4**). This suggests that variation in the nodule dry weight attributed to nodule count was between 85.1% at Angonia 2018 to 91.6% at Ruace 2017.

Environment	Correlation coefficient	Significance level
Angonia 2017	0.926	<0.0001
Nampula 2017	0.935	<0.0001
Ruace 2017	0.957	<0.0001
Angonia 2018	0.922	<0.0001
Ruace 2018	0.938	<0.0001

Table 4.
The correlation between nodule count and nodule dry weight of soybean.

3.2 Nitrogen uptake in non-promiscuous soybean safari

Nitrogen is important in soybean production. Soybean has the ability of obtaining nitrogen from the atmosphere through BNF. The proportion of nitrogen derived from the atmosphere denoted as %Ndfa by soybean used as an indicator of nitrogen fixed through BNF. The %Ndfa was as low as 3.8% for control treatment in Angonia 2017 to as high as 69.8% for soybean that were inoculated with Cell-Tech liquid inoculant at Ruace 2018 (**Figure 1**). Our study showed that inoculating soybean seed with Soycap-powder could derive as high as 50.8% of the nitrogen from the atmosphere across the environments compared to 14.1% for the uninoculated soybean. The proportion of N derived from the atmosphere significantly varied with treatment for each environment. Therefore, the highest %Ndfa was 44.0% for soil Cell-Tech peat in Nampula 2017, 46.9% for seed Soycap-powder in Angonia 2017, 66.4% for seed Soyflo-liquid and 69.8% for soil Cell-Tech liquid inoculant at Ruace 2018. In each environment, % Ndfa due to inoculation was significant ($p \leq 0.05$) between the treatments resulting in average %Ndfa of 27.5% for Nampula 2017, 36.4% for Angonia 2017, 59.1% for Angonia 2018 and 47.1% for Ruace 2018 (**Figure 1**). Consequently, the proportion of N derived from the atmosphere was higher at Angonia in 2018.

Nitrogen uptake associated to BNF by the Safari variety per hectare was also calculated across the seasons for each site. Inoculating soybean increased the amount of plant N uptake at all the three sites. Plant N uptake was highest at Angonia with 235 kg N ha^{-1}, followed by Ruace with 150 kg N ha^{-1} and at Nampula with 137 kg N ha^{-1} for the inoculated soybean against the uninoculated counterparts at 113 kg N ha^{-1}, 46 kg N ha^{-1} and 98 kg N ha^{-1} correspondingly for all the sites (**Table 5**). Different treatments had significantly high amount of plant N uptake at

