DIVISÃO 2 - PROCESSOS E PROPRIEDADES DO SOLO

Comissão 2.1 - Biologia do solo

SEEDS WITH HIGH MOLYBDENUM CONCENTRATION IMPROVED GROWTH AND NITROGEN ACQUISITION OF RHIZOBIUM-INOCULATED AND NITROGEN-FERTILIZED COMMON BEAN PLANTS[(1)]

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SUMMARY

Seeds of common bean (*Phaseolus vulgaris*) with high molybdenum (Mo) concentration can supply Mo plant demands, but to date no studies have concomitantly evaluated the effects of Mo-enriched seeds on plants inoculated with rhizobia or treated with N fertilizer. This work evaluated the effects of seed Mo on growth and N acquisition of bean plants fertilized either by symbiotic N or mineral N, by measuring the activities of nitrogenase and nitrate reductase and the contribution of biological N_2 fixation at different growth stages. Seeds enriched or not with Mo were sown with two N sources (inoculated with rhizobia or fertilized with N), in pots with 10 kg of soil. In experiment 1, an additional treatment consisted of Mo-enriched seeds with Mo applied to the soil. In experiment 2, the contribution of N_2 fixation was estimated by ^{15}N isotope dilution. Common bean plants grown from seeds with high Mo concentration flowered one day earlier. Seeds with high Mo concentration increased the leaf area, shoot mass and N accumulation, with both N sources. The absence of effects of Mo application to the soil indicated that Mo contents of Mo-enriched seeds were sufficient for plant growth. Seeds enriched with Mo increased nitrogenase activity at the vegetative stage of inoculated plants, and nitrate reductase activity at late growth stages with both N sources. The contribution of N_2 fixation was 17 and 61 % in plants originating from low- or high-Mo seeds, respectively. The results demonstrate the benefits of sowing Mo-enriched seeds on growth and N nutrition of bean plants inoculated with rhizobia or fertilized with mineral N fertilizer.

Index terms: *Phaseolus vulgaris*, nodulation, biological N_2 fixation, ^{15}N isotope dilution, ontogeny.

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RESUMO: *SEMENTES COM ALTA CONCENTRAÇÃO DE MOLIBDÊNIO AUMENTARAM O CRESCIMENTO E A AQUISIÇÃO DE NITROGÊNIO DE PLANTAS DE FEIJOEIRO INOCULADAS COM RIZÓBIO OU SOB FERTILIZAÇÃO NITROGENADA*

Sementes de feijoeiro (Phaseolus vulgaris) *com alto teor de molibdênio (Mo) podem suprir as demandas vegetais; entretanto, estudos prévios não avaliaram concomitantemente os efeitos do Mo da semente em plantas inoculadas com rizóbio ou sob fertilização nitrogenada. Este trabalho avaliou os efeitos do Mo da semente, no crescimento e na aquisição de N de plantas de feijoeiro, sob N simbiótico ou N mineral, pela mensuração das atividades da nitrogenase e da nitrato redutase e da contribuição da fixação biológica de N_2, em diferentes estádios de crescimento. Sementes enriquecidas ou não com Mo foram semeadas sob duas fontes de N, inoculadas com rizóbio ou fertilização nitrogenada, em vasos com 10 kg de solo. No experimento 1, um tratamento adicional consistiu de sementes enriquecidas com Mo e aplicação de Mo ao solo. No experimento 2, a contribuição da fixação biológica de N_2 foi estimada pela diluição isotópica de ^{15}N. Plantas de feijoeiro originadas de sementes com alto teor de Mo floresceram um dia antes. Sementes com alto teor de Mo aumentaram a área foliar, a massa de parte aérea e a acumulação de N, nas duas fontes de N. A ausência de efeitos do Mo adicional aplicado ao solo indicou que as sementes enriquecidas com esse nutriente foram suficientes para o crescimento vegetal. Sementes enriquecidas com Mo elevaram a atividade da nitrogenase, no estádio vegetativo de plantas inoculadas, e da nitrato redutase, em estádios tardios nas duas fontes de N. A contribuição da fixação biológica de N_2 foi de 17 e 61 %, em plantas oriundas de sementes com baixo e alto teor de Mo, respectivamente. Os resultados evidenciaram os benefícios das sementes enriquecidas com Mo para o crescimento e a nutrição nitrogenada do feijoeiro, quando inoculado com rizóbio ou sob adubação nitrogenada.*

