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Evaluating the feasibility of late nodulation in common beans

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ABSTRACT. The nodulation of common beans occurs continuously until the flowering stage, followed by nodule senescence. However, reports have indicated the potential for late nodulation in this species, contributing to increased grain production. This study aimed to evaluate the occurrence of late nodulation in common beans and its contribution to plant growth. Experiments were carried out by testing two inoculation strategies: rhizobial inoculation (1) in different sections of the root system and (2) at different phenological stages. Plants were harvested at flowering and the beginning of pod filling. When the first strategy was applied, both inoculation on the seeds and throughout the pot volume resulted in greater nodulation compared to the uninoculated control. However, shoot biomass accumulation remained unaffected. When the second strategy was applied, supplementary inoculation at different stages did not improve nodulation or plant growth compared to seed inoculation. We conclude that neither method promoted effective late nodulation of common beans and that seed inoculation was sufficient to promote good vegetative development of common beans.

Keywords: symbiosis, inoculation, *Phaseolus vulgaris* L.

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Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most important crops in the world, with Brazil being among the largest global producers in 2022 (Food Agriculture Organization of the United Nations [FAO], 2024; Soares, Trejo, Veloso, & Castro, 2016). This legume can form a symbiotic relationship with rhizobia, which supply part of its nitrogen demand (Allito, Ewusi-Mensah, & Logah, 2020; Rufini et al., 2011). This symbiotic interaction results in the formation of root nodules, which start from 7 to 15 days after emergence and extend until plant flowering, when the nodulation peak usually occurs (Carneiro, Paula Júnior, & Borém, 2014). Subsequently, there is a sharp decline in nodulation as well as in the rates of biological nitrogen fixation during the pod-filling period (Hungria, Campo, & Mendes, 2003).

Some studies observed the potential of common beans to maintain nodulation after flowering, influenced by several factors such as the genetic characteristics of the plant, rhizobial strains, and environmental conditions (Andraus, Cardoso, & Ferreira, 2016; Ayra et al., 2021; Graham, 1981; Lima, Boldt, Kava, Gali-Terasawa, & Adamoski, 2022; Rufini et al., 2011; Vickman & Vessey, 1993). Late nodulation, with the formation of new nodules after the peak of nodulation, has been observed in soybeans and contributed to increased grain production (Danso, Kapuya, & Hardarson, 1990; Moretti et al., 2018). Nevertheless, late nodulation may not be effective for nodulation and development because most distal regions of the root system are not in contact with the strains introduced through commercial inoculants applied to the seed and are infected by native soil strains that develop ineffective nodules in some cases (Allito et al., 2020; Hungria et al., 2003; Mwenda et al., 2023).

Based on the work of Vickman and Vessey (1993), this study hypothesized that the common bean can carry out late nodulation and that these nodules can contribute to plant growth and productivity through higher rates of biological nitrogen fixation (BNF). Therefore, the objective of this study was to evaluate the occurrence of late nodulation in common beans and assess its contribution to providing a crop with better plants.

Material and methods

To evaluate the occurrence of late nodulation and its contribution to plant growth, common bean plants were subjected to two inoculation strategies: (1) the spatial distribution of *Rhizobium tropici* in different portions of the soil and (2) supplementary inoculation at different stages of development. The first strategy was based on Vickman and Vessey (1993) and aimed to inoculate rhizobia into different sections of the root system using charcoal inoculated at different depths. The premise was that the roots would nodulate early when the inoculum was at the surface of the pot and later when it was available at the bottom. The second strategy involved the application of a liquid inoculant at different stages of the vegetative and reproductive cycles. The cultivar Pérola was used. It is in the commercial group of Carioca grain and has a high productive potential and a normal cycle (85-95 days).

Inoculation at different depths of the root system

The experiment was conducted in October 2018 following a randomized block design with six treatments and eight replicates. Four replicates were harvested at the flowering stage (R6, 42 days after sowing) and the remaining four replicates were harvested at the beginning of pod filling (R8, 57 days after sowing). The treatments were as follows: (1) uninoculated control, (2) peat inoculated on seeds, (3) peat inoculated on seeds and charcoal inoculated in the upper third of the pot, (4) peat inoculated on seeds + charcoal inoculated in the middle third of the pot, (5) peat inoculated on seeds + charcoal inoculated in the lower third of the pot, and (6) peat inoculated on seeds and charcoal inoculated throughout the pot. All pots contained charcoal throughout the pot to standardize the possible effects of charcoal on nodulation among all treatments.

