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Early nitrogen supplementation stimulates the nodulation and growth of common bean plants inoculated with rhizobium

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ABSTRACT. The initial development of the common bean plants (*Phaseolus vulgaris* L.) that rely on symbiotic nitrogen (N) can be limited when active N_2 fixation is delayed. Thus, adequate plant growth with rhizobium inoculation could require supplemental mineral N, which in turn may inhibit the symbiosis. Five experiments were performed using hydroponics to identify the initiation of nodulation and nitrogenase activity in common bean cultivars, and effects of mineral N addition on plant nodulation and growth. Three experiments evaluated the initial growth of five inoculated bean cultivars in the absence or presence of mineral N, while two experiments evaluated the effect of mineral N addition until the beginning of the reproductive stage. The first root nodules appeared 10 days after transplanting (DAT), while nitrogenase activity was initiated at 11 DAT. Large seed cultivars had lower levels of initial nodulation and nitrogenase activity than those of small seeds. Inoculated plants showed limited shoot growth that lasted until 21-25 DAT relative to the inoculated plants that received mineral N. Moreover, adding mineral N greatly reduced the nodule mass more than the nodule number, and caused an even greater reduction in nitrogenase activity. Mineral N that was applied until 15 DAT enhanced nodulation and nitrogenase activity without limiting shoot growth relative to the plants that received N throughout their growth. In contrast, plants that received N after 15 DAT had lower levels of nodule mass and nitrogenase activity than those of the plants that were only inoculated. Hence, these results indicate that symbiotic N was not sufficient for an adequate initial growth of the common beans. Therefore, some supplemental N is necessary, which should be added at sowing to stimulate plant growth without inhibiting further nodulation and N fixation.

Keywords: *Phaseolus vulgaris*; rhizobia; nitrogen fixation; nitrogen fertilization; inoculation.

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Introduction

Common bean (*Phaseolus vulgaris* L.) plants can associate symbiotically with several types of bacteria from the rhizobia group, forming nodules in the roots where the conversion of atmospheric N_2 to ammonium occurs. Poor plant nodulation and a lack of responses to seed inoculation of selected rhizobium strains have been frequently reported in common bean crops under field conditions, particularly in soils with high native rhizobia populations, raising doubts regarding the potential of the symbiosis to properly fulfill the plant demand for N (Graham et al., 2003; Hungria, Campo, & Mendes, 2003; Brito et al., 2015).

The establishment of the symbiosis between the rhizobia and legumes requires the successful infection of legume roots, which is a multifaceted developmental process that is driven by bacteria, but it is ultimately under the control of the host (Murray, 2011). Plants may primarily regulate nodulation via a systemic mechanism called the autoregulation of nodulation in response to existing infection events (Reid, Ferguson, Hayashi, Lin, & Gresshoff, 2011). Additionally, legumes also regulate nodulation in response to environmental N availability as a means of preferentially obtaining N from sources that are energetically favorable relative to the energy costs of both nodulation and nitrogenase activity (Streeter & Wong, 1988; Reid et al., 2011). In turn, nitrate can inhibit N_2 fixation by many factors, such as through a decreased nodule number and mass, restricted N fixation activity as well as the acceleration of nodule senescence or disintegration (Nanjareddy et al., 2014; Saito et al., 2014).

In seedlings of common bean, there is a lack of synchronization between the depletion of N reserves of the cotyledons and the beginning of active N_2 fixation, which causes a transient yellowing of the leaves that can last up to 15 and 20 days after plant emergence and can limit initial plant growth (Sprent & Thomas, 1984; Hungria, Barradas, & Wallsgrove, 1991). Therefore, seed inoculation associated with low levels of mineral N fertilization at sowing, which is referred to as 'N start', is often suggested to stimulate initial plant growth and improve common bean yields (Hungria et al., 2003; Pelegrin, Mercante, Otsubo, & Otsubo, 2009; Barros, Oliveira, Magalhães, Médici, & Pimentel, 2013). Indeed, a level of combined N is required for the maximum growth of legume crops, and under specific circumstances, plants that received low N additions could actually fix more N_2 than plants without N, because of the growth stimulus of the supplemental N (Streeter & Wong, 1988; Nanjareddy et al., 2014). However, the effects of mineral N additions at the beginning of the bean growth cycle still remain controversial, since high N levels might negatively affect nodulation and the subsequent N fixation (Müller & Pereira, 1995; Pelegrin et al., 2009).

Nitrogen fixation ability can largely vary according to the physiological characteristics of the distinct common bean genotypes (Hardarson et al., 1993; Graham et al., 2003). There is evidence of differences in the contributions of N_2 fixation between common bean plant growth habits, with indeterminate and climbing genotypes requiring more symbiotic N than those with a determinate or bush type of architecture (Rennie & Kemp, 1983; Kumarasinghe, Danso, & Zapata, 1992; Ramaekers, Galeano, Garzón, Vanderleyden, & Blair, 2013; Pacheco et al., 2020). Additionally, the symbiotic ability may differ across gene pools, with Mesoamerican bean genotypes presenting greater nodulation and fixing more N_2 than the Andean genotypes (Knupp, Ferreira, & Araújo, 2017; Wilker et al., 2019). Hence, identifying differences in the nodule initiation of common bean cultivars originating from distinct gene pools could highlight their potential for acquiring N through symbiosis at early growth stages. Therefore, understanding the mineral N supply effects on nodule initiation and plant growth across distinct common bean cultivars under controlled conditions can be insightful for field crop management. In this study, we aimed to identify the initiation of nodulation and nitrogenase activity as well as the effects of adding mineral N on the nodulation and development of hydroponically grown common bean plants.

Material and methods

Experimental designs

Five experiments were performed in a greenhouse at Embrapa Agrobiologia, Seropédica, Rio de Janeiro State, Brazil, where common bean plants of five commercial cultivars were grown in a hydroponics system. In the first three experiments (named I, II, and III), the common bean plants were grown in plastic trays, while in experiments IV and V, the plants were cultivated in pots. Selected characteristics of the evaluated cultivars have been presented in Table 1.

Table 1. Selected characteristics of the evaluated common bean cultivars.