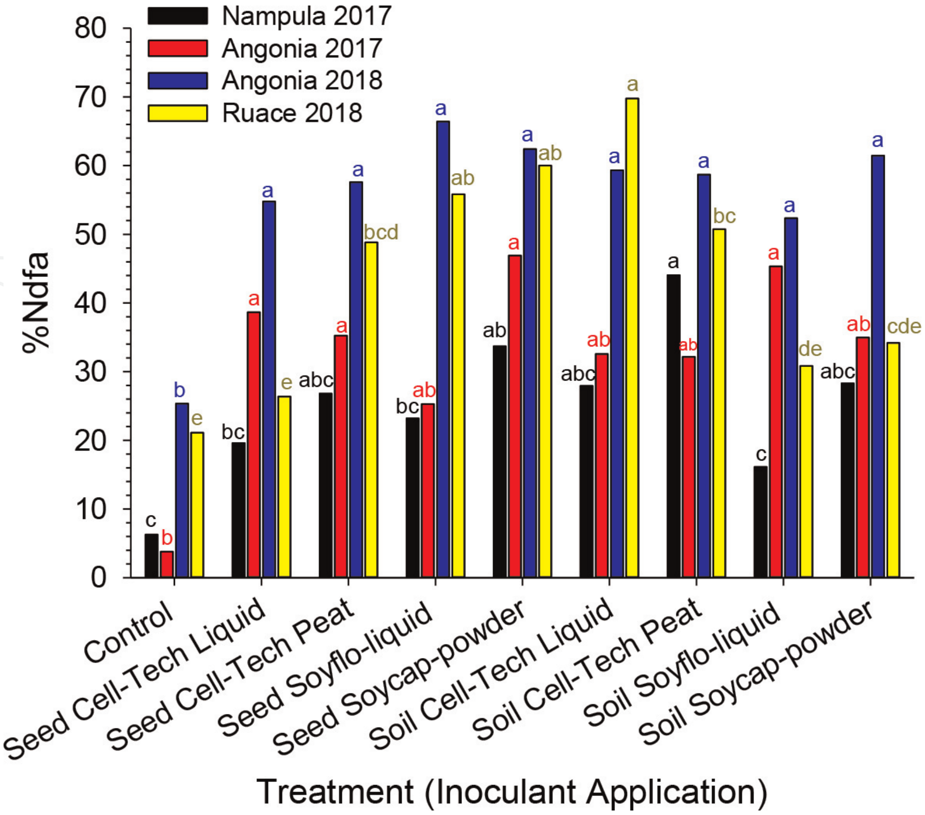

Figure 1.
Proportion of nitrogen derived from the atmosphere (%Ndfa).

Treatment (inoculant application)	Nampula	Angonia	Ruace
Control	98^{b}	113^{c}	46^{d}
Seed Cell-Tech liquid	110^{ab}	181^{abc}	112^{c}
Seed Cell-Tech peat	137^{ab}	313^{a}	129^{bc}
Seed Soyflo-liquid	110^{ab}	261^{ab}	144^{ab}
Seed Soycap-powder	136^{ab}	213^{abc}	144^{ab}
Soil Cell-Tech liquid	149^{ab}	221^{abc}	194^{a}
Soil Cell-Tech peat	154^{ab}	178^{bc}	171^{ab}
Soil Soyflo-liquid	134^{ab}	202^{abc}	120^{bc}
Soil Soycap-powder	158^{a}	307^{ab}	188^{ab}
%CV	24.1	32.1	18.8
p-Value	0.0257	0.0519	<0.0001

The subscripts signify statistical differences at p < 0.05. Same letters indicate no differences while different letters show significance in the treatments within the season.

Table 5.
Amount of plant nitrogen derived from BNF (kg ha^{-1}) by soybean in 2018 growing season following inoculant application.

each site. The highest plant N uptake was 158 kg N ha^{-1} at Nampula, 307 kg N ha^{-1} at Angonia for soil Soycap-powder and 194 kg N ha^{-1} for soil Cell-Tech liquid at Ruace when averaged across the seasons. Like the nodulation data, the amount of plant N uptake per ha for liquid based inoculant was numerically lower than the solid form at every application method (seed or soil) at Nampula. Since the form of inoculant also affected the amount of plant N uptake per ha at each site, solid-based inoculants resulted in more N absorbed by the plant than liquid-based at 146 vs. 126, 253 vs. 216 and 158 vs. 143 kg N ha^{-1} for Nampula, Angonia and Ruace respectively (**Table 5**).

3.3 ^{13}C isotope discrimination and water use efficiency

Water-use efficiency at growth level (WUE$_g$), an indicator of biomass accumulation over water transpired was calculated based on the assimilation of carbon at R3 growth stage. Before the calculations, the C:N ratio of plant biomass was also determined. Our data indicate that no significant differences existed for the C:N ratio values across the treatments with an average of 13.6 (data not presented). Similarly, ^{13}C isotope discrimination (a fraction of carbon isotope of soybean leaves during CO_2 uptake and fixation) was not significant with an average of 20.1‰ across treatments within environments except for Ruace 2018 where seed Cell-Tech peat inoculant had the lowest significant ($p \leq 0.05$) value of 19.7‰ than the other treatment (**Figure 2**). The highest numerical ^{13}C isotope discrimination at Ruace 2018 was soil Soyflo-liquid application with 20.93‰. Like the ^{13}C isotope discrimination, WUE$_g$ was not significant among the treatments within each environment averaging at 11.8 g C kgH_2O^{-1} except at Ruace in 2018 where seed Cell-Tech peat inoculant had the highest significant ($p \leq 0.05$) value of 12.0 g C kgH_2O^{-1} (**Figure 2**). The WUE$_g$ average ranged from 11.6 g C kgH_2O^{-1} at Nampula 2017 to 13.3 g C kgH_2O^{-1} at Angonia 2018. There were also no significant differences in applying either of the inoculants on seed or soil with a mean of 11.0 g C kgH_2O^{-1}. However, application of the inoculant in a solid form