We used inoculated charcoal as a strategy to inoculate different sections of the root system through the distribution of the inoculum at different depths of the substrate (Figure1), based on the work of Vickman and Vessey (1993). Because of its high absorption, the inoculum would be more concentrated in the section of interest. The pots were composed of three plastic segments mounted on top of each other, with diameters of 10 cm and depth of 13 cm, totaling 39 cm depth (Figure 1). Charcoal was used as the inoculation vehicle owing to its high absorption capacity and availability.

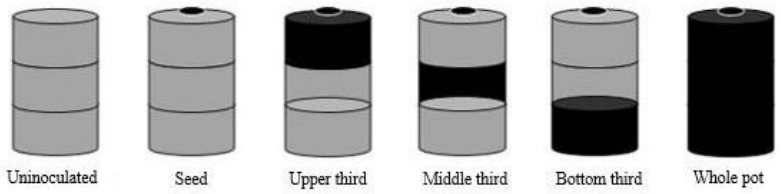

Figure 1. Schematic representation of the experiment of inoculation at different depths of the root system. The black color indicates the inoculation of charcoal and seeds. From left to right, uninoculated control; peat inoculation on seeds (1.2×10^6 cells $seed^{-1}$); inoculation of the upper, middle, and bottom thirds of the pots; and inoculation of the whole pot. The seeds were inoculated with peat inoculant in all pots that received inoculated charcoal.

The substrate was composed of a subsurface layer (20–40 cm deep) of Argisol (type Hapludult) and fine gravel 1:1 (v/v). Soil was collected from the experimental area of 'Embrapa Agrobiologia,' the municipality of Seropédica, RJ State. The chemical attributes were pH 5.03, 0.6 $cmol_c$ dm^{-3} Ca, 0.6 $cmol_c$ dm^{-3} Mg, 0.4 $cmol_c$ dm^{-3} Al, 13 mg L^{-1} K, and 1.9 mg L^{-1} P. The native rhizobia population was quantified using the most probable number technique (Woomer, Singleton, & Bohlool, 1988). Plants of the cultivar Pérola were grown in glass bottles supplemented with 200 mL Norris nutrient solution without N. The seeds were disinfected and inoculated with 1 mL of the suspension from serial dilutions of the soil (10^{-1} to 10^{-8}). The plants were harvested 30 d after emergence, and nodulation was detected to estimate the number of nodule-forming colonies in the soil.

The soil was limed with 0.5 g $CaCO_3$ kg^{-1} soil and mixed with fine gravel 1:1 (v/v). The substrate was combined with charcoal at a proportion of 108 g of charcoal for each 3 kg of substrate, inoculated or not inoculated. The substrate was fertilized once with a nutrient solution containing the following amounts of salts per kg of substrate: 101.4 mg of $MgSO_4.7H_2O$, 7.9 mg of $CuSO_4.5H_2O$, 4.4 mg of $ZnSO_4.7H_2O$, 0.6 mg of H_3BO_3, 0.5 mg of $Na_2MoO_4.2H_2O$, 526.8 mg of KH_2PO_4, and 0.1 mL of Fe-EDTA.

The inoculant *Rhizobium tropici* CIAT 899 (Semia 4077), one of the strains recommended by Ministério da Agricultura e Pecuária [MAPA] (2011) for successful inoculation, was grown in yeast–mannitol broth. Six

hundred (600) milliliters of the inoculant at a concentration of 10^8 cells mL^{-1} was diluted in filtered water 1:1 (v/v) and mixed with 2.6 kg of charcoal. The inoculated charcoal was distributed at different depths, following the treatments described above (Figure 1). Each pot received five seeds and two plants remained per pot after thinning. The pots were irrigated periodically and neem oil was applied to control trips, according to the needs of the plants.

Four replicates were collected at the flowering stage and four replicates were collected at the pod-filling stage. Shoots were separated from the root system. The pots were sectioned into three segments to assess nodulation in different root system sections. The roots were washed and the nodules were detached and counted. Each part of the plants (shoot, roots, and nodules) was dried at 65ºC and weighed.