Cultivar	Grain type	Plant architecture	100-seed mass (g)	Life cycle (days)
Jalo Precoce	Cream yellowish	Semi-erect	35	< 75
BRS Radiante	Beige with purple speckled	Semi-erect	44	< 75
Ouro Negro	Black	Prostrate	24	85 - 95
BRS Pontal	Cream with brown stripes	Prostrate	26	85 - 95
BRS Valente	Black	Erect	23	85 - 95

In Experiments I and II, the initiation of nodulation and nitrogenase activity were evaluated across the common bean cultivars without the supplemental mineral N. In Experiment I, the inoculated plants of five cultivars (Jalo Precoce, Radiante, Ouro Negro, Pontal, and Valente) were grown in eight hydroponic trays, where each tray contained four plants per cultivar. The plants were harvested at 10, 11, 12, and 13 days after transplanting (DAT), two trays per day. In Experiment II, the inoculated plants of four cultivars (Radiante, Ouro Negro, Pontal, and Valente) were grown in four trays, each tray containing five plants of each cultivar. Plants were sampled at 10 and 12 DAT, one tray per day. In Experiment III, the nodule initiation of four cultivars (Radiante, Ouro Negro, Pontal, and Valente) was evaluated in the absence or presence of mineral N. Each of the eight trays had five inoculated plants per cultivar, with four trays with and four without N. One tray per N treatment and per day was harvested at 10, 11, 12, and 13 DAT.

Next, Experiments IV and V were performed in pots using 1 L of the nutrient solution with two plants per pot. The Ouro Negro cultivar, which has a high level of nodulation and contribution of N_2 fixation (Pacheco et al., 2020), was used. In Experiment IV, the ontogeny of the nodulation was evaluated in plants supplied with three different N sources (rhizobia inoculation, mineral N, and rhizobia inoculation with the mineral N). Plants across five pots per treatment were harvested at 11, 15, 25, 35, and 45 DAT, which approximately corresponded to the phenological stages of Ouro Negro cultivar in terms of 15 DAT reflecting the fully expanded V4 third trifoliate, 25 DAT reflecting the R5 pre-flowering, 35 DAT reflecting the R6 plentiful flowering, and 45 DAT reflecting the R7 pod setting. In addition, Experiment V consisted of evaluating the nodulation ontogeny in response to different time points of mineral N supplementation. Plants were grown under four distinct regimes of N supplementation, which included inoculation without mineral N, inoculation with mineral N during the entire growth period, inoculation with supplemental N from the time of seedling transplantation until 15 DAT, and inoculation with supplemental N after 15 DAT. Plants were harvested at 14 and 21 DAT, but only for the treatments without N and with N during the entire growth period, since the treatments in which N supply was interrupted had not yet been introduced. For the four N regimes, plants were harvested at 28, 35, and 42 DAT. Five pots were harvested per treatment at each date.

Inoculation and plant growth

The seeds were disinfected with 70% ethanol for 30 seconds, with hydrogen peroxide for 3 min., then washed in sterile distilled water. Seeds were placed in sterile petri dishes containing humid absorbing paper. After 5 days, when the radicles emerged, the seedlings were inoculated by immersing the roots in the liquid inoculant for 30 min. The inoculant contained a mixture of the rhizobium strains that were recommended for the commercial common bean crops in Brazil, including CIAT 899 (BR 322 syn SEMIA 4077) of *R. tropici* that was isolated from tropical acid soils, PR-F81 (BR 520 syn SEMIA 4080) of *R. freirei* that was isolated in southern Brazil, and CPAC H12 (BR 534 syn SEMIA 4088) of *R. tropici* that was isolated from the Brazilian Cerrado region. The strains were obtained from the Embrapa Agrobiologia strain collection and grown in a yeast-mannitol broth.

In Experiments I, II, and III, the inoculated seedlings were transferred to plastic trays of 50 x 30 x 15 cm and 12 L of solution, with 20 plants per tray, while in Experiments IV and V, two seedlings were transferred to each pot that contained 1 L of solution. Seedlings were held with a cotton wool at the hypocotyl level to maintain the root systems suspended in the solution. The nutrient solution contained (Araújo, Plassard, & Drevon, 2008): 0.25 mmol L^{-1} KH_2PO_4, 0.70 mmol L^{-1} K_2SO_4, 1.00 mmol L^{-1} $MgSO_4$, 1.65 mmol L^{-1} $CaCl_2$, 4 µmol L^{-1} H_3BO_3, 6 µmol L^{-1} $MnSO_4$, 2 µmol L^{-1} $ZnSO_4$, 1 µmol L^{-1} $CuSO_4$, 0.2 µmol L^{-1} $Na_2MoO_4.2H_2O$, and 8 µmol L^{-1} Fe-EDTA. Each tray received 10 g of $CaCO_3$, while each pot received 1 g of $CaCO_3$ to buffer the solution to a pH of 7. Urea was added at 2 mmol L^{-1} as the N source in the respective treatments. The nutrient solution in each recipient was replaced weekly and the aeration was driven by a compressor pump for 15 min. every 2h.

Assays

During the harvesting, the shoots and roots were separated by cutting the stems at the hypocotyl level. The nitrogenase activity was estimated in the root systems by the acetylene reduction assay. Whole root systems were placed in 250 mL closed glass recipients and 30 mL of acetylene was injected using a syringe. After 30 min. of incubation, 1 mL of the air inside the recipient was sampled and used to measure the ethylene concentration by gas chromatography using the Flame Ionization Detector (PerkinElmer Inc., USA). The values of the produced ethylene were converted to µmol h^{-1} C_2H_4 per plant, representing the nitrogenase activity.

Root nodules were detached and counted. The nodules, roots, and shoots were oven-dried at 70°C and weighed. The unit nodule mass was calculated as the ratio of nodule mass and number, while the specific nitrogenase activity was determined by the ratio between nitrogenase activity and nodule dry mass. The shoots and roots that were harvested 35 and 45 DAT in Experiment IV, and 35 and 42 DAT in Experiment V, were ground and the N concentration was determined by the semi-micro Kjeldahl method. The amount of N in the shoots and roots was determined by the product of the N concentration and the dry mass of each plant tissue.

Statistical analysis

The generated data across the five experiments was subjected to an analysis of variance per individual sampling time. Experiments I and II were analyzed using the cultivars as a single factor in completely randomized design, while Experiments IV and V used the N sources as a single factor in block design. Experiment III included a double factor between the cultivars and N sources in randomized design. In

Experiments I, II, and III, each plant was considered a replicate, while in Experiments IV and V each pot was the replicate. The error mean square that was obtained by the analysis of variance was used to determine the least significant differences between the means by Tukey test at the 5% significance level.