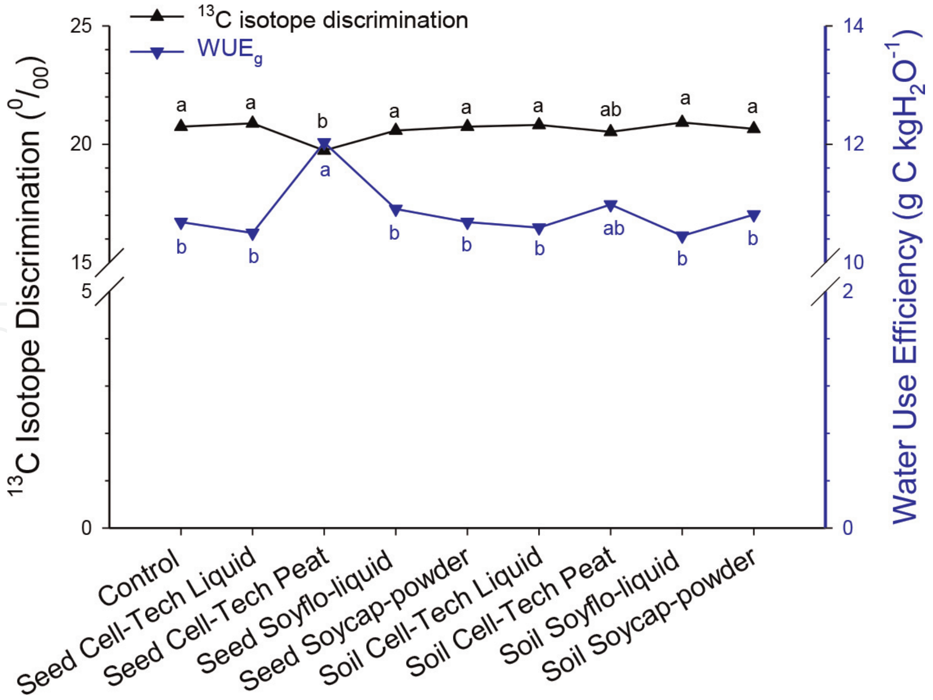

Figure 2.
Relationship between ^{13}C isotope discrimination and WUE in Ruace 2018 growing season.

resulted in a numerically higher WUE_g of 11.1 g C kgH_2O^{-1} against the liquid counterpart with 10.6 g C kgH_2O^{-1}. There is an inverse relationship between the ^{13}C isotope discrimination and WUE_g (**Figure 2**). A treatment with higher isotope discrimination had a corresponding lower WUE_g value. For instance, soybean inoculated with seed Cell-Tech peat inoculant has an isotope discrimination value of 20.89‰ which corresponded to WUE_g of 12.0 g C kgH_2O^{-1}.

3.4 Soybean yield

Inoculation treatment yield was determined within each environment. Significant differences ($p \leq 0.05$) were observed between treatments in all environments except for Ruace 2017 (p-value = 0.9851) with a mean yield of 2186 kg ha^{-1} and Angonia 2018 (p-value = 0.0883) averaging at 2572 kg ha^{-1} (**Figure 3**). However, in these two environments, mean yield of the inoculated soybean 2248 kg ha^{-1} at Ruace 2018 and 2413 kg ha^{-1} at Angonia 2018 were significantly higher than the uninoculated with 1685 and 1756 kg ha^{-1} respectively. In Nampula 2017, seed Soycap powder gave the highest significant yield of 2194 kg ha^{-1} over the uninoculated production of 978 kg ha^{-1} representing over 2.3-fold increase in yield due to inoculation (**Figure 3**). Soybean production increased in the second season at the Nampula site. In Nampula 2018, the highest yield at 2059 kg ha^{-1} was 81% more than the uninoculated treatment with 1140 kg ha^{-1}. Soybean yielded better in Angonia and Ruace sites that are in high soybean production potential agroecologies. For instance, in Angonia 2017 environment, the highest statistical yield was from soil Soycap powder at 3439 kg ha^{-1} against a check of 1646 kg ha^{-1} while in Ruace 2018 was from Cell-tech liquid inoculant applied on the seed before planting with 2684 kg ha^{-1} compared to the uninoculated fields with 1439 kg ha^{-1} (**Figure 3**). From the data, we deduce that applying inoculant in solid form either on seed or soil was better than using the liquid formulation. Mean yield of solid over the liquid inoculants in the different environments were Nampula

