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The effect of rhizobium inoculation with nitrogen fertilizer on growth and yield of soybeans (*Glycine max* L.)

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Abstract

The greatest success in terms of modified agricultural practices arising from scientific research on biological nitrogen fixation (BNF) has certainly been the development of rhizobial inoculants. Soybean (*Glycine max* L.) has been the only widely adopted by farmers, mainly due to the relative specificity of the plant for rhizobia. Soybeans demand high amount of nitrogen, which are mainly obtained from biological nitrogen fixation. In view of this, field experiment was conducted to evaluate the response of soybean (SC Siesta) to *Bradyrhizobium japonicum* inoculation and nitrogen fertilizer applied at four rates (0, 50, 100 and 200 kg N (34.5%) ha^{-1})). The experiment was laid out in randomized complete block design with three replications during 2015 - 2016 growing season at Africa University Farm, Mutare, Zimbabwe. Plant height, nodulation and nodule dry weight, pods number and pods dry weight, stem dry weight and yield were measured. Increasing nitrogen fertilizer in non-inoculated plants decreased nodule number and nodule dry weight but enhanced plant height, the pods number and pod dry weight, stem dry weight and grain yield. In the inoculated seeds, the application of 50 kg N ha^{-1}increased plant height, pods number, pods dry weight and soybean yield which were as much as the application of 200 kg N ha^{-1} for the non-inoculated plants. Thus, on the obtained results the study suggests applying nitrogen fertilizer at the rate of 200 kg N (34.5%)ha^{-1} + non-inoculation or 50 kg N (34.5%)ha^{-1} fertilizer + inoculation of soybean seeds under field conditions in Mutare, Zimbabwe.

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Introduction

Soybean (*Glycine max* L. Merrill) is one of the main oil seeds produced in Zimbabwe. Nutritionally, it contains about 37 to 42% high quality protein (Wilcox and Shibles, 2001), 6% ash, 29% carbohydrate and 17-24% oil (Ali *et al.*, 2015) and this make the crop to be economically important. In addition, the seed also contains vitamins, minerals and excellent amount of dietary fiber. Besides, it is easy to grow and particularly adapted to various ecological areas (Tran *et al.*, 2015).

Soybean plants require a large amount of nitrogen as the seeds contain high concentrations of protein and the total amount of nitrogen accumulating in the shoot is proportional to the seed yield (Board, 2013). The plants obtain nitrogen requirements by either soil mineral nitrogen or symbiotic nitrogen fixation. Furthermore, the crop has a relative specificity for rhizobia (Seneviratne *et al.*, 2000) among other leguminous crops. Soybeans can suffer from nitrogen deficiency under field conditions, particularly at flowering when the nodules start to senescence or when seeds are either planted without inoculation of soil with proper symbiotic bacteria, particularly in areas where soybean has not been grown before, or on acid soils that prevent successful nodulation (Mengel and Diaz, 2012). Yet, excess use of N-fertilizer wastes non-renewable resources and often results in environmental pollution. In contrast, BNF uses photosynthetically produced energy and is environmentally cleaner (Albareda *et al.*, 2009). Janagard and Ebabi- Segherloo (2016) emphasized on the importance of BNF as part of sustainable agriculture and pointed that growers often use it as bio-fertilizers. Whiele other studies found that inoculation of seeds with rhizohium increases nodule number (Salih *et al.*, 2015) and its dry matter (Mohamed and Hassan, 2015).

The bacterial strain of rhizobium used by soybean is *Bradyrhizobium japonicum.* This bacterial strain plays a pivotal role in the nitrogen cycle of agro-ecosystems by infecting the roots of soybean plants and inducing the formation of nitrogen fixing nodules.

These nodules provide an appropriate environment for atmospheric nitrogen fixation and its conversion into ammonium by nitrogenase activity (NA), a bacterial enzyme sensitive to high oxygen concentration. Rhizobial bacteria convert atmospheric nitrogen to ammonium (NH_4^+), which is a form of nitrogen available to the plant, and in turn, the plant provides carbohydrates to the bacteria.

Inoculation of legume crops is an advisable agricultural practice when there are no specific rhizobia in soil capable to nodulate the cultivated legume and when the levels of soil nitrogen are low (Catroux*et al.*, 2001). This practice is always done in areas which are free of soybean specific rhizobia. When soybean is grown on land for the first time, inoculation with soybean rhizobia is essential for higher yields. Furthermore, applying inoculant to the seed and soil can potentially increase nodulation and yield in a first year soybean crop.