Results

Nodule initiation across the different cultivars

Common bean plants of different cultivars were inoculated with the rhizobium and grown in trays with nutrient solution to identify the initiation of nodulation and nitrogenase activity. In Experiments I and II, no supplemental N was applied, whereas in Experiment III, plants were grown in the absence or presence of added mineral N in the nutrient solution.

In Experiment I, the shoot dry mass of the small-seeded cultivars (Ouro Negro, Pontal, and Valente) decreased by 51% from 12 to 13 DAT, while in the case of the large-seeded cultivars (Radiante and Jalo Precoce), it decreased by 14% (Figure 1). In Experiment II, the shoot mass decreased by 27% from 10 to 12 DAT on average across the four cultivars (Figure 2).

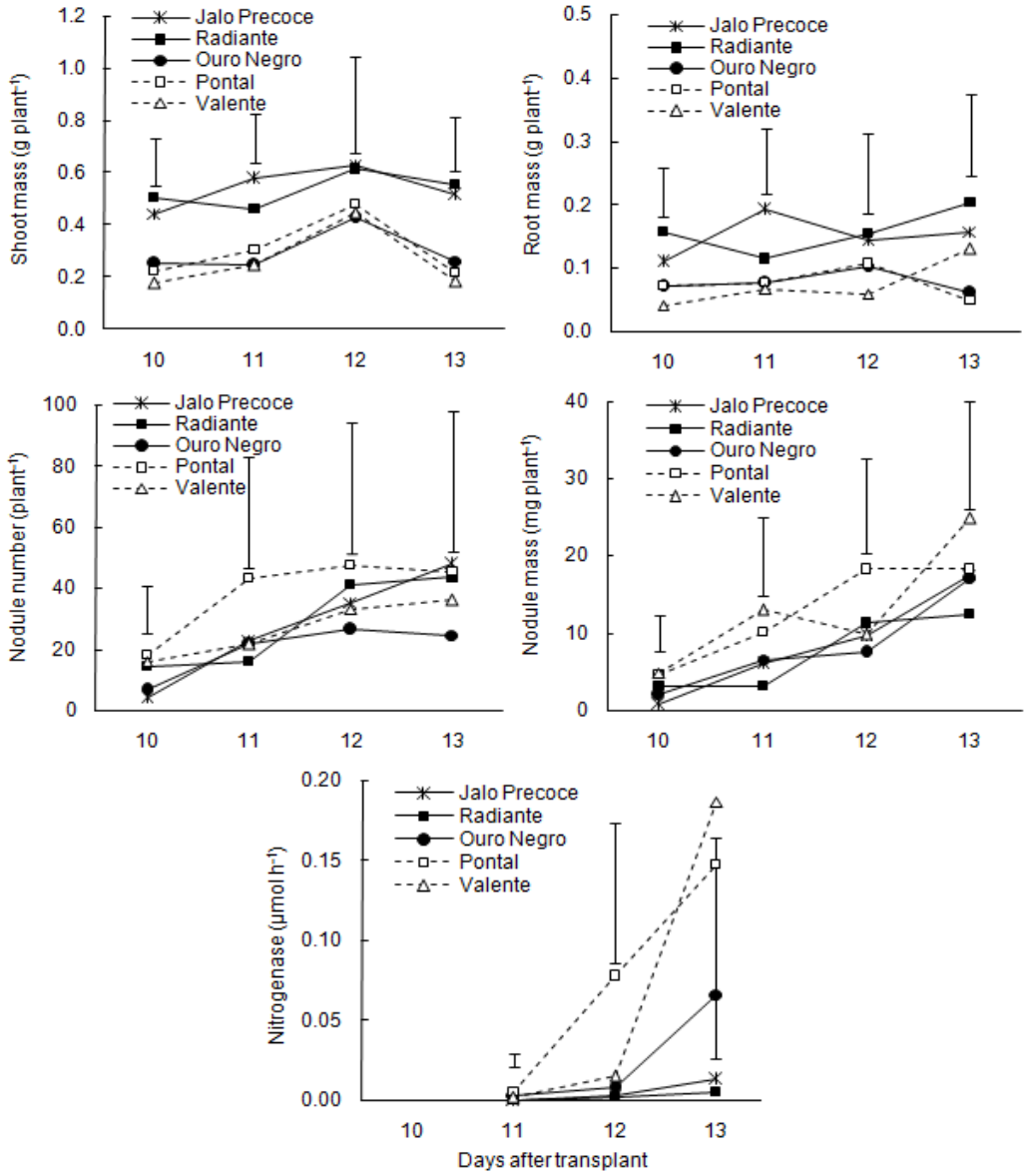

Figure 1. Shoot dry mass, root dry mass, nodule number, nodule dry mass, and nitrogenase activity in the root system of five common bean cultivars (Jalo Precoce, Radiante, Ouro Negro, Pontal, and Valente) that were inoculated with rhizobium and grown in a nitrogen-free nutrient solution at harvesting dates of 10, 11, 12, and 13 days after transplanting in Experiment I. The vertical bars represent the least significant differences by Tukey test at 5% significance and compare the cultivars within each harvesting date.