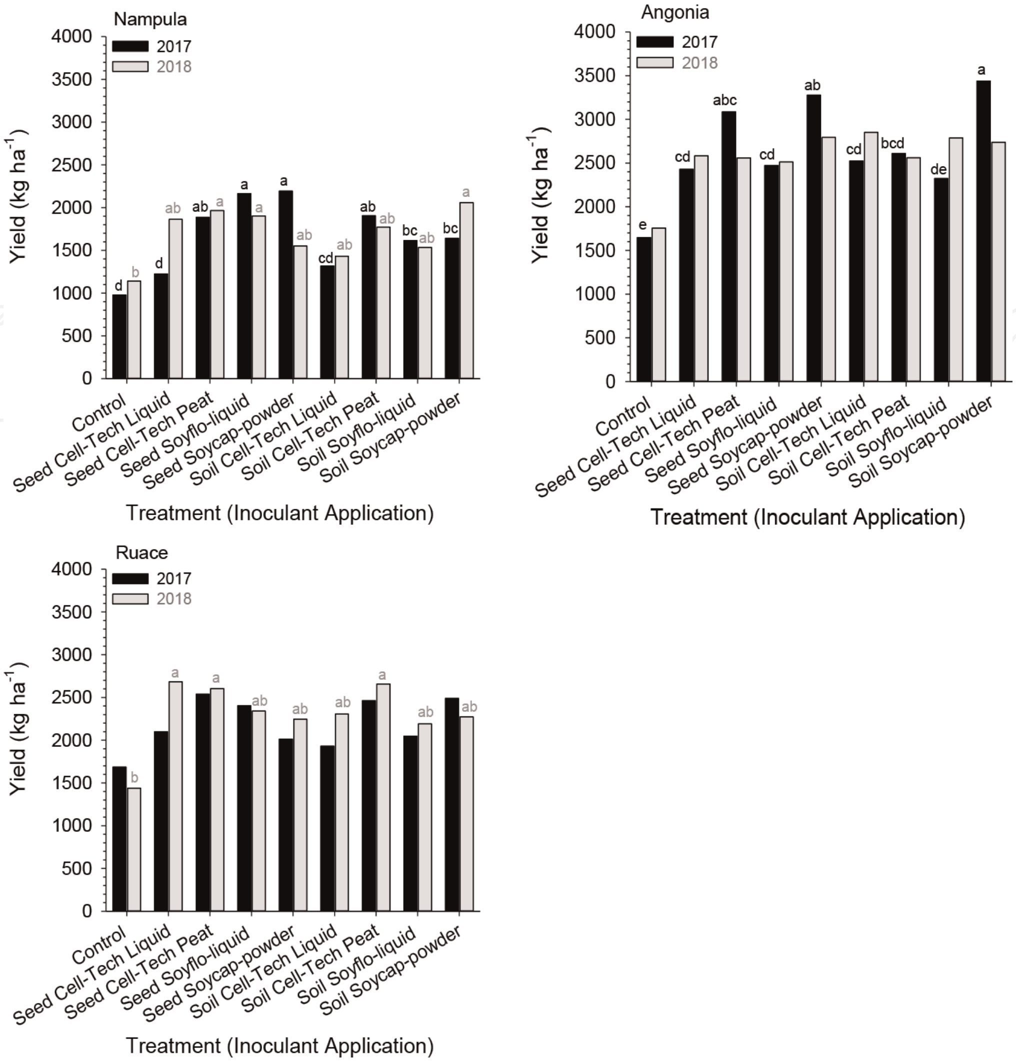

Figure 3.
Yield of inoculated soybean at three experimental sites of Nampula, Angonia and Ruace in 2017 and 2018 growing seasons.

