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Assessing Liquid Inoculant Formulation of Biofertilizer (*Sinorhizobium meliloti*) on Growth, Yield, and Nitrogen Uptake of Lucerne (*Medicago sativa*)

Auges Gatabazi [1,2,*], Martin Botha [1] and Mireille Asanzi Mvondo-She [2]

[1] In Line Trading 112, Cedar Lake Industrial Estate, Suite # 114 Private Bag X 27 Kempton Park, Johannesburg 1620, South Africa
[2] Department of Plant and Soil Sciences, University of Pretoria, Pretoria 0002, South Africa
* Correspondence: auges2012@gmail.com

Abstract: Lucerne is regarded as the best legume crop for forage to be cultivated in South Africa. It is commonly used to produce good quality hay. It also plays an important role in soil conservation, regeneration, and crop rotation systems as it supplies substantial amounts of nitrogen to succeeding crops through symbiotic N_2 fixation, which makes it the preferable choice for intensive forage production systems. Fertilizer in liquid inoculant formulations has demonstrated to contribute growth and yield increase for leguminous crops. Therefore, the aim of this paper was to determine the effects of *Sinorhizobium meliloti* liquid formulation inoculation on the growth, yield, and nitrogen content in lucerne. The strain RF14 (*Sinorhizobium meliloti*) was collected from the Agricultural Research Council at Roodeplaat (Plant Health and Protection located (East), Pretoria (South Africa). The liquid inoculant contained 6.5×10^9 viable cells mL^{-1}. According to the Kooen–Gieger climatic classification, the experiments were conducted on two different climatic zones. The first site was in Bronkhorspruit (Blesbokfontein farm) in the Gauteng province and the second was in Hartbeesfontein (Rietfontein Farm) in the Northwest province. The results showed that lucerne inoculation with liquid inoculant formulation of *Sinorhizobium meliloti* significantly increased nodule number, size, growth, and yield in both bioclimatic zones. The significantly increased were compared to the negative control. The *Sinorhizobium meliloti* inoculant increased nitrogen accumulation in all inoculated treatments compared to the control. The finding of this research provides important information on the impact of rhizobium microbial inoculant application in the improvement of soil fertility through nodule formation. In addition, seed vigor improvement was translated in overall growth and yield increase in lucerne plants.

Keywords: liquid formulation; nitrogen content; nodule; rhizobium

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1. Introduction

Lucerne, also known as alfalfa (*Medicago sativa*), is a perennial fodder crop suited for grazing, silage, and hay that can be productive for six years or more [1,2]. It is also used in animal production systems incorporated for selecting grazing for cattle, sheep, ostriches, and other livestock [2].

It is classified globally as one of the most reliable forage crops due to the high source of protein, Ca, Mg, P, carotene, and vitamin D [3]. It enhances microbial activities within the soil; therefore, the nutrient availability increases results of high yields of forage [3]. It also plays an important role in soil conservation, regeneration, and crop rotation systems as it supplies substantial amounts of nitrogen to succeeding crops through symbiotic N_2 fixation, which makes it the preferable choice for intensive forage production systems [4]. In South Africa, lucerne is one of the pasture hay plants mostly used due to its high-quality roughage hay and high protein content in comparison to other legumes and tropical grasses [4,5].

Furthermore, its resilience and tolerance to wide varieties of climatic and soil conditions makes it more suitable for dryland South African production areas [1].

Lucerne is a leguminous plant species that forms a symbiotic relationship with the rhizobium bacteria *Ensifer meliloti* and *E. medicae* [6,7]. Through this symbiotic relationship, the rhizobium bacteria facilitate the fixation of atmospheric N_2 in their root nodules into NH_3 [8]. Then the fixed N is further assimilated to supply organic N compounds, amino acids, and proteins to plants. The N fixation process is important for the lucerne plant due to its high N requirement because of its high productivity and nutritional value [2–4].