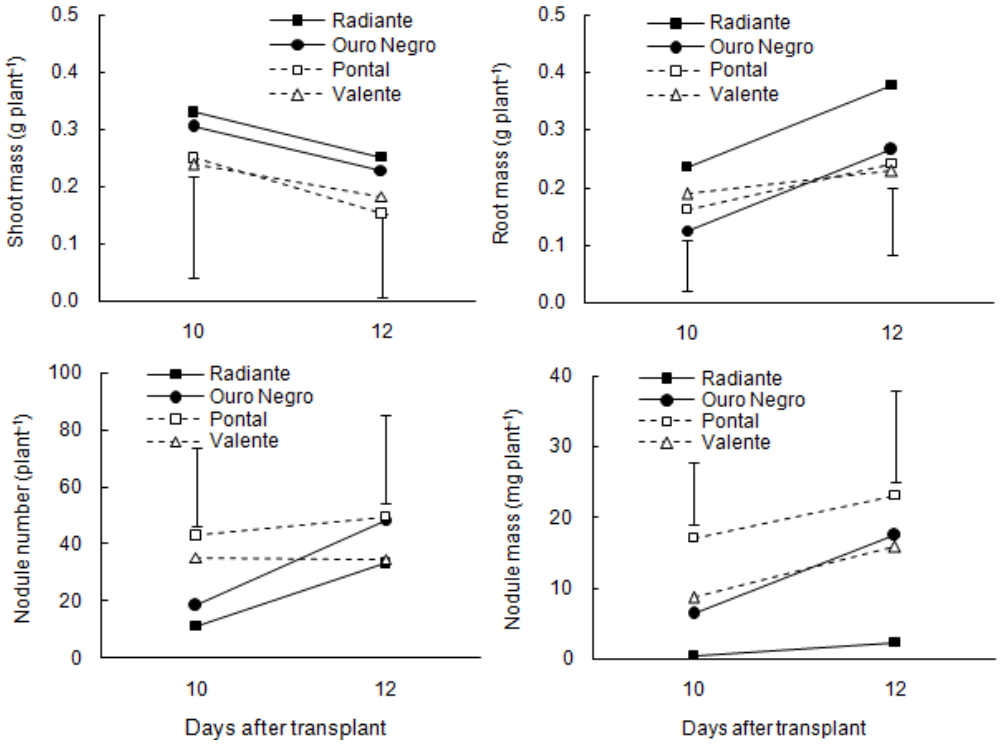

Figure 2. Shoot dry mass, root dry mass, nodule number, and nodule dry mass of four common bean cultivars (Radiante, Ouro Negro, Pontal, and Valente) that were inoculated with the rhizobium and grown in a nitrogen-free nutrient solution at harvesting dates of 10 and 12 days after transplanting in Experiment II. The vertical bars represent the least significant differences by Tukey test at the 5% significance level and compare cultivars within each date.

Despite these shoot diminutions, the root mass remained relatively stable in Experiment I (Figure 1) and increased from 10 to 12 DAT in Experiment II (Figure 2). Therefore, the root to shoot ratio increased from 264 to 860 mg g^{-1} between 10 and 13 DAT in Experiment I, averaged across the cultivars. The Jalo Precoce and Radiante cultivars had greater shoot mass than the other cultivars in Experiment I (Figure 1), while the Radiante cultivar had greater root mass in Experiment II (Figure 2).

In Experiments I and II, the first nodules were observed at 10 DAT, which was irrespective of the cultivar (Figures 1 and 2). In Experiment I, the number of nodules increased between 10 and 12 DAT but not at 13 DAT, whereas the nodule mass increased continuously between 10 and 13 DAT. Hence, the unit nodule mass increased from 0.30 to 0.49 mg between 12 and 13 DAT. In Experiment I, the Radiante cultivar had a lower nodule mass than Valente at 11 DAT and Pontal at 12 DAT (Figure 1). In Experiment II, the Radiante cultivar had a lower nodule number and mass than Pontal at 10 DAT and a lower nodule mass than the other cultivars at 12 DAT (Figure 2). In Experiment I, the nitrogenase activity began at 11 DAT across all cultivars but was very low (Figure 1) and remained low for the large-seeded cultivars (Jalo Preconce and Radiante), but increased after 12 DAT in the small-seeded cultivars (Ouro Negro, Pontal, and Valente). In Experiment II, the Radiante cultivar had a lower nitrogenase activity relative to the other cultivars at 12 DAT (data not shown).

In Experiment III, the shoot mass declined slightly between 11 and 13 DAT in the plants that were inoculated of the Ouro Negro and Valente cultivars (Figure 3). The shoot mass of the inoculated plants that received mineral N increased continuously from 10 to 13 DAT. Therefore, the inoculated plants that received N produced more shoot mass than those that were only inoculated after 11 DAT. Moreover, the Radiante cultivar had a greater shoot mass than Valente at 13 and 14 DAT when inoculated, but with the mineral N, the Ouro Negro cultivar had more shoot mass than Valente at 13 and 14 DAT (Figure 3).

The first nodules were observed at 10 DAT in Experiment III, except in the case of the Radiante cultivar, which developed its first nodules at 13 DAT (Figure 3). Interestingly, the addition of N to the nutrient solution did not affect the appearance of the first nodules, but it strongly reduced the nodule number and mass (Figure 3). Moreover, the mineral N limited the nodule mass more severely than the nodule number. Hence, the unit nodule mass was lower in the plants that received mineral N than the plants that were only inoculated (0.34 and 0.43 mg, respectively, at 14 DAT). The Pontal cultivar had the highest nodule number and mass at 13 and 14 DAT, whereas Radiante had the lowest. In plants that received the mineral N, the Ouro Negro cultivar showed a higher nodule number and mass than the other cultivars at 14 DAT (Figure 3).