During soybean growth, the plants often go through a period when leaves are light green or even pale yellow; this is the period just before the nodules start supplying adequate nitrogen to the leaves and is an important phase in the development of a healthy crop. When the nodules have been established and start providing nitrogen to the plant, the leaves will turn into a dark-green color. Rhizobia able to nodulate soybean include six species belonging to three different genera, *Bradyrhizobium, Mesorhizobium*and *sinorhizobium.*

Biological nitrogen fixation of up to 300 kg N ha^{-1} supplying up to 94% of the crop's needs has been reported (Hungria *et al.*, 2006). Recently, it has been reported that BNF can supply nitrogen which may increase relative growth rate (Salih *et al.*, 2015) and yield of soybean. Several studies reported significant increase in soybean growth parameters and grain yield due to inoculation of bradyrhizobial isolates (Soe *et al.*, 2010; Kala *et al.*, 2011). Zuffo *et al.* (2015) reported higher plant height and shoot dry matter of soybean after being subjected to application of *Bradyrhizobium japonicum.* In addition, Mohamed and Hassan (2015) reported that inoculated plants produced higher nodule dry weight, grain yield, number of pods, seed number than the uninoculated plants. The study aims at reducing the use of nitrogen fertilizer when soybean seeds are inoculated with the bacterial *Bradyrhizobium japonicum* under field conditions of Mutare, Zimbabwe.

Materials and methods

Study site

To investigate the effects of rhizobium inoculation on soybean growth and development, a trial was conducted at Africa University Farm (18°53'70, 3" S: 32°36'27.9" E, 1,131 m above sea level), Mutare, Zimbabwe during 2015-2016 farming season. Average day length was 14 hours in summer and 11 hours in winter and rainfall ranged from 750 to 1,200mm. Usually rains start from November to March although heavy showers may be experienced before and after this period. The average maximum temperature ranged from 18°C in July to 32°C in October. The soils are classified as red sandy clay loamy of the red orthoferallitic 7E soils according to the Zimbabwe soil classification(Nyamapfene, 1991).In the last two previous years, the field had been alternatively planted with potatoes in 2013 and maize in 2014.

Field experiment set up

The experiment was laid out in randomized complete block design (RCBD) with four treatments and three replications. Treatments were 0 kg N ha^{-1}+ inoculation, 50 kg N ha^{-1} + inoculation, 100 kg N ha^{-1} + inoculation, 200 kg N ha^{-1}; 0, 50, 100 and 200 kg N ha^{-1} as shown in Table 1. Ammonium nitrate (34.5%) was applied as top dressing. After harrowing, the land was divided into 20 equal plots, each having 4 rows measuring 0.45 m × 5 m.

The two center rows were used as the net plots. All plots were separated by 0.5 m long rows, and 1.5m wide terraces to avoid cross contamination due to surface run-off, which might carry bacteria and fertilizers. The area has never been inoculated with *Rhizobium*. The plants were irrigated using sprinkler irrigation at 48mm/12 h as a net discharge per cycle.

Inoculation procedure

The inoculation procedure was done just before planting under shade to maintain the viability of bacterial cells. *Rhizobium japonicum* used in this study was supplied by the Seed Company Ltd (Seed Co) and applied at the rate of 10g inoculant/kg seed. A cup size 22 of rhizobial inoculant was added to a spoon (table spoon) of brown sugar. Thereafter, inoculant and sugar were mixed thoroughly with water in a bucket to ensure that all the seeds had been inoculated and sown immediately. In order to ensure that all the applied inoculum stick to the seed, the required quantity of inoculant was suspended in 1:1 ratio in 10% sugar solution. The tick slurry of the inoculant was gently mixed with dry seed so that all the seeds received a thin coating of the inoculant. Seeds were allowed to air dry for a few minutes and were then sown at the required rate and spacing. Plots with uninoculated seeds were planted first to avoid contamination. To have maximum efficacy of *B. japonicum* and to avoid death of cells due to the sun's radiation, the seeds were immediately covered with soil after sowing.

Soil sampling

At the onset of experiment, 10 soil subsamples (0-15cm depth) were taken from the site using soil auger to evaluate soil chemical and physical characteristics (Table 2). Before being analyzed, soil samples were dried at 60°C for 48 h and pulverized in 2-mm sieve. The level of nitrogen, phosphorus, potassium, Calcium, magnesium, pH and cation exchange capacity (CEC)in the sample were determined.