The European expert panel recommend achieving a Nitrogen Use Efficiency (NUE) between 50–90% [9]. This target is only attainable under the high N fertilizer level. However, due to the low income of smallholder farmers and low soil fertility in the sub-Saharan Africa region, this is not possible [10]. Moreover, considering the current conflict between two major fertilizer producers, Russia, and Ukraine, this has resulted in a significant increase in fertilizer price [11]. Therefore, there is a great need to explore biological nitrogen fixation among other techniques to improve soil fertility, NUE, and provide N benefits for crop rotation systems [10]. Moreover, rhizobium is known to improve growth development and yield in many plants [12–16]. Little is known concerning the natural prevalence of *rhizobium meliloti* in natural soils of South Africa. *Rhizobium S. meliloti* inoculation is recommended in soils where lucerne has not been grown for at least a minimum of 3 years [17]. To our knowledge, there are few studies that have investigated the impact of *S. meliloti* on lucerne. A study conducted in two lucerne cultivars in two Canterbury soils provided only an indication of the increase in nodulation by 50% compared to the uninoculated plants [18]. A gap in the understanding of the impact of *S. meliloti* inoculation on the growth development and yield of lucerne plants was evident in previous research. Therefore, the hypothesis formulated states that the liquid inoculant formulation of Biofertiliser *Sinorhizobium meliloti* will have significant impact on the growth, nodulation, yield, and nitrogen content of lucerne. Consequently, the aim of this study was to assess the impact of the liquid inoculant formulation of Biofertilizer (*S. meliloti*) on the growth, nodulation, yield, and nitrogen content of lucerne.

2. Materials and Methods

2.1. Preparation of Liquid Inoculant

Sinorhizobium meliloti, Strain RF14 was collected from the Plant Health and Protection Unit at Agricultural Research Council, Roodeplaat East, Pretoria (South Africa). Bacterial cultures were grown on agar complemented with congo red, then 10 mL of inoculum were transferred into 750 mL of yeast extract mannitol and shaken for 3–5 days at 150 r.p.m. at 30 °C till the sample reached a concentration of 6.5×10^9 cells/mL. The yeast mannitol broth was composed of (g/L) 10.0 mannitol, 0.5 K_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.1 NaCl, 1.0 yeast isolate, 1.0 glucose, 0.5 arabinose, 200 mM/L FeEDTA, and 4 mL/L glycerol. A mixture of additives was added and shaken at 150 r.p.m. at 30 °C for 4 h. This procedure provided 1 L of liquid *S. meliloti* biofertilizer; however, the final formulation product was stabilized and presented as per g kg^{-1} on the formulation. The yeast mannitol broth was made up using the methods described by [19]. The liquid inoculant contained *S. meliloti* 6.5×10^9 viable cells mL^{-1}.

2.2. Experimental Location

Experimental sites were performed in two climatic zones according to the Koppen–Gieger climate classification [20]. The first site was in Blesbokfontein farm (Bronkhorspruit) in the Gauteng province of South Africa. The location is categorized under a humid subtropical climate defined as C-wa (Bronkhorspruit/Gauteng, South Africa) and situated at 25°48′30″ S latitude and, 28°44′26″ E. Sandy clay loam was used in the site.

Hartbeesfontein (Rietfontein Farm) in the Northwest province of South Africa was used as the second site and is categorized as an arid climate represented by BWwh. Ri-

etfontein farm is situated at 26°47′16″ S latitude and 26°53′59″ E longitude with a sandy loam soil. The annual seasonal rainfall of the area is 670 mm.

2.3. Analysis of Soil and Biofertilizer Application

Soil samples were taken from depths of 0–40 cm randomly to assess physical properties and chemicals according to [21]. The soil of the experiment site was described as a Hutton soil [22] form with a clay content of 36% at 0–40 cm soil depth similar to [16]. No additional fertilizer was incorporated in both bioclimatic zones. Chemical and physical properties of different bioclimatic zones of the experiments at a soil depth of 0–40 cm of the soil horizon was similar to [16].

Weather data for Bronkhorspruit/Gauteng and Hartbeesfontein/Northwest weather data during the year 2019/2020 were similar to [16].

2.4. Trial Design and Treatment

The field trials were laid down in randomized trip plot designs. Each plot consisted of four lucerne rows, 50 m long, and four treatments and six replicates were used. The used treatments were liquid inoculant dosages for *Sinorhizobium meliloti* (T0 = 0 mL, T1 = 150 mL, T2 = 300 mL, and T3 = 150 mL was also used for a registered standard). Inoculant was applied onto 50 kg of lucerne seeds. The experimental site was ploughed and harrowed to a depth of 20 cm and separated into plots before sowing. Planter machine equipment was used for sowing. Irrigation was performed by using a sprinkler.

Trial sites in both climatic zones were conducted in the summer cropping season of December 2019 to April 2020. Sowing was carried out on the 17 November 2019 and the measurement date for growth parameters was completed on 2 February 2020, while the lucerne harvest was conducted on 20 May 2020.