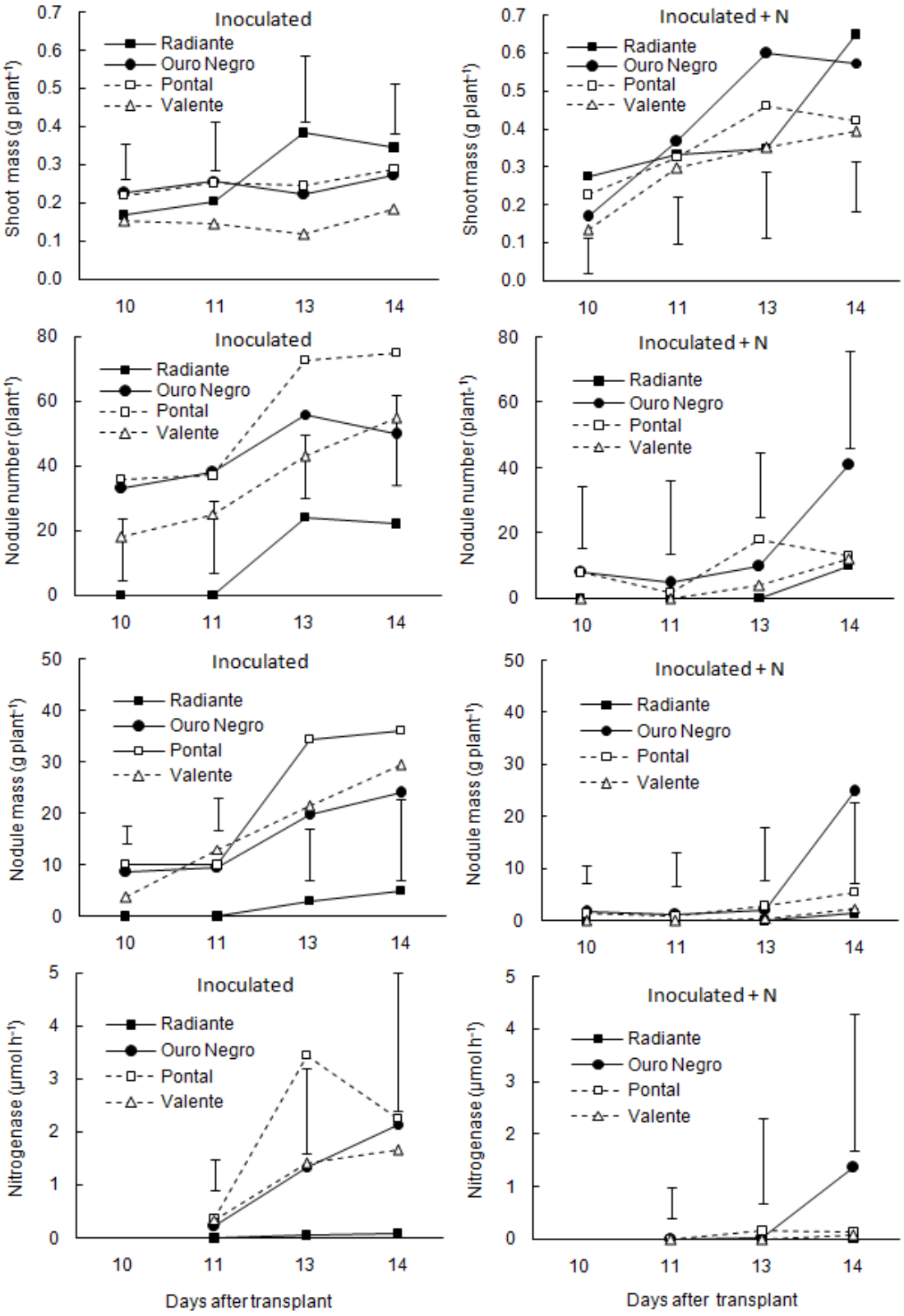

Figure 3. Shoot dry mass, nodule number, nodule dry mass, and nitrogenase activity in the root system of four common bean cultivars (Radiante, Ouro Negro, Pontal, and Valente) that were grown in a nutrient solution with two nitrogen sources (inoculated with the rhizobium or inoculated + mineral nitrogen), at harvesting dates of 10, 11, 12, and 13 days after transplanting in Experiment III. The vertical bars represent the least significant differences by Tukey test at the 5% significance level and compare the cultivars within each date.

The nitrogenase activity started at 11 DAT but was very low and increased in plants that were only inoculated afterwards (Figure 3). The Radiante cultivar showed the lowest nitrogenase activity. The addition of N strongly reduced the specific nitrogenase activity, which was 57 and 30 µmol h^{-1} g^{-1} nodule at 14 DAT, in inoculated plants with or without N, respectively.

Time point of nitrogen supplementation

Plants of the Ouro Negro cultivar were grown using different N sources (Experiment IV) or at different time points of mineral N application (Experiment V) in pots with nutrient solution. In Experiment IV, a treatment without plant inoculation (i.e., mineral N) was also evaluated.

In Experiment IV, the shoot dry mass increased throughout the experiment but less intensively in the plants that were only inoculated (Figure 4).