Planting method

The inoculated and uninoculated seeds were hand planted at a depth of 4 cm and at a spacing of 5 cm between plants and 45 cm between rows making 6 rows per plot. Basal fertilizer compound D was applied at sowing at the rate of 200kg per hectare. The fertilizer was applied at a distance of 5cm away from soybean seed to avoid seed injury. The seeds emerged at 8 days after planting. Since the cultivar is susceptible to soybean rust, Punch®Xtra fungicide was used at 52, 72 and 95 days after planting against soybean rust which is caused by the fungus *Phakopsora pachyrhizi* and *Phakospora meibomiae*.

The fungicide is mostly used by farmers and known to be the most effective fungicides against soybean rust in Africa(Miles *et al*., 2004).Weeding was carried out at 3, 6 and 9weeks after emergence. The common weeds in the field were *Galinsoga parviflora*, *Tradescantia pallida*, *Elymus repens*, *Acanthospermum hispidum* and *Cyperus esculentus* (Ntambo *et al*., 2015).

Data collection and variables measured

Harvesting was carried out when the crop was physiologically mature. Yield was measured by randomly selecting 10 plants from each net plot for all the treatments. Grain yield was then weighed and recorded to get yield per hectare. Randomly selected plants were carefully uprooted from the gross area in each plot using a spade in order to obtain data on nodulation. Uprooting was done by exposing the whole-root system to avoid loss of nodules. The adhering soil was then removed by washing the roots gently with water over a metal sieve.

The nodules from the roots of each plant were separated collected and counted. The shoots, nodules and pods were first air-dried and then oven-dried at 65 ^{o}C for 72 hours and the dry weights were recorded respectively.

Statistical analysis

The statistical package SPSS 17TH Edition was used for data analysis. The data was subjected to Analysis of variance (ANOVA) to determine the effects of different treatments. The least significant difference (LSD) test (α = 0.05) was used to separate the means.

Results and Discussion

Soybean growth parameters

Plant height

In the inoculated plants with nitrogen fertilizer, the highest plant height was produced at 50 kg N ha^{-1}, which is of a significant advantage as compared to 0, 100 and 200 kg N ha-^{1}. Plant height decreased with increasing rates of nitrogen fertilizer and the lowest plant height was obtained at 200 kg N ha^{-1}. Increased uses of fertilizer in uninoculated plants increased plant height, though the difference between 0, 50 and 100 kg N ha^{-1} was not significant. But, 200 kg N ha^{-1} produced significantly higher height than 0, 50 and 100 kg N ha^{-1}. Overally, plants from inoculated seeds had significantly higher height compared to the uninoculated plant at 0 and 50 kg N ha^{-1}but at 100 and 200 kg N ha^{-1} both inoculated and uninoculated plants had almost similar height (Table 3).

Table 1. Different soybean treatments.

Treatment	Inoculation	Nitrogen fertilizer (34.5%) (kg/ha^{-1})
1	Inoculation	0
2	Inoculation	50
3	Inoculation	100
4	Inoculation	200
5	Uninoculation	0
6	Uninoculation	50
7	Uninoculation	100
8	Uninoculation	200

The inoculation of soybean seeds with *Rhizobium* sp. produced greater plant height than on inoculated plants (Alam *et al*., 2015; Janagard and Ebadi-Segherloo, 2016). In addition, Tahir *et al.* (2009) reported that the highest amount of plant height produced in the inoculation of seeds with Rhizobium + 25 Kg N ha^{-1} + 90 kg P ha^{-1}. Janagard and Ebadi-segherloo (2016) concurred with Tahir *et al.* (2009) but add on that the application of 50 kg ha^{-1} urea + inoculation has significant effect on soybean growth and yield.

However, Mishra *et al.* (2010) reported that the greatest plant height was produced when all the chemical fertilizers such as nitrogen, phosphorus and potassium are integrated along with seed inoculation with Rhizobium + PSB + Plant growth promoting rhizobacteria. Nitrogen fertilizer has significant effect on plant height, however, Argaw (2012) observed significant effect of nitrogen fertilizer on soybean plants but noted that adding nitrogen fertilizer at the rate of46 kg ha^{-1} had not significant effect on plant height compared to the control. According to Tahir *et al.* (2009) resulted that there was significant increase in plant height in relation to increase in nitrogen application rates.

Table 2. Chemical and physical characteristics of soil.