2.5. Data Collection

Growth and Yield Parameters

Emergence parameters were recorded from five to eleven days after planting in both sites. The final emergence rate was recorded when 80% of lucerne had emerged in all treated plots and replicates. The growth measurements, which consist of plant height and root length, were taken at flowering growth stage (R3). The two factors were established by randomly selecting and thoroughly uprooting forty plants selected randomly from the center of plots. The weight was then measured from the selected 40 plants, and the lucerne weight was determined and the yield was expressed in tons per hectare ($t \cdot ha^{-1}$). The number and size of the nodules were determined by randomly sampling 40 plants in the center of the rows of each plot. The nodule size was determined by measuring the vertical length of the cylindrical nodule using a caliper. This was completed at mid flowering by uprooting the whole plant carefully with a spade. The normal average nodules number and size were performed from the above 40 plants and were measured and calculated. The practice of uprooting the plant was also thoroughly determined using a spade to avoid nodules damage.

However, the Kjeldahl method as described by [22] was used for the determination of the nitrogen content; plant samples were taken at mid-flowering from each plot and then samples were oven dried at 70 °C to a constant weight, ground, and passed through a 1 mm sieve, then analyzed for their nitrogen concentrations.

2.6. Statistical Analysis

Analysis of variance was performed using the Statistical Analysis Software R Version 4.2.1. Fisher's Least Significant Difference (LSD) at 5% level of significance was used to determine the differences between treatments.

3. Results

3.1. Lucerne Growth, Yield and N Uptake Parameters

3.1.1. Lucerne Emergence

In bioclimatic zone A, the lucerne emergence was higher in the inoculated treatments (T1 and T3) compared to the control (T0) (Figure 1). Meanwhile, no significant differences were observed between T0 and T2. In bioclimatic zone B, significant differences were shown between the control and the treatment (T1). Thus, the data show that there were no significant differences between T0, T2, and T3. In both bioclimatic zones (A and B), there were no significant differences detected among the inoculated treatments.

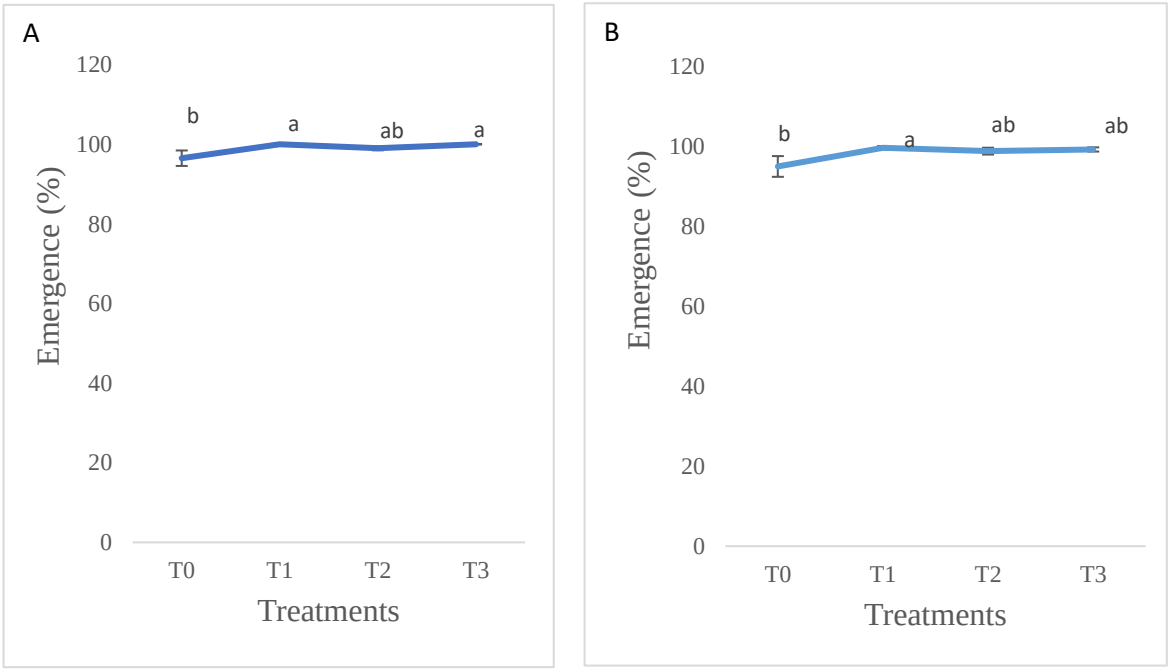


Figure 1. Rate of lucerne emergence under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti* in climatic zone (**A**) = Bronkhorspruit/Gauteng and (**B**) = Hartbeesfoein/Northwest Province). Bars sharing a letter are not significantly different (Lsd Zone A) = 2.940 and (Lsd Zone B) = 4.286. Data are means ± standard errors.