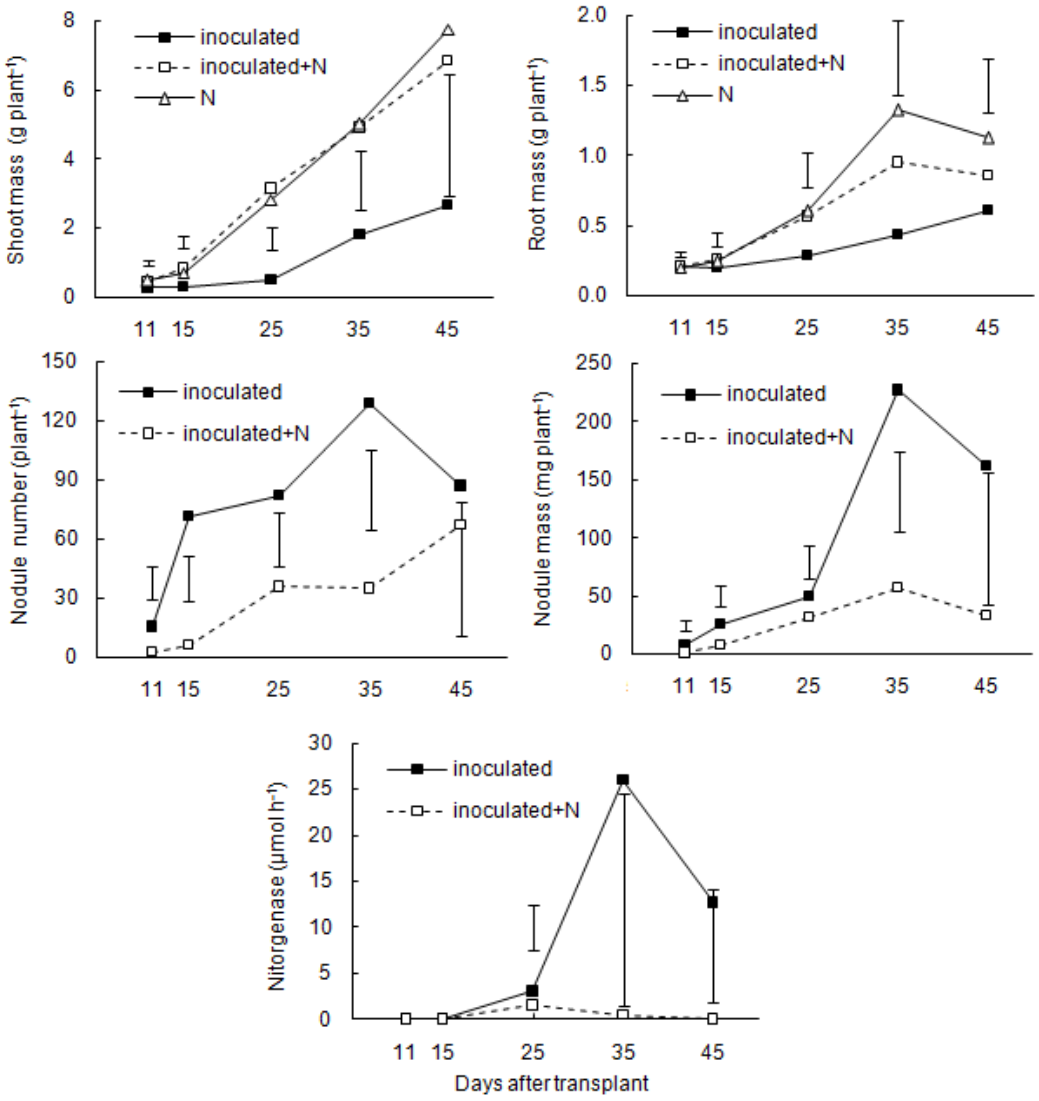

Figure 4. Shoot dry mass, root dry mass, nodule number, nodule dry mass, and nitrogenase activity in the root system of the common bean Ouro Negro cultivar, that was grown in a nutrient solution with three nitrogen sources (inoculated with the rhizobium, inoculated plus mineral N, and mineral N), at five harvesting dates of 11, 15, 25, 35, and 45 days after transplanting during Experiment IV. The vertical bars represent the least significant differences by Tukey test at the 5% significance level and compare the N sources within each date.

Therefore, plants only inoculated had a lower shoot mass across the entire experiment, but without any differences across treatments that received N (Figure 4). The root mass was also lower in the plants that were only inoculated from 15 to 45 DAT. Plants that received mineral N, inoculated or not, invested proportionally more biomass in their shoot growth, resulting in a lower root to shoot ratio than the plants that were only inoculated (data not shown). Inoculated plants that received mineral N had a lower nodule number and mass than the plants that were only inoculated; non-inoculated plants did not form any nodules (Figure 4). Nitrogenase activity remained very low during the experiment in the inoculated plants that received N, whereas plants that were only inoculated had high nitrogenase activity at 35 and 45 DAT (Figure 4). At 35 DAT, the specific nitrogenase activity was found to be 114 and 8 µmol h^{-1} g^{-1} in plants that were inoculated with or without N, respectively. Plants that were only inoculated accumulated less N in the shoots and roots at 35 and 45 DAT relative to the plants that received N (Table 2). When

comparing the plants that received N, the non-inoculated plants accumulated more N in the roots than the inoculated plants (Table 2).

Table 2. Amount of nitrogen (N) accumulated in the shoots and roots of the common bean plants Ouro Negro cultivar using three N sources (inoculated with the rhizobium, inoculated + N, and mineral N) during Experiment IV, as well as using four N sources (inoculated, inoculated + N, inoculated + N after 15 days after transplanting, and inoculated + N until 15 days after transplanting) during Experiment V, at two harvesting dates in days after transplanting (DAT). Means followed by the same letter within a column and within each experiment do not differ significantly according to Tukey test at the 5% significance level.

N source	Amount of N in shoots (mg plant^{-1})		Amount of N in roots (mg plant^{-1})	
	Experiment IV			
	35 DAT	45 DAT	35 DAT	45 DAT
Inoculated	47 b	56 b	7.9 c	12.9 c
Inoculated + N	102 a	133 a	23.7 b	21.2 b
Mineral N	104 a	139 a	34.0 a	28.4 a
	Experiment V			
	35 DAT	42 DAT	35 DAT	42 DAT
Inoculated	77 b	77 b	16.6 a	15.5 a
Inoculated + N	107 a	109 a	14.7 a	18.9 a
Inoculated + N after 15 DAT	94 ab	97 ab	17.8 a	21.4 a
Inoculated + N until 15 DAT	77 b	109 a	19.0 a	18.3 a

In Experiment V, the shoot mass increased up to 35 DAT, and then decreased slightly between 35 and 42 DAT (Figure 5). The inoculated plants that received N had more shoot mass than the inoculated plants at 21 and 28 DAT. Plants that received N up to 15 DAT had more shoot mass than the plants inoculated at 28 and 45 DAT. Furthermore, the plants that received N up to 15 DAT had more root mass between 28 and 42 DAT, but without any differences among the other treatments (Figure 5). Moreover, it was observed in every evaluation that the plants that were only inoculated had a higher root to shoot ratio than the plants that were inoculated and received N during the experiment or received N after 15 DAT (data not shown). Additionally, the plants that received N up to 15 DAT had a higher root to shoot ratio at 35 and 45 DAT relative to the other treatments with N (data not shown).

The nodule number increased between 14 and 28 DAT and remained stable thereafter, whereas the nodule mass increased until 35 DAT (Figure 5). Hence, the unit nodule mass was at its maximum value at 35 DAT, with 2.11 mg in the inoculated plants and 0.63 mg in the inoculated plants that received N during the experiment. Across the entire experiment, the nodule number and mass, as well as the unit nodule mass (data not shown), were much greater in the inoculated plants than in the plants that received N (Figure 5).

Plants that received N up to 15 DAT increased their nodulation after 35 DAT, whereas plants that received N after 15 DAT showed decreased nodulation after 35 DAT (Figure 5). Nitrogenase activity remained very low in the inoculated plants that received N during the experiment or that received N after 15 DAT (Figure 5). In plants that were only inoculated as well as in plants that received N until 15 DAT, the nitrogenase activity increased between 21 and 35 DAT, which was higher relative to the other treatments. At 35 DAT, the specific nitrogenase activity was 17 and 38 μmol h^{-1} g^{-1}, respectively, in the plants that were inoculated with or without N up to 15 DAT, while in the plants that received N after 15 DAT, the specific activity was 0.7 μmol h^{-1} g^{-1}. At 42 DAT, the plants that were only inoculated accumulated less N in the shoots relative to the plants that received mineral N at any point during the growth cycle, while the amount of N in roots did not differ across the N treatments (Table 2).