		Nutrient level in the soil						
Site collected	Texture	pH* ($CaCl_2$)	Ca (%)	Mg (%)	K (%)	P_2O_5 (ppm)	Total N (ppm)	CEC (cmol kg^{-1})
AU research block	Clay loam	7.29	5.01	2.42	0.87	29	57	22.44

*pH was measured using the 0.01 mol $CaCl_2$ Method.

Nodule formation and dry weight

The control and 50kg N ha^{-1} treatments from inoculated seeds produced the highest nodule number and nodule dry weight per plant which was significantly different from 100 and 200 kg N ha^{-1}. Increasing nitrogen levels decreased nodule number and nodule dry weight per plant in the inoculated plants. Increasing the use of nitrogen fertilizer in inoculated seed treatments reduced significantly nodule number and nodule dry weight per plant. The highest nodule number and nodule weight was in the control while the lowest nodule number and nodule weight per plant was recorded in the highest amount of nitrogen application. The decrease in nodule dry weight per plant was insignificant and the treatments were almost statistically the same. However, in the inoculated plants the application of 0, 50, 100 and 200 kg N ha-1 resulted in high nodule number and nodule dry weight per plants which was statistically significant than the uninoculated plants at the same nitrogen levels (Table 3).

Table 3. Plant height, Nodule number, nodule dry weight, stem dry weight, total shoot N content at 79 days after sowing (DAS) at Africa University farm in response to seed inoculation with *Bradyrhizobium* + nitrogen fertilizer(Mean± s.d; n = 10).

Treatments	Plant height	Number of nodules/plant	Nodule dry weight/plant	Number of pods/plant	Pod dry weight/plant	Stem dry weight
Inoculation N	63.70±0.95^{b}	5.40±0.58^{a}	1.74±0.19^{a}	12.30±0.29^{bc}	8.90±2.4^{bc}	5.40±19^{ab}
Inoculation +50 kg Nha^{-1}	72.26±0.98^{a}	5.18±0.51^{a}	1.72±2.7^{a}	16.67±0.18^{a}	11.50±.0.31^{a}	5.83±0.44^{a}
Inoculation +100 kg N ha^{-1}	58.1±0.29^{c}	4.68±1.8^{ab}	1.56±0.40^{ab}	12.4±0.42^{bc}	8.77±0.50^{bc}	4.75±0.55^{abc}
Inoculation +200 kg N ha^{-1}	54.7±1.02^{cd}	3.10±2.4^{bc}	1.39±0.32^{abc}	11.30±0.33^{cd}	7.90±3.2^{cd}	4.15±0.13^{bcd}
Uninoculation without N fertilizer	46.7±1.34^{d}	2.45±0.26^{cd}	0.84±0.24^{bc}	7.56±0.38^{e}	3.70±0.40^{ed}	2.40±0.10^{e}
Uninoculated + 50 kg N ha^{-1}	47.6±0.56^{d}	2.60±0.18c	0.82±0.32^{bc}	10.40±0.29^{de}	5.50±0.15^{d}	3.20±0.17^{dc}
Uninoculation +100kg N ha^{-1}	51.4±0.44^{cd}	2.04±0.50cd	0.77±0.42^{c}	11.58±1.66^{bc}	7.46±0.20^{c}	3.80±0.23^{cd}
Uninoculation +200 kg Nha^{-1}	59.66±0.27^{c}	1.79±0.22^{d}	0.75±0.15^{c}	15.4±0.18^{ab}	9.40±0.20^{b}	5.20±0.44^{bc}

*N: Nitrogen fertilizer (34.5%). Means followed by the different letters in a column are significantly different at 5% level according to the LSD's test followed ANOVA.

The current results corroborated with Hungria *et al.* (2015) and Janagard and Ebadi-segherloo (2016), who observed that nodule growth and dry weight increased in inoculated seeds as compared to the non inoculated plants. The symbiotic association between rhizobium and soybean plants usually leads to initiation and development of the root nodules (Tirichine *et al.*, 2006) resulting in increased amount of nitrogen fixation in the pant. Recently studies by Solaiman and Hossain, 2005, Hungria *et al.,* 2015, Janagard and Ebali-segherloo, 2006 revealed that nodulation and nitrogen fixation are more affected when large amount of nitrogen fertilizer is applied. To have a better understanding, Tahir *et al.* (2009) demonstrated that high nodule weight of soybean was obtained when soybean seeds were inoculated with rhizobium + 25 kg N + 90 kg P ha^{-1}. However, Mishra *et al.* (2010) disagreed and reported that higher nodule dry weight was obtained in integrated application of all chemical fertilizers such as nitrogen, phosphorus and potassium with seed inoculated with Rhizobium +PSB+ plant growth promoting rhizobacteria.