3.1.2. Plant Height

In both climatic zones (A and B), the plant height was significantly higher ($p < 0.01$) under inoculation with *S. meliloti* (T1, T2, and T3) compared to the control (T0) (Figure 2).

3.1.3. Root Length

In both climatic zones (A and B), the root length was highly significant at ($p < 0.01$) under inoculation with *S. meliloti* (T1, T2, and T3) compared to the control (T0) (Figure 3). Among the inoculated treatments, T1 was significantly higher than T2 and T3 in both bioclimatic zones.

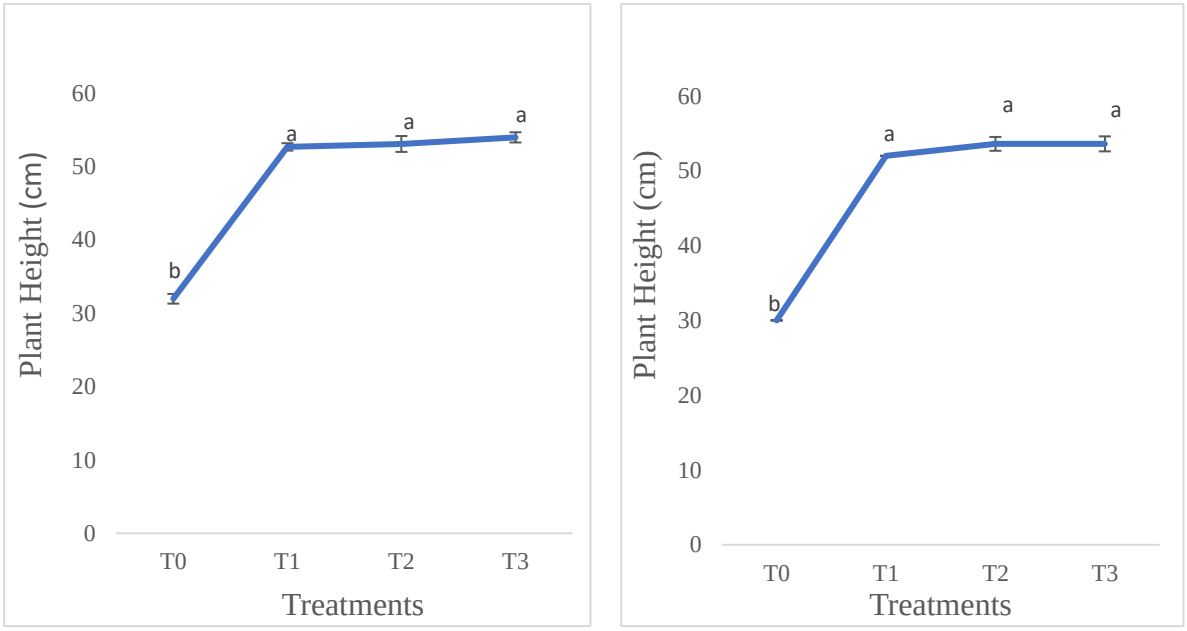


Figure 2. Plant height of lucerne under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti* in climatic zone (**A**) = Bronkhorspruit/Gauteng and (**B**) = Hartbeesfoein/Northwest Province). Bars sharing a letter are not significantly different (Lsd Zone A) = 20.148 and (Lsd Zone B) = 20.094. Data are means ± standard errors.