Discussion

Common bean plant cultivars with distinct architectures and seed sizes were hydroponically-grown to identify the effects of N supply on the nodule formation and function as well as its impacts on improving plant growth. The effects of N supply on plant nodulation depend on the type of combined N that is applied to each legume species. In general, the inhibitory effects of nitrate were greater than those of ammonium, while urea was slightly inhibitory in certain legumes at least (Streeter & Wong, 1988; Guo, Silsbury, & Graham, 1992). It is currently recognized that plants possess effective urea transporters, hydrolyze urea very efficiently, and can use urea as sole N source (Witte, 2011). Therefore, in order to avoid the confounding effect of N in its

ionic form, urea was added to growth media to study the symbiosis of the common beans and the rhizobium (Vadez, Beck, Lasso, & Drevon, 1997; Araújo et al., 2008). Despite the limitations of the acetylene reduction assay in measuring N_2 fixation over entire growth periods, this technique can be effective in studying the symbiosis at specific time points under defined experimental conditions (Unkovich & Pate, 2000) to topically represent the nitrogenase activity within nodules.

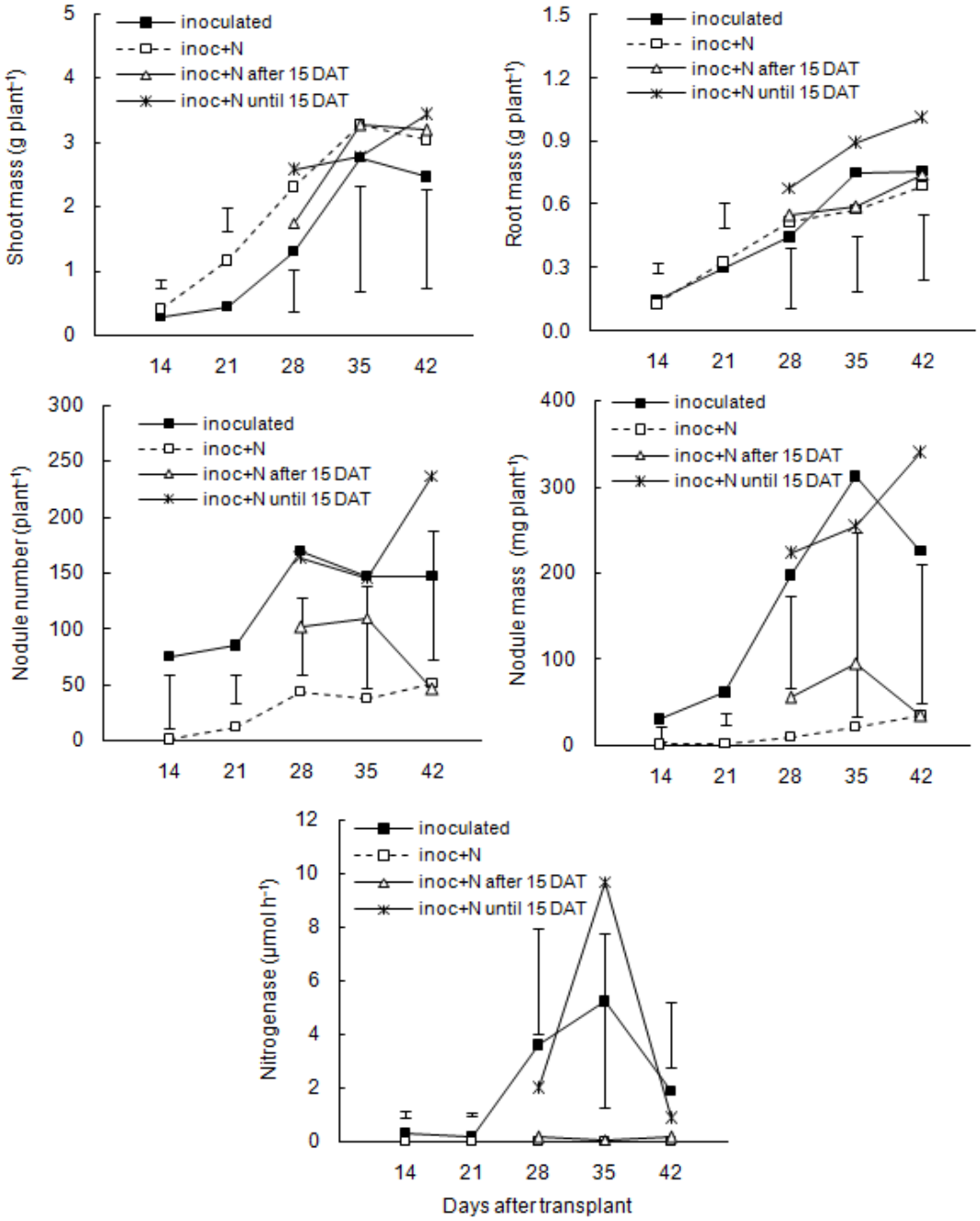

Figure 5. Shoot dry mass, root dry mass, nodule number, nodule dry mass, and nitrogenase activity in the root system of the common bean Ouro Negro cultivar, that was grown in a nutrient solution with four nitrogen sources (inoculated with the rhizobium, inoculated plus mineral N during the experiment, inoculated plus mineral N until 15 days after transplanting, and inoculated plus mineral N after 15 days after transplanting) at five harvest dates of 14, 21, 28, 35, and 42 days after transplanting during Experiment V. The vertical bars represent the least significant differences by Tukey test at the 5% significance level and compare N sources within each date.

The germplasm of *Phaseolus vulgaris* was divided into two main centers of domestication, the Andean and the Mesoamerican, which can be distinguished by plant morphology as well as molecular approaches (Singh, Gepts, & Debouck, 1991; Bitocchi et al., 2013). The Mesoamerican gene pool predominately has small seeds (100-seed mass < 25 g), while the Andean gene pool has medium to large seeds (Singh et al., 1991). Despite lacking a genetic background of the cultivars evaluated in the present work, the Radiante and Jalo Precoce

cultivars were supposed to pertain to the Andean group, while the Valente, Ouro Negro, and Pontal cultivars represent the Mesoamerican group (Table 1). The large-seeded cultivars, Radiante and Jalo Precoce, showed very low initial nodulation and nitrogenase activity (Figures 1 and 2). In one experiment, the first nodules appeared in Radiante cultivar three days after the other cultivars (Figure 3). This confirms the evidence that the common bean germplasm of Andean origin has a lower potential for nodulation than the Mesoamerican group (Knupp et al., 2017; Pacheco et al., 2020).