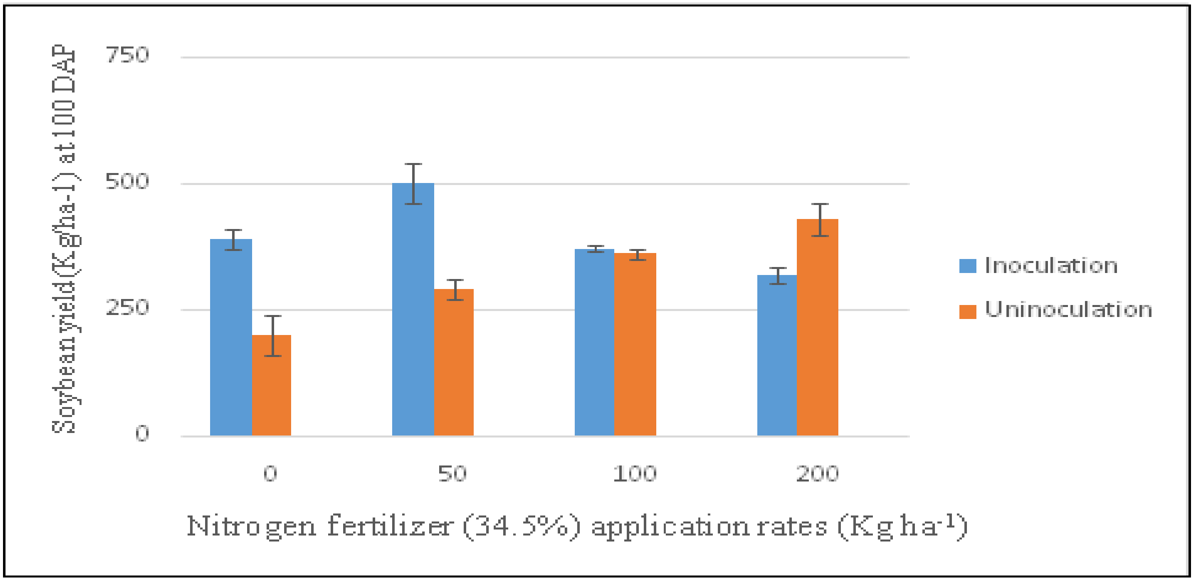

Fig. 1. Effect of inoculation and nitrogen fertilizer (34.5%)application on soybean yield (Kg ha^{-1}). The standard error of the mean is presented as error bar.

Pod formation and dry weight

The application of 50 kg N ha^{-1} in the plants inoculated with bradyrhizobium produced the highest pod formation and pod dry weight per plant as compared to the uninoculated plants. However, the application of nitrogen fertilizer at 100 and 200 kg ha^{-1} resulted in lower pod number and pod dry weight and the lowest value was obtained at 200 kg N ha^{-1}. Furthermore, the difference between 0, 100 and 200 kg N ha^{-1} was insignificant.

In the uninoculated plants treatments, pod number and pod dry weight increased with nitrogen levels though that the difference between 0 and 50 kg N ha^{-1} as well as 100 and 200 kg N ha^{-1} was insignificant. However, the application of 200 kg N ha^{-1} increased both pod number and pod dry weight which was almost similar to the application of 0 and 50 kg N ha^{-1} in inoculated plants with *B. japonicum*. Besides, it is important to note that inoculated plants with bacterial *B. japonicum* produced higher pod number and pod dry weight which was significantly different from uninoculated plants at 0 and 50 kg N ha^{-1}.

The results of the present study are consistent with Janagard and Ebadi-Segherbo (2016) who reported higher pod dry weight per plant in the plants from seeds inoculated with Biosoy. After conducting experiments in both 2010 and 2011, Alam *et al.* (2015) reported higher pod yield from inoculated soybean with *Rhizobium* sp. than uninoculated plants. Further studies suggested that the integration of the bacterial *B. japonicum*a t lower nitrogen level rather than nitrogen alone improved the growth and yield of soybean and the maximum effectiveness of the *B. japonicum* on pod dry weight was observed at lower nitrogen levels. Futher studies by El-Shaarawi *et al.* (2011), stated that the integrated application of *B. japonicum* at smaller amounts of nitrogen rather than the application of nitrogen alone improved the growth and yield of soybean. In addition, the beneficial effect of *B. japonicum* on pods dry weight at low levels of nitrogen fertilizer improved and the lowest amount of fertilizer had maximum effectiveness on soybean growth. Their study also reported that increasing nitrogen fertilizer produced high biomass of soybean leaves, stems and pods (El-Shaarawi *et al*., 2011).