2017 (1907 > 1580 kg ha^{-1}), Angonia 2017 (3103 > 2437 kg ha^{-1}), Nampula 2018 (1838 > 1683 kg ha^{-1}) and Ruace 2018 (2445 > 2380 kg ha^{-1}).

Contrast analysis of yield on whether to apply inoculant or not and using which placement (seed or soil) were conducted at $p \leq 0.05$ (**Table 6**). Inoculation increased yield in all the environments except Angonia 2018. Yield increase in 2017 due to inoculation was 82% in Nampula, 68% in Angonia and 35% in Ruace (**Table 6**). During the second season of 2018 inoculation increased yield from 1140 to 1760 kg ha^{-1} in Nampula and 1439 to 2413 kg ha^{-1} in Ruace. Generally, across all the environments, it was advantageous to apply inoculant on seed than the soil (**Table 6**). The differences in yield due to the inoculant form (liquid or solid), source (Cell-Tech or Soygro) and placement were also determined through contrast analysis (**Table 7**). Soygro inoculants performed statistically better than Cell-Tech counterparts in Nampula and Angonia 2017. In the same locations, solid-based inoculants enhanced yield more than the liquid-based application. Results also show that it is more beneficial to apply inoculants on the seed that the soil directly. For instance, 1868 and 2817 kg ha^{-1} yield obtained from applying inoculant on seed was more than soil placements with 1620 and 2725 kg ha^{-1} in Nampula 2017 and Angonia 2017 respectively (**Table 7**).

Contrasts	Nampula	*p*-Value	Angonia	*p*-Value	Ruace	*p*-Value
			2017			
Control	978		1646		1685	
Control vs. inoculant	1779	<0.0001	2770	<0.0001	2270	0.0251
Control vs. seed	1903	<0.0001	2817	<0.0001	2285	0.0268
Control vs. soil	1655	0.0004	2724	<0.0001	2254	0.0350
Contrasts	**Nampula**	***p*-Value**	**Ruace**	***p*-Value**		
			2018			
Control	1139		1439			
Control vs. inoculant	1753	0.0047	2413	0.0005		
Control vs. seed	1821	0.0029	2469	0.0005		
Control vs. soil	1684	0.0137	2357	0.0014		

Table 6.
Yield gains of inoculation and inoculant application place (seed or soil).

Contrasts	Nampula	*p*-Value	Angonia	*p*-Value
Cell-Tech	1584		2662	
Cell-Tech vs. Soygro	1904	<0.0001	2878	0.0501
Liquid	1580		2437	
Liquid vs. peat	1907	<0.0001	3103	<0.0001
Seed	1868		2817	
Seed vs. soil	1620	0.0002	2725	0.3894

Table 7.
Yield of soybean due to source, grade, and placement of inoculant in 2017 season.

4. Discussions

4.1 Nodulation and plant nitrogen uptake

Inoculation increased the number of nodules and dry weight. Inoculants have been shown to increase the number of nodules per plant in soybean production regardless of the source and stage of plant growth at application ranging from planting time to V6 [38]. Use of the inoculants with compatible rhizobia strain for non-promiscuous varieties [39, 40] and availability of right strain resident rhizobia for promiscuous genotypes [41] leads to formation of more nodules in soybean. In our study, on average, the number of nodules increased by 5.1 times in Angonia 2017, 5.5 times in Ruace 2017 and 3.9 times in Ruace 2018 due to inoculation with liquid and solid inoculants either in seed or direct soil application. Solid based inoculants had high number of nodules and dry weight than the liquid inoculants. Our results corroborate with the findings from a study conducted in the Eastern Region of the south of Vietnam where nodulation of the liquid inoculants was less than the peat-based inoculants for similar rhizobia strains [15, 42]. Solid based inoculants better protect the rhizobia strains from harsh environmental conditions hence leading to increased viability than the liquid inoculants. In addition, solid carrier inoculants attach better onto the seed during inoculation. Also, our data indicated that although crown nodules were fewer in number than the lateral nodules, individual nodules of the former were heavier than the later. It has been reported that crown nodules can account for up to 82% and above of the total nodule count or dry weight in soybean [43]. Crown nodules from our study accounted for 41.7–64.0% of the total nodule dry weight. More crown nodules are formed early in the season following inoculation than the lateral nodules that are formed later after development of lateral roots.