Reductions in shoot biomass were observed during the initial growth of the inoculated plants (Figures 1, 2, and 3), which reflected the 'N-hunger' of inoculated plants that exhibited a transient period of apparent N deficiency that occurred after most seed N was distributed to the young seedlings but before significant amounts of fixed N have been exported from the nodules (Atkins, Pate, Sanford, Dakora, & Matthews, 1989). This reduction in shoot growth was more severe in small-seeded cultivars than in large-seeded common bean (Figures 1 and 3). The small-seeded Mesoamerican genotypes have a smaller fraction of seed weight that is invested in the cotyledon and a greater fraction in the embryonic axis and seed coat (Sexton, Boote, White, & Peterson, 1997), thereby, possessing lower seed reserves for initial growth and becoming more susceptible to the 'N-hunger'. Atkins et al. (1989) also observed that a cowpea cultivar of small seeds showed physiological symptoms of 'N-hunger', which in turn, were not identified in a large-seeded cultivar.

This reduced shoot growth of the common bean plants that rely on symbiotic N lasted up to 25 DAT in Experiment IV and 21 DAT in Experiment V (Figures 4 and 5), which was during the vegetative stage. When facing a limited availability of nutrients in the growth medium, bean plants preferentially invest carbohydrates in the root systems, thereby increasing the root to shoot mass ratio (Nanjareddy et al., 2014), which was observed in the inoculated plants. In Experiment IV, plants that received the mineral N produced more shoot mass than the inoculated plants across the entire experiment, whereas in Experiment V, this difference did not persist after flowering (i.e., 35 DAT). Plants relying on the symbiotic N also accumulated less N in the shoots than the plants that received the mineral N at the stages of flowering and pod setting (Table 2). This indicates that in N-free growth conditions, the N arising from the symbiosis was not sufficient in achieving adequate plant growth and that supplemental N should be added. However, adding mineral N limited the development of the nodulation and the nitrogenase activity.

Streeter and Wong (1988) didactically proposed that the effects of combined N on the legume-rhizobium symbiosis should be divided into three categories. One negative effect of combined N is on the infection of roots by rhizobium, which is measured by the number of nodules and this effect often requires a relatively high N level. A second effect of combined N is on the nitrogenase activity, which can be depressed over a period of a few days, but it can recover after N withdrawal. The third effect of combined N is on the nodule mass per plant, which is more sensitive to N than the infection response (Streeter & Wong, 1988). Following this interpretative scheme, we can conclude that adding mineral N limited the nodule development more severely than the nodule initiation, thereby reducing nodule size, which was reflected by the lower unit nodule mass, in the plants that received N in Experiments III and V. Nanjareddy et al. (2014) also observed that high nitrate concentrations in the growth media inhibited nodule development but not the process of rhizobial infection in the common beans. Nodule functioning was affected even more severely, as denoted by the feeble nitrogenase activity and specific nitrogenase activity in the plants that received the mineral N. Nova-Franco et al. (2015) also observed that 3 days after nitrate addition, the nitrogenase activity decreased drastically and the nodules had senesced in the common beans.

Treatments in which mineral N supply was interrupted provided further insights into the effects of exogenous N on the symbiosis (Figure 5). The N withdrawal after 15 DAT stimulated nodule growth and subsequent functioning, where these plants had more nodule mass and nitrogenase activity than the plants without N or that received N during the experiment. Moreover, this interruption of N supply did not limit the shoot growth. In contrast, when mineral N was only supplied after 15 DAT, nodule growth and functioning was subsequently restricted; these plants had a lower nodule mass and nitrogenase activity than the plants that were only inoculated and without any enhancement in the shoot growth. This indicates that the supply of combined N at the beginning of plant growth could benefit the symbiosis, but later N additions could inhibit nodule function, as was pointed out by Müller and Pereira (1995). Imsande (1986) also observed in hydroponically-grown soybeans that the presence of nitrate for 3 to 6 days temporarily delayed nodule development, then subsequently stimulated nodule mass and nitrogenase activity. However, the nitrate that was applied for 14 days reduced the nodule mass, which blocked both the early and late steps of nodule development (Imsande, 1986).

These results indicate that some initial N supply is necessary to guarantee adequate common bean plant growth that were inoculated with rhizobium, but the continuous addition of the mineral N could be detrimental to the symbiosis. Under field conditions, an initial N supply can be added using a small fertilization during sowing or even by the mineralization of organic matter in more fertile soils. Since the common bean plants have an efficient uptake of N from the soil, this could be associated with its lower N_2 fixation capacity relative to soybeans (George & Singleton, 1992). Therefore, the host-rhizobium symbiosis components that are tolerant to high nitrate levels have been sought through conventional breeding (Bliss, 1993) or molecular approaches (Nova-Franco et al., 2015) to enhance the contribution of N_2 fixation to the common bean crop. Thus, field experiments should be performed in soils with different fertility levels to identify whether the N supply from the soil is sufficient for the initial development of plants that were inoculated with rhizobium, while maintaining that the N fertilization levels are not detrimental to the symbiosis. Notably, our evaluations continued only to the phenological stage of pod setting in the common beans (Figures 4 and 5). Hence, further investigations are required regarding the plant reproductive development and whether the symbiosis can fulfill the N demand for filling the grains.

Conclusion

The first nodule appeared in the common bean roots 10 days after seedling inoculation, while the nitrogenase activity was initiated after 11 days, which was irrespective of the presence of mineral N. Cultivars of large seeds showed lower levels of initial nodulation and nitrogenase activity relative to cultivars of small seeds. Shoot biomass decreased during the initial growth period of the inoculated plants, which was more pronounced in the small-seeded cultivars than in the large-seeded. Moreover, the N arising from the symbiosis did not suffice for inducing adequate plant growth, reflecting the need for adding supplemental N. However, adding mineral N across the vegetative growth period was detrimental to the symbiosis, greatly limiting nodule development more than nodule initiation as well as strongly inhibiting the nitrogenase activity. Alternatively, when the mineral N supply was withdrawn after 15 days of growth, the bean plants invested in their subsequent nodulation without limiting further shoot growth. Therefore, the rhizobium inoculation in combination with low additions of mineral N at the beginning of growth can stimulate the development of the common bean plants without limiting their symbiosis.

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