Termos de indexação: Phaseolus vulgaris*, nodulação, fixação biológica de N_2, diluição isotópica de ^{15}N, ontogenia.*

INTRODUCTION

Besides being a component of the nitrogenase enzyme of diazotrophic microorganisms, the transition element molybdenum occurs in four enzymes catalyzing diverse redox reactions in plants (Mendel & Hänsch, 2002). Nitrate reductase catalyzes the key step in N assimilation, aldehyde oxidases catalyze the last step in biosynthesis of abscisic acid, xanthine dehydrogenase is involved in purine catabolism including ureide synthesis in legume nodules, and sulphite oxidase is probably involved in detoxifying excess sulphite (Mendel & Hänsch, 2002). Molybdenum deficiency can occur in very weathered soils due to continuous cropping, soil erosion, reduction of soil organic matter, and adsorption by soil colloids particularly at low pH (Kaiser et al., 2005). Legumes that depend on N_2 fixation for their N supply require more Mo than plants fertilized with mineral N, since more Mo is needed for symbiotic N_2 fixation than for general plant metabolism (Parker & Harris, 1977). Root nodules of common bean act as a strong sink for Mo derived from seed or external sources in order to maintain adequate rates of N_2 fixation (Brodrick & Giller, 1991).

Since crops require low amounts of Mo, this nutrient can be provided by seed pellets, while on the other hand, seed pelleting with molybdate can impair seed respiration, reduce the survival of the rhizobia inoculated on seeds, and reduce plant nodulation and the efficiency of N_2 fixation (Campo et al., 2009). Alternatively, Mo-enriched seeds can be harvested from crops treated with Mo foliar applications, since the translocation of Mo from leaves occurs rapidly and efficiently (Brodrick & Giller, 1991). Bean yields are not affected by high Mo rates applied to foliage, and Mo fertilizer in the amount required to increase seed content is relatively inexpensive (Vieira et al., 2005; Campo et al., 2009).

In legumes, seeds at maturity contain a large proportion of the plant-accumulated Mo, often in amounts much higher than plant demand over an entire growth cycle, indicating that seed may supply enough Mo to crops to achieve high yields without additional Mo fertilization (Meagher et al., 1952; Jongruaysup et al., 1997). Common bean plants raised from seeds with high Mo concentration accumulated more biomass and N in shoots, had a higher root nitrogenase activity (Brodrick et al., 1992; Kubota et al., 2008) and also yielded more grain in soil with low N content (Vieira et al., 2005). Moreover, sowing bean seeds with high Mo contents did not require additional Mo supply via foliar fertilization to ensure adequate grain yields (Vieira et al., 2011). Nevertheless, in these previous studies the effects of Mo-enriched seeds on bean plants simultaneously inoculated with rhizobia and N-fertilized were not evaluated. Surveying the ontogenetic variations of the activities of nitrate reductase and nitrogenase enzymes, associated with

estimations of the biological N_2 fixation by techniques such as the ^{15}N isotope dilution, could contribute to establish the relevance of the Mo supply provided by enriched seeds for growth and N metabolism of bean plants relying either on symbiotic or mineral N.

Therefore, this study aimed to evaluate the effects of Mo- enriched seeds on growth and N acquisition of common bean plants inoculated with rhizobia and/or fertilized with mineral N, by measuring the nitrate reductase activity in leaves, the nitrogenase activity in the root system, and the contribution of biological N_2 fixation, at different growth stages.

MATERIALS AND METHODS

Experimental conditions

Two experiments in pots were carried out at the National Research Center in Agrobiology (Embrapa Agrobiologia), in Seropédica - RJ, Brazil. Experiment 1 was conducted from August to October 2008, and Experiment 2 from May to July 2009. Seeds of the common bean cultivar Carioca were obtained in a previous field experiment, in which the leaves were sprayed with 120 g Mo ha^{-1} as $(NH_4)_6Mo_7O_{24}.4H_2O$, 52 and 71 days after emergence (DAE). Samples of harvested seeds were dried, ground, nitric-perchloric digested and analyzed for Mo concentration by plasma emission spectrometry (ICP-EAS, Perkin-Elmer). Seeds used in the experiments were sieved to uniform size, with an average weight of 317 mg/seed and Mo concentrations of 0.2 and 10.9 μg g^{-1}, respectively, for low- and high-Mo seeds. Thus, Mo-enriched seeds contained 3.5 μg Mo/seed.