Sources of nitrogen for soybean in our study were either BNF or absorption from soil. The BNF process was enhanced by introduction of compatible rhizobia strain through inoculation. More nitrogen was fixed from the atmosphere for inoculated soybean in Angonia and Ruace relative to Nampula. Nampula lies in a semi-arid region of Mozambique with frequent incidences of drought leading to low soil moisture. High temperatures, drought and low soil moisture has been shown to reduce the effectiveness of rhizobia in BNF process leading to low nodulation hence reduced %Ndfa [44]. In Angonia and Ruace the large share of plant N was from the atmosphere representing as high as 69.8%. Other studies have reported high percentages of plant N in soybean to be associated with atmospheric nitrogen though BNF [45, 46]. As earlier indicated, plant N uptake associated with BNF varies with the biotic factors such as soybean and rhizobia characteristics as well as abiotic factors largely controlled by the environment and management. Due to the differences in the interaction levels of these factors, variations were observed in the amount of N uptake by soybean [47]. For instance, soybean in Angonia a more humid environment, absorbed more N from the atmosphere than Nampula site that is in a drier ecology. A similar trend of N fixed in wet versus drier environment was reported on farmer's fields in humid Dowa (88.9 kg N ha^{-1}) and drier Salima location (47.1 kg N ha^{-1}) in Malawi [48]. Soil moisture that depends on the rainfall amount has been reported to greatly affect amount of N fixed. The amount of N uptake was determined at R3 growth stage in soybean. This growth stage falls within the peak N demand period of flowering to podding in soybean production. Like the amount of N derived from the atmosphere, plant tissue N was enhanced by inoculation [14]. Soybean had accumulated as high as 307 kg N ha^{-1} in Angonia. These findings are like those reported for inoculated TGx 1660-19F soybean with 306 kg N ha^{-1} at Mokwa in the southern Guinea savanna of Nigeria [49]. Although we did not monitor plant N over the growing season, the amount of N in plant tissue varies with the growth stage due to the translocations that occur between plant parts.

4.2 ^{13}C isotope discrimination and water use efficiency

Both ^{13}C isotope discrimination and WUE_g were not significant among the treatments within each environment except at Ruace in 2018. This suggests that these two parameters measured at the R3 stage were not dependent on the application of the inoculant. Like our findings at R3 growth stage in soybean, Zhao et al. [50] also reported that no significant difference existed in C isotope discrimination and corresponding WUE_g at wheat harvest time. Also, Yang et al. [51] reported no clear significance differences in the amount of carbon isotope composition among C3 plants in the Yellow River region in China. For the case of Ruace in 2018 a negative relationship was observed between ^{13}C isotope discrimination and WUE_g. Values of ^{13}C isotope discrimination generally decrease with reductions in water availability. Reduced water availability leads to a decline in transpiration rate hence increased water-use efficiency [22, 35, 52]. Also earlier reported was a negative relationship between ^{13}C isotope discrimination in wheat at tillering stage and WUE of above ground biomass measured over the seedling to tillering period [50]. The change in ^{13}C isotope discrimination in relation to the environment may differ with plant growth stages due to variation in physiological processes within the plant that define its functionality requirements [53]. Since we measured ^{13}C isotope discrimination and WUE_g at one stage for all the treatments, the likelihood of soybean functionality being comparable was high and more dependent on the environment. Therefore, ^{13}C isotope discrimination can be used to determine differences in WUE_g of different soybean growth stages rather than a variation associated to inoculation at a single stage [54].