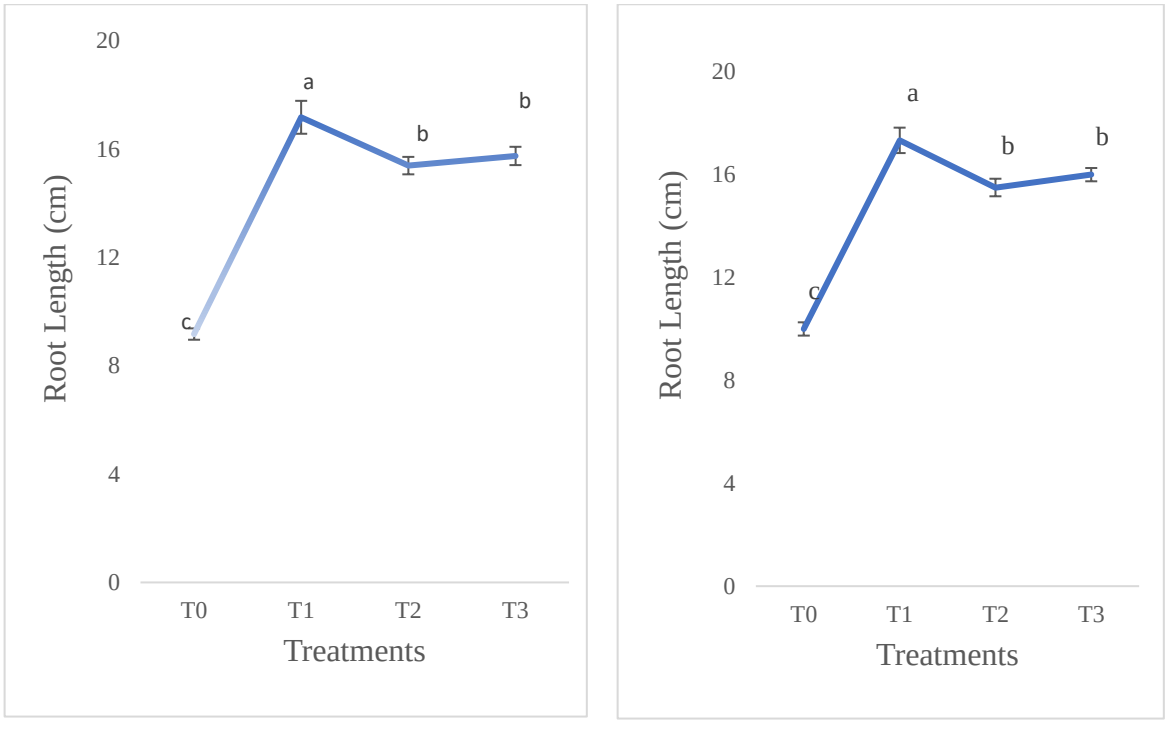


Figure 3. Root length of lucerne under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti* in climatic zone (**A**) = Bronkhorspruit/Gauteng and (**B**) = Hartbeesfoein/NorthWest Province). Bars sharing a letter are not significantly different (Lsd Zone A) = 12.322 and (Lsd Zone B) = 10.949. Data are means ± standard errors.

3.1.4. Size of Nodules

In bioclimatic zone A, the size of the nodules of lucerne was significantly higher in the inoculated treatments (T2 and T3) compared to the control (T0) (Figure 4). Meanwhile, no significant differences were registered between T0 and T1. In bioclimatic zone B, the size of the nodules of lucerne was significantly higher in the inoculated treatments (T1 and T2) compared to the control (T0) (Figure 5). Meanwhile, no significant differences were registered between T0 and T3.

3.1.5. Number of Nodules

In both climatic zones (A and B), the number of nodules was significantly higher ($p < 0.01$) under inoculation with *S. meliloti* (T1, T2, and T3) compared to the control (T0).

3.1.6. Yield

In both climatic zones (A and B), the number of nodules was significantly higher ($p < 0.01$) under inoculation with *S. meliloti* (T1, T2, and T3) compared to the control (T0) (Figure 6).

3.1.7. Nitrogen Content

The nitrogen content was higher by 50% in T1 compared to the control, T0. Meanwhile, T2 and T3 were higher compared to T0, respectively, by 9.3% and 21% (Figure 7).