Stem dry weight

Higher stem dry weight was recorded from 50 kg N ha^{-1}. Treatment in plant from seeds inoculated with the bacterial *B. japonicum*. High amount of nitrogen fertilizer than 50 kg ha^{-1} resulted in reduced plant height as observed in Table 3. The lowest plant height was recorded where high amount of nitrogen fertilizer was applied. Though the differences between 0, 50 and 100 kg N ha^{-1} were not substantial.

Plant from seeds uninoculated with *B. japonicum* increased application of fertilizer resulted in increase stem dry weight with the highest stem dry weight recorded at 200kg N ha^{-1} while the lowest stem dry weight at 0 kg N ha^{-1}. There were not significant differences among the control (0) and the plants where 50 kg N ha^{-1} was applied. This was also the same for plants where 100 and 200 kg N ha^{-1} was applied.

The current study concurred with Janagard and Ebadi-Segherloo *et al*. (2016), who reported higher stem dry weight in plant from seeds inoculated with *B. japanicum* and increased use of urea fertilizer in their study resulted in increase stem dry weight for non-inoculated plants. Alam *et al*. (2015) also reported a significant increase in soybean shoot biomass when the plants were inoculated with Rhizobium. Further study by Elkoca *et al*. (2007) reported a significant increase in pea plant height and stem biomass in plant inoculated with *Rhizobium* as compared to non inoculated plants. Their study also reported that this increase was equal to or greater than nitrogen (N), phosphorus (P) and nitrogen+ phosphorus treatments. Significant increase in plant biomass was also reported when there was increase in soybean fertilization (Tahir *et al*., 2009).

Soybean yield

Soybean yield was significantly affected by the treatments applied. In plants that were inoculated with the bacterial *B. japonicum*, the application of 0 and 50 kg N ha^{-1} produced higher yield. Highest yield was recorded in 50 kg N ha^{-1} treatment and was statistically similar to nitrogen application of 200 kg ha^{-1} in the uninoculated plants (Fig 1). Application of nitrogen fertilizer at 50 and 100 kg ha^{-1} significantly increased yield in treatments that were not inoculated compared to the control. However, the application of 200 kg N ha^{-1} significantly increased yield than 0, 50 and 100 kg N ha^{-1}. The use of nitrogen in non inoculated plants increased yield and the lowest yield was obtained in the control. In the inoculated plants the application of 50 kg N ha^{-1} produced the highest yield than uninoculated plants (Fig 1).

Increase in soybean yield resulting in increased use of nitrogen fertilizer has been already reported (Starlin *et al.,* 2000; Mbah *et al*. 2007; El- Shaarawi *et al*., 2011; Usman *et al*., 2015). Despite this, Argaw (2012) reported that the addition of nitrogen fertilizer at a rate of 46 kg ha^{-1} urea did not significantly increase soybean yield compared to the control. Hungria *et al*. (2015) reported higher soybean yield in plants that were inoculated with *Rhizobium* than the control suggesting that the co- inoculation promotes yield increases without adding any chemical N fertilizers even in soils where established populations of soybean bradyrhizobial exist. El-Shaarawi *et al*. (2011) reported also that the bacterial *B. japonicum* had greatest effect on soybean yield in the least amount of chemical fertilizer. Despite this, Javaid *et al.,* (2002), reported no significant effect of *B. japonicum* in soybean yield

Conclusion

The treatments in the current study significantly influenced the parameters that were measured. Increasing nitrogen fertilizer in uninoculated plants insignificantly reduced nodule number and nodule dry weight but increased plant height, number of pods, pod dry weight, stem dry weight and soybean yield. The application of 0 and 50 kg N ha^{-1}for inoculated seeds produced more plant height, number of nodules and nodule dry weight as compared to the application of 100 and 200 kg N ha^{-1} for uninoculated plant. Therefore based on this study we recommend to apply 200 kg N (34.5%) ha^{-1} when soybean are not inoculated and 50 kg N (34.5%) ha^{-1} when soybean seeds are inoculated with *B. japonicum* in clay loam soils region of Africa University Farm in Old Mutare.

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