4.3 Soybean yield

Inoculation increased yield in all the three sites between an average of 602–1124 kg ha^{-1}. Our results agree with a study conducted in 2013 and 2014 in the same locations using storm a non-promiscuous variety that recorded an increase of 523–989 kg ha^{-1} [6]. These results of yield increase due to inoculation also confirms previous report [5, 8] where inoculation alone led to higher soybean yield that uninoculated. Although numerical average increase in yield due to inoculation was higher in Angonia and Ruace than Nampula across the seasons, percent rise in production was higher at Nampula 620–766 kg ha^{-1} (65%) than Angonia 918–1124 (60%) and Ruace 602–974 (52%). Chibeba et al. [6] reported and increased of 47% in yield of inoculated over the uninoculated soybean variety storm. Association between the introduced rhizobia strain and soybean was enhanced in the humid environments of Angonia and Ruace than the drier Nampula. Adequate moisture is required to take full advantage of the BNF process in inoculated soybean. The numerical rise in yield is also a pointer to the earlier reported enhanced nodulation in the inoculated soybean regardless of the placement on seed or soil. Across the sites, average soybean yield of 1440 kg ha^{-1} for the uninoculated fields is above the Mozambique national average of 1216 kg ha^{-1} [55, 56]. Therefore, use of inoculation in this study indicated that soybean yield can be increased by 1052 kg ha^{-1} over the national average figure. Our study observed that inoculant application on seed (2308 kg ha^{-1}) gave higher yield than soil application (2228 kg ha^{-1}) agrees with the report by [57] where seed inoculation 2842 kg ha^{-1} was greater than 2678 kg ha^{-1} for soil inoculation on planting line. Seed inoculation plus good adhesive agent and proper mixing of the seeds in the bag enables better distribution of the rhizobia cells per seed-grain. As a result, the rhizobia cells remain close to the seed and can attach to the root as soon as it germinates leading to better nodulation and BNF process that promote increased yield production. Seed inoculation led to a difference in yield was also noted between the liquid and solid inoculants. Solid inoculants (peat or powder) gave higher yield of 2389 kg ha^{-1} than the liquid inoculant 2147 kg ha^{-1} across the environments. Similar results where solid inoculants gave higher yields than liquid inoculants were reported from a study comparing the two forms of inoculants in Vietnam on promiscuous soybean varieties where identical rhizobia strains of in peat inoculant outyielded the liquid counterparts between 40 and 60 kg ha^{-1} [42]. These results demonstrates that farmers in Mozambique have a basket of inoculation options to choose from in enhancing soybean yield on their fields. However, selection of suitable inoculant should be made with consideration of environmental site conditions especially soil moisture availability over the growing season and the easiness of application.

5. Conclusions

Inoculation improved soybean nodulation by increasing the number of nodule count and its dry weight. Increase in nodulation could be associated to improved soybean productivity through high plant N uptake and yield. Nitrogen uptake and yield increased with application of inoculants. Farmers in Mozambique are likely to produce more soybean through using of solid cased inoculants applied on the seed than the liquid inoculants plus soil application. Although WUE_g related to ^{13}C isotope discrimination at R3 stage did not vary with inoculation, it is recommended that further study be conducted to determine cumulative WUE of the whole plant for the complete growing season while segregating for different growth stages. This could offer information on how to time soybean planting to take advantage of shifting growing seasons characteristics due to climate change. As such, soybean varieties could be selected for adaptability and resilience in specific agroecologies based on carbon assimilation, WUE and plant N uptake that affect yield. Data on inoculation and ^{13}C isotope discrimination could be utilized by breeders in selection of high yielding soybean varieties adapted to drought conditions like those found in Mozambique. The varieties developed would have high transpiration efficiency and WUE.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial benefits that could be construed as a potential conflict of interest.

Author details

Canon E.N. Savala[1]*, David Chikoye[2] and Stephen Kyei-Boahen[1]

1 International Institute of Tropical Agriculture (IITA), Nampula, Mozambique

2 IITA-Zambia, Lusaka Province, Zambia

*Address all correspondence to: c.engoke@cgiar.org

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