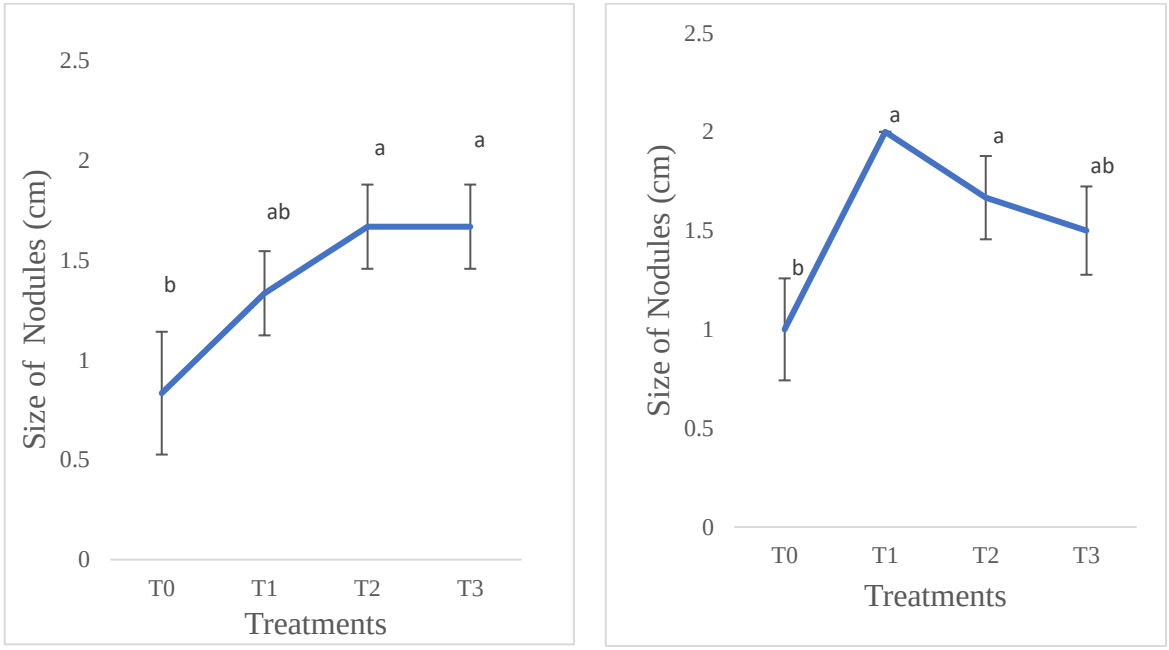
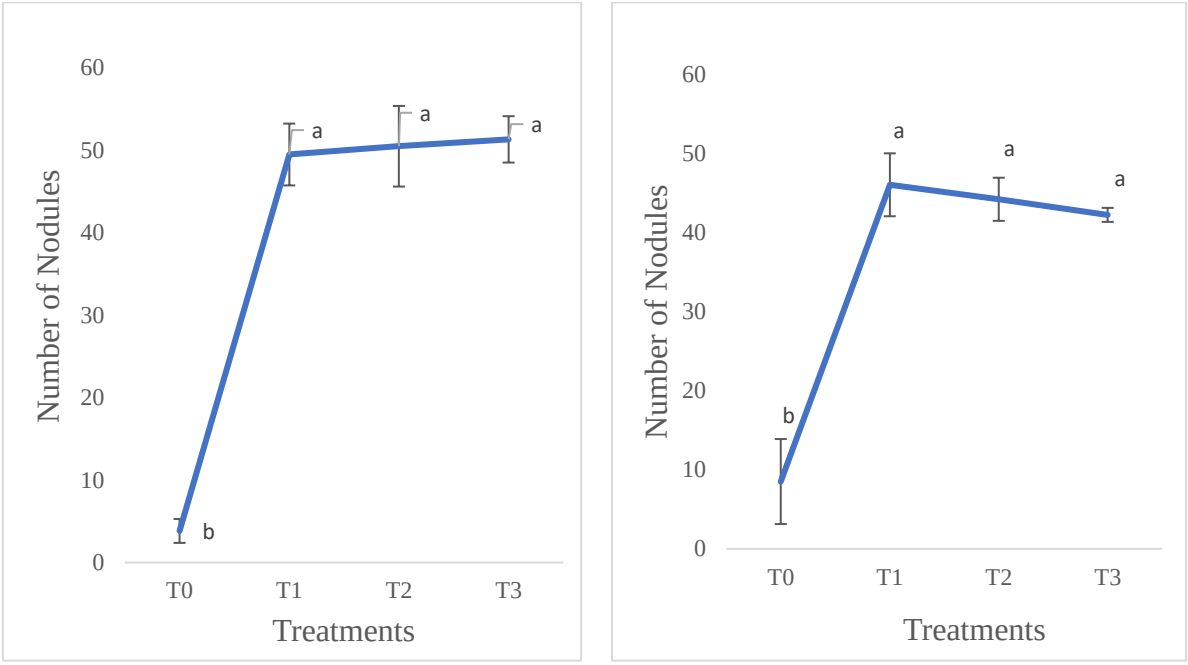
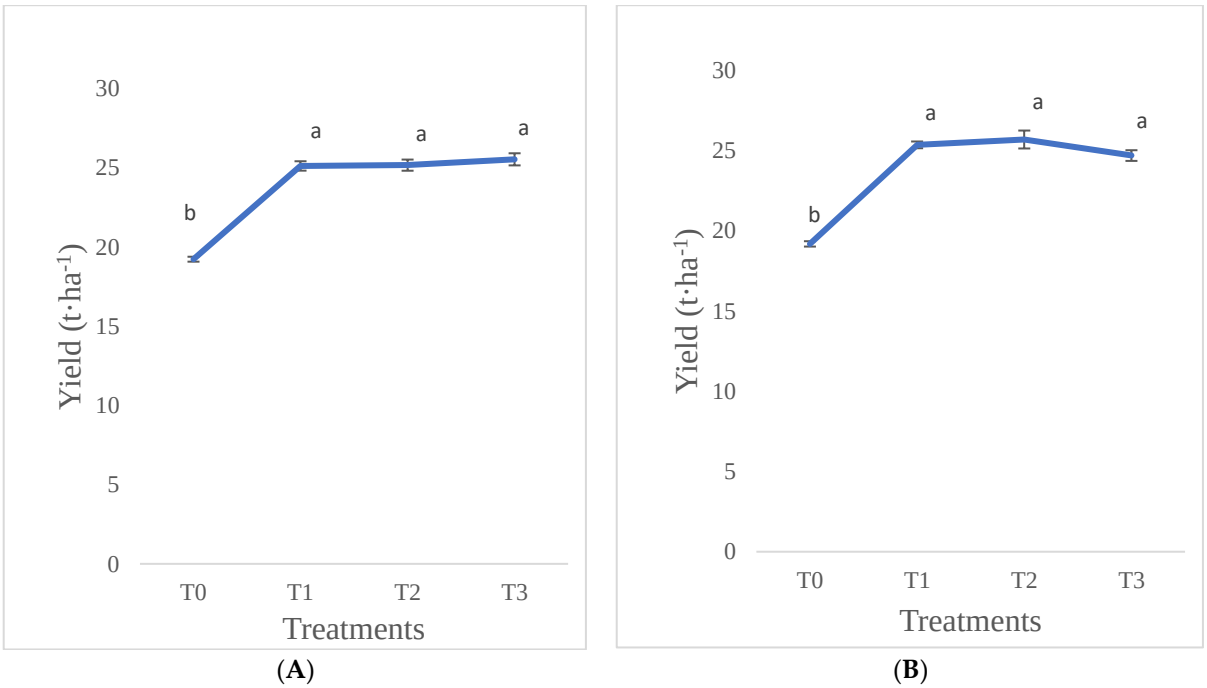