Supplementary inoculation at different phenological stages

In May 2019, two greenhouse experiments were conducted to evaluate the strategy of supplementary inoculation at different phenological plant stages. The experiment used a randomized block design with four replicates. In the first experiment, five treatments were harvested at flowering (R6, 47 days after sowing) and in the second experiment, seven treatments were harvested at the beginning of pod filling (R8, 62 days after sowing). The treatments in the first experiment were (1) uninoculated control; (2) peat inoculant on seeds; (3) peat inoculant + liquid inoculant at the V3 stage (first expanded trifoliate); (4) peat inoculant + liquid inoculant at the V4 stage (third expanded trifoliate); and (5) peat inoculant + liquid inoculant at the V3 and V4 stages. The treatments of the second experiment were (1) uninoculated control; (2) peat inoculant on seeds; (3) peat inoculant + liquid inoculant at V3 stage; (4) peat inoculant + liquid inoculant at V4 stage; (5) peat inoculant + liquid inoculant at R5 stage (flowering); (6) peat inoculant + liquid inoculant at stage R6 (full flowering); (7) peat inoculant + liquid inoculant at stages V3, V4, R5, and R6.

The pots contained 5 L of a mixture of vermiculite and gravel-crushed stone 1 a 1:2 (v/v) ratio. Each pot received five seeds that were inoculated with peat inoculated with *Rhizobium tropici* CIAT 899 (SEMIA 4077), except for the control. The peat inoculant was applied to achieve a dose of 1.2×10^6 CFU per seed (Hungria, Araujo, Júnior, & Zilli, 2017). The same nutrient solution used in the first experiment was applied at 7 and 15 days after sowing.

After thinning, two plants remained in each pot. Irrigation was carried out according to plant needs. At the moment of supplementary inoculation, each pot received 2 mL of Yeast-Mannitol broth containing strain CIAT 899 (optical density 1.0, $\lambda = 600$ nm), five-fold diluted with water with a final concentration of 10^8 cells per milliliters. Supplementary inoculation was performed according to the description of the treatments.

The two experiments were conducted at 47 and 62 days after sowing. Shoot, root, and nodule dry weights as well as the number of nodules were evaluated. The number of pink nodules, considered active nodules, was determined during the pod-filling harvest. The roots were washed and the nodules were detached and counted. Each part of the plants (shoot, roots, and nodules) was dried at 65ºC and weighed.

Data analysis

The data from each experiment were subjected to analysis of variance, and the means were compared using Duncan's test at 5% significance ($p < 0,05$). Statistical analysis was performed using the ExpDes.pt package (Ferreira et al., 2021) in R (R Core Team, 2017).

Results and discussion

Inoculation at different depths of the root system

No significant differences were observed among the treatments for any of the traits evaluated at the flowering stage (Figure 2A, B, and C). Significant differences in the number of nodules were observed during the pod-filling stage. The inoculation of rhizobia on the seeds and throughout the pot induced the greatest number of nodules and was the only treatment that significantly differed from the uninoculated control (Figure 2C). However, shoot dry weight was not significantly affected by the inoculation treatments (Figure 2A). The soil presented 1.2 UFC g^{-1} soil, as estimated using the most probable number technique, which was a relatively low native rhizobial population but did not hinder the uninoculated control from nodulating. No differences in nodulation were observed among the different root system depths.