Both experiments were arranged in randomized blocks with five replications. Experiment 1 was arranged in a 5 x 4 factorial design, consisting of five treatments (low-Mo seed inoculated with rhizobia; high-Mo inoculated seed; low-Mo seed plus mineral N; high-Mo seed plus mineral N; high-Mo inoculated seed with additional Mo in the soil) were harvested at four growth stages (20, 35, 45, and 55 DAE). These periods corresponded to the developmental stages: fully expanded third trifoliate, flowering, early pod filling, and mid pod filling. Experiment 2 had a 4 x 2 factorial design with four treatments (low-Mo inoculated seed; high-Mo inoculated seed; low-Mo seed plus mineral N; high-Mo seed plus mineral N) and two harvest times (38 and 51 DAE, corresponding to pod setting and mid-pod filling). In experiment 2, a non-nodulating bean genotype NORH-54, sunflower and sorghum were used as non-fixing control crops, with three replications per species at each harvest. A previous test with the same soil showed abundant nodulation in common bean plants without rhizobia inoculation, thus control treatments without inoculation were not run in either experiment.

The substrate was sieved soil (< 6 mm) from the Ap horizon of a Typic Ultisol (Red Yellow Podzolic) in 10 kg pots. Soil chemical analysis, as described by Embrapa (1997), showed: water pH 5.3, Al: 1 $mmol_c$ dm^{-3}, Ca: 17 $mmol_c$ dm^{-3}, Mg: 16 $mmol_c$ dm^{-3}, K: 33 mg dm^{-3}, P: 8 mg dm^{-3} (Mehlich-1), and C: 9.1 g kg^{-1}. The soil in each pot was limed with 500 mg kg^{-1} $CaCO_3$ and wetted for three weeks to allow enough time for the lime to react. In experiment 2 the soil received 2.5 mg kg^{-1} N as urea enriched with 5 atom% ^{15}N excess 100 days before liming, to estimate the contribution of N_2 fixation by isotope dilution. In both experiments, the following water-diluted nutrients were applied (in mg kg^{-1} soil): 80.0 P as KH_2PO_4, 10.0 Mg as $MgSO_4.7H_2O$, 2.0 Cu as $CuSO_4.5H_2O$, 1.0 Zn as $ZnSO_4.7H_2O$, and 0.05 B as H_3BO_3. Pots of the inoculated and mineral N treatments received 30 and 60 mg kg^{-1} N as $(NH_4)_2SO_4$, respectively. In experiment 1, pots of the treatment with soil-applied Mo received 0.5 mg kg^{-1} Mo as $(NH_4)_6Mo_7O_{24}.2\,H_2O$. After nutrient additions, the soil of each pot was homogenized. At sowing, the soil contained 0 $mmol_c$ dm^{-3} Al, 26 $mmol_c$ dm^{-3} Ca, 17 $mmol_c$ dm^{-3} Mg, 192 mg dm^{-3} K, and 36 mg dm^{-3} P, at a pH 5.5.

Seeds were sown six days after soil fertilization. In the inoculated treatments, each seed received 1.0 mL of liquid inoculant containing the strains CIAT899 (or BR322) and PR-F81 (or BR520) of *Rhizobium tropici* from the collection of Embrapa Agrobiologia. After thinning, three plants were left to grow. Pots were placed in the open air, on tiles distributed on a grass sward, and irrigation was provided whenever necessary. Treatments with mineral N also received two applications of 300 mg N per pot as urea (23 and 37 DAE in experiment 1 and 24 and 41 DAE in experiment 2).

Assays

The trait days to flowering, i.e., when the three plants of each pot had one fully opened flower, was evaluated daily in both experiments. At each harvest, nitrate reductase activity in leaves was measured *in vivo* by the method described by Jaworski (1971), with adaptations. In the morning, the first fully expanded trifoliate of each plant was cut, placed on ice and transferred to the laboratory. A sample of 200 mg of 2-cm leaf discs were placed in vials containing 5 mL incubation medium, consisting of 0.1 mol L^{-1} K-phosphate buffer (pH 7.5), 20 mmol L^{-1} KNO_3 and n-propanol 1 %, and then incubated for 1 h at 30 ºC. After incubation, 0.4 mL of the medium was sampled, and the nitrite released was measured by colorimetric reaction with the addition of 0.3 mL sulfanilamide