Figure 4. Lucerne size of nodules under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti* in climatic zone (**A**) = Bronkhorspruit/Gauteng and (**B**) = Hartbeesfoein/Northwest Province). Bars sharing a letter are not significantly different (Lsd Zone A) = 0.742 and (Lsd Zone B) =0.645. Data are means ± standard errors.



(**A**) (**B**)

Figure 5. Lucerne nodules number under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti* in climatic zone (**A**) = Bronkhorspruit/Gauteng and (**B**) = Hartbeesfoein/NorthWest Province). Bars sharing a letter are not significantly different (Lsd Zone A) = 9.126 and (Lsd Zone B) = 7.418. Data are means ± standard errors.



(**A**) (**B**)

Figure 6. Yield of lucerne under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti* in climatic zone (**A**) = Bronkhorspruit/Gauteng and (**B**) = Hartbeesfoein/Northwest Province). Bars sharing a letter are not significantly different (Lsd Zone A) = 0.940 and (Lsd Zone B) = 1.035. Data are means ± standard errors.

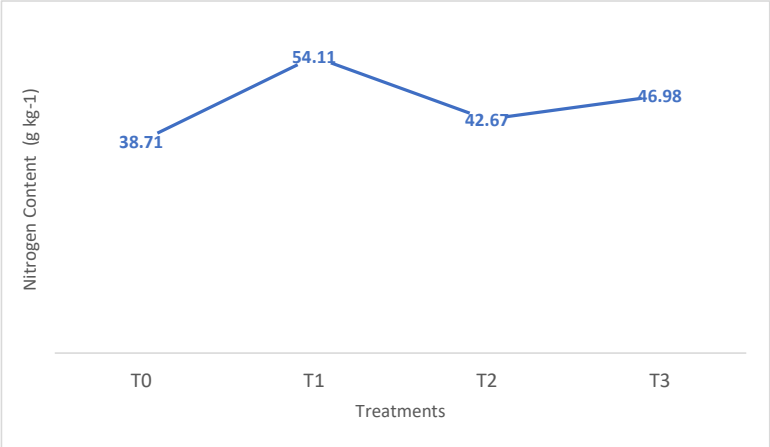


Figure 7. Lucerne nitrogen uptake under inoculation with liquid formulation (T0, T1, T2, and T3) of *Sinorhizobium meliloti*.

4. Discussion

Sinorhizobium meliloti inoculation in both bioclimatic zones revealed a positive effect on growth and yield. In bioclimatic zone A, the emergence percentage was significantly higher in inoculated treatments (T1 and T3) compared to the un-inoculated treatment (T0) (Figure 1). Meanwhile, in bioclimatic zone B the emergence percentage was significantly higher in T1 compared to the control. This corroborates with previous studies in soybean inoculated with *Bradyrhizobium japonicum* that demonstrated a significant increase in emergence % in inoculated treatments compared to the control [16]. These results demonstrated that some of the applied inoculant concentrations are more effective in increasing the emergence percentage compared to others because of the increased seed vigor due to the soil health condition improvement [23]. The underlining mechanism of seed vigor improvement under rhizobia inoculation has been demonstrated to be due to the production of phytohormone indoleactic acid (IAA), which improves sugar metabolization, therefore supplying energy for subsequent growth development [24].

With respect to plant height, the treatments inoculated with *S. meliloti* (T1, T2, and T3) were significantly higher compared to the un-inoculated treatment in both climate zones (Figure 2). This agrees with our previous studies conducted on soybean [16].

In both climatic zones (A and B), the root length was highly significant at ($p < 0.01$) under inoculation with *S. meliloti* (T1, T2, and T3) compared to the control (T0) (Figure 3). However, T1 was the optimum absorption concentration and resulted in a significantly higher root length.

The significant higher growth was recorded consistently in comparison to the untreated control corroborates with findings on soybean, which is justified by the fact that the soil was under regenerative practices [16].

The size of the nodules was influenced positively by inoculated *S. meliloti* compared to the un-inoculated treatment in both bioclimatic zones (Figure 4). The inoculant liquid formulation produced a significant increase in the nodule size compared to the control. Meanwhile, the registered standard demonstrated no significant difference compared to the control. This implies that the liquid formulation inoculant could provide a competitive advantage with respect to soil structure and fertility improvement compared to the registered product.

This study shows that the number of nodules and the yield were significantly greater in inoculated plants compared to the control (Figures 5 and 6). Similarly, in another study conducted on lucerne crop inoculated with *S. meliloti*, the results demonstrated that seeds coated with the inoculant had the highest nodulation (of about 45%) compared to the uninoculated plants [18]. This also concurs with our previous study conducted on soybean [16], which also substantiates a study performed by [25] that reported *B. japonicum*

to significantly increase soybean seed yield and yield components, such as the number of pods per plant, number of seeds per pod, and yield. In other studies, conducted by [26], it was stated that the number of nodules were enhanced in plants inoculated with *B. japonicum* compared to the control treatment, as was the case in the research performed on the growth and yield of soybean varieties inoculated with *Bradyrhizobium* spp. Strains [27,28]. In a previous study, the enhancement in growth development demonstrated by the soybean, sweet pea, rice, spinach, and maize plants inoculated with the biofertilizer *Streptomyces griseoflavus* P4 was attributed to the production of Indoleacetic acid (IAA) which stimulates energy for microorganisms production, therefore supplying energy for plant growth and development. [29–31] reported that Biofertilizers competitively colonize plant root systems, which, in turn, enhance nutrient uptake, increase productivity, and crop yield, improve plants' tolerance to stress and their resistance to pathogens, and improve plant growth through mechanisms such as the mobilization of essential elements, nutrients, and plant growth hormones.

Findings from our research regarding nitrogen content (Figure 7) demonstrated that uptake was higher in the inoculated treatments compared to the control. It also demonstrated that T1 (150 mL) was the optimum inoculant concentration of 50 kg of lucerne seed. This was evident in a phytotoxicity test conducted at the onset of the experiment, which determined T2 to be a suboptimal level [16].

5. Conclusions

This study determined T1 (150 mL) with concentration of 6.5×10^9 cells/mL as the optimum concentration of *S. meliloti* inoculant to induce a significant increase in growth, the yield of lucerne, and nitrogen content in both bioclimatic zones. We can infer that the improvement in the growth and development of lucerne could be due to the supply of energy for growth and development from phytohormone IAA production. The liquid inoculant formulation provided a significant higher nodule size compared to the registered product. Therefore, it has better potential in improving soil structure and fertility. In addition, the nitrogen content increases due to *S. meliloti* inoculation compared to the control treatment can be explored to meet the high fertilizer requirement of lucerne. Moreover, phytohormone production in crops inoculated with biofertilizer has a great potential in alleviating environmental stress responses, therefore warranting further investigation.

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