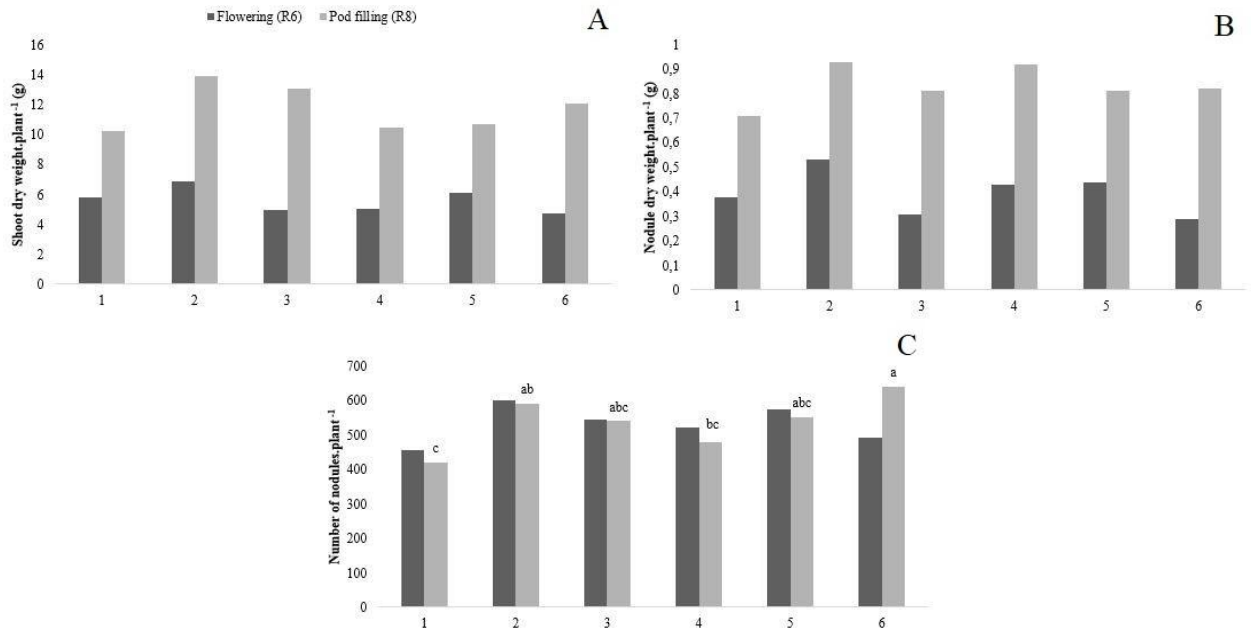

Figure 2. Inoculation experiments at different depths of the root system. Shoot dry weight (A), nodule dry weight (B), and number of nodules (C) in common bean cv. Pérola during flowering and early stages of pod filling. From left to right, uninoculated control; peat inoculation on seeds (1.2 x 10^6 cells $seed^{-1}$); inoculation of the upper, middle, and bottom thirds of the pots; inoculation of the whole pot. Seeds were inoculated with peat inoculants in all the pots that received the inoculated charcoal. Bars with the same letter indicate that the means did not differ according to Duncan's test at the 5% significance level.

Supplementary inoculation at different phenological stages

In the experiment harvested at full flowering (Figure 3A, B, and C), seed inoculation and reinoculation at the V3 stage resulted in a greater number of nodules than the uninoculated control (Figure 3 C). Shoot and nodule dry weights were similar among treatments (Figure 3A and B).

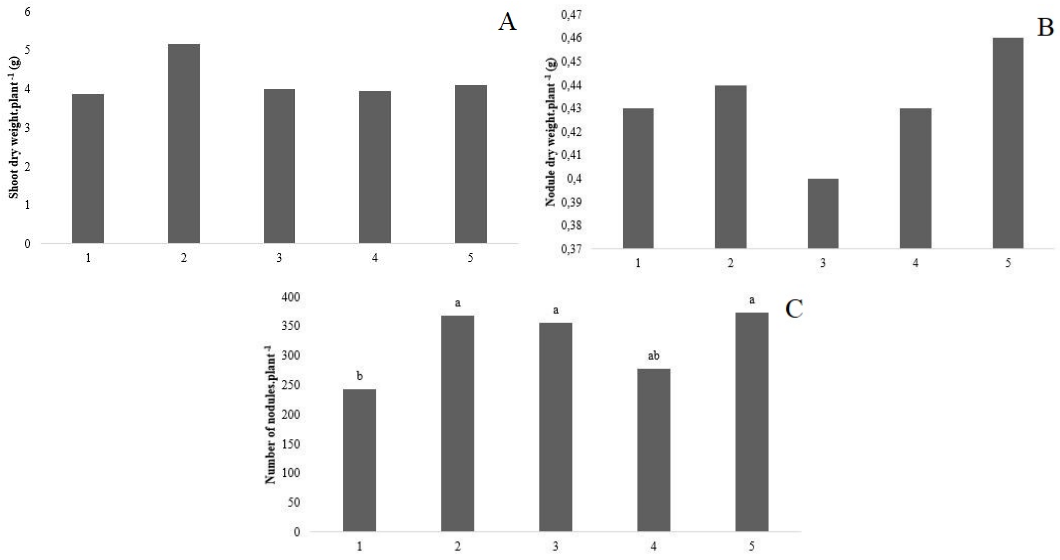

Figure 3. First supplementary inoculation experiment. Shoot dry weight (A), nodule dry weight (B), and number of nodules (C) in common bean cv. Pérola at the flowering stage. From left to right, uninoculated control; peat inoculant on seeds (1.2 x 10^6 cells $seed^{-1}$); peat inoculant + supplementary inoculation at V3 stage (first expanded trifoliate); peat inoculant + supplementary inoculation at V4 stage (third expanded trifoliate); peat inoculant + supplementary inoculation at V3 and V4 stages. Bars with the same letter indicate that the means did not differ according to Duncan's test at the 5% significance level.

No differences between treatments were observed in the experiment harvested at the pod-filling stage (Figure 4A, B, and C), although some of the treatments presented shoot biomass (Figure 4A) and number of nodules (Figure 4C) close to twice those observed in the control. The numbers of pink, green, and white nodules were similar among all treatments at pod-filling harvest (data not shown).

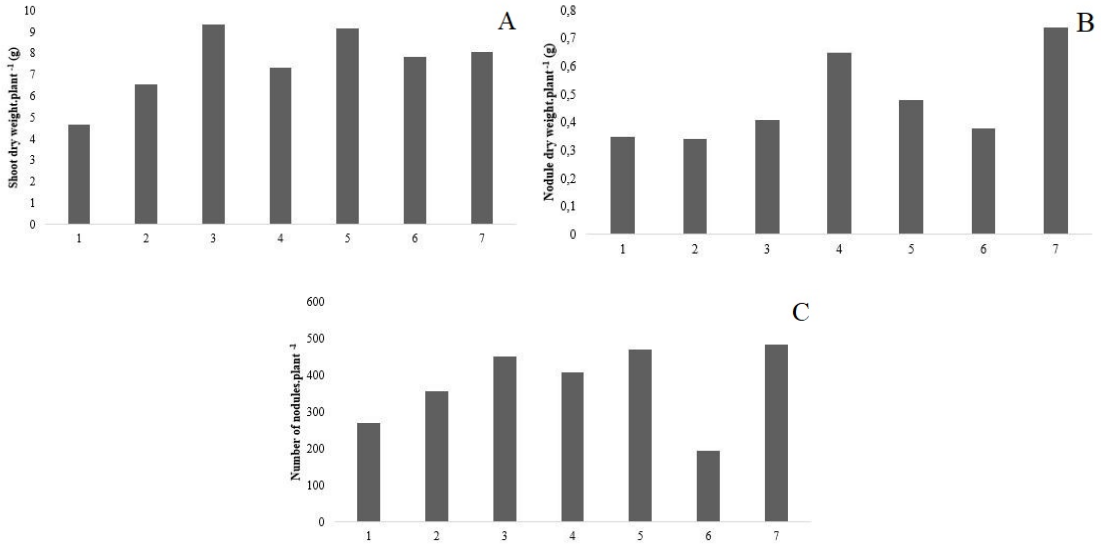

Figure 4. Second supplementary inoculation experiment. Shoot dry weight (A), nodule dry weight (B), and number of nodules (C) in common bean cv. Pérola at the pod-filling stage. From left to right, uninoculated control, peat inoculant on seeds, peat inoculant + supplementary inoculation at V3 stage (first expanded trifoliate); peat inoculant + supplementary inoculation at V4 stage (third expanded trifoliate); peat inoculant + supplementary inoculation at R5 stage (flower bloom); peat inoculant + supplementary inoculation at stage R6 (full flowering); peat inoculant + supplementary inoculation at stages V3, V4, R5, and R6. Bars with the same letter indicate that the means did not differ according to Duncan's test at the 5% significance level.

Seed inoculation with selected rhizobial strains has been used as an alternative to provide nitrogen to several legumes of high economic importance (Hungria et al., 2003). Inoculation enables the formation of a sufficient number of nodules that contribute to vegetative development and crop productivity (Brito et al., 2015; Fonseca et al., 2013; Hungria et al., 2003). This technology has been successfully disseminated but is still underexplored for common bean production. Among the reasons are the common bean's short phenological cycle, which results in a short period for the contribution of biological nitrogen fixation, and the early senescence of nodules during the pod-filling stage (Fernandez-Luqueño, Cabrera-Lazaro, Méndez-Bautista, López-Valdez, & Dendooven, 2012; Andraus et al., 2016). However, some studies have indicated that common bean can form new nodules even after the flowering stage, pointing to the possibility of maintaining the continuity of biological nitrogen fixation even at a lower rate (Hungria et al., 2003; Vickman & Vessey, 1993).

The results of the experiment with inoculation at different pot depths (sections of the roots) showed that a greater number of nodules obtained with inoculation throughout the pot at the pod-filling stage was not reflected in an increase in shoot dry weight, and did not show any difference in root dry weight (data not shown), as verified by Matoso and Kusdra (2014). The inoculation of distal regions of the root system was not superior for development and nodulation to the already widespread seed inoculation procedure. The results of other studies suggest that these effects may be related to genetic self-regulation of nodulation in common beans (Andraus et al., 2016; Graham, 1981; Vickman & Vessey, 1993). Another possible explanation is that plants invest less in vegetative growth and more in production during the reproductive period, making it necessary to investigate grain yield in future studies (Oliveira et al., 2018). In developing the method used in the present study, Vickman and Vessey (1993) observed the occurrence of a smaller number of nodules at the post-flowering stage. These nodules were smaller and senesced quickly without any contribution to the vegetative development of the plant, as observed here.

Supplementary inoculation at different growth stages, applied in the two other experiments, showed a pattern of nodulation similar to that observed in the first experiment. However, at this time, significant differences in nodulation were observed at the flowering stage. Nodulation was superior in all inoculated treatments compared to that in the uninoculated control. Although supplementary inoculation at the V4 stage did not show any statistical difference compared to the other treatments in the first experiment, it was the only treatment that presented a smaller number of nodules. This indicates that inoculation during this stage may have resulted in a delay in the formation of new nodules. However, shoot biomass accumulation was similar among all treatments, further reinforcing the genetic limitations of the species. Moretti et al. (2018) analyzed the effects of supplementary inoculation on soybeans in the field and observed that supplementation increased the number of nodules without affecting the accumulation of plant biomass.

However, supplementary inoculation contributed to a greater grain yield in one of the two harvests evaluated (Moretti et al., 2018). As common bean grain yield was not assessed here, whether the observed increase in the number of nodules leads to greater productivity requires further study.

Although Vickman and Vessey (1993) indicated the potential contribution of late nodulation in common beans, the approaches presented herein did not contribute significantly to the vegetative growth of this legume. This pattern was confirmed by Moreira, Oliveira, and Ferreira (2017) in their study of 17 new isolates of *Rhizobium tropici* in soil samples and three (3) reference strains. They observed that an increase in the number of nodules did not reflect vegetative characteristics. According to Graham (1981), the response of the variables analyzed in this study may vary according to the cultivar and strain adopted (Moreira et al., 2017; Toso, Andriolo, Lerner, Schmitt, & Cardoso, 2017). Thus, further studies with different cultivars, strains, and cultivation conditions are necessary to better understand the possible contribution of late nodulation (Andraus et al., 2016; Facco, Andrade, Capristo-Silva, Silva Junior, & Souza, 2019). Proposals for the re-inoculation of common beans under other conditions showed different results from those presented in our study. Teixeira, Lopes, Sousa, and Teixeira (2018) performed reinoculation experiments at different phenological stages of common bean (V4 and R5) and observed an increase in nodulation and biomass production of the plants studied. Other alternative inoculation methods proposed in previous studies have obtained good results in terms of increasing effective nodulation. Kanonge-Mafaune, Chiduwa, Chikware, and Pisa (2018) reported an increase in nodule mass, plant biomass production, and grain yield with an increase in the dose of inoculant applied to the plants. Samago, Anniye, and Dakora (2018) observed an increase in the morphological and productive parameters of common beans when combined with inoculation and phosphorus application in Ethiopia. Thus, new studies performed under the same conditions presented in this study but with different proposals for re-inoculation should be performed for a better understanding of the promotion of efficient nodulation in common bean.

Conclusion

The supplementary inoculation methods and conditions proposed in this study did not significantly contribute to efficient nodulation during common bean development. Seed inoculation was sufficient to promote satisfactory nodulation and plant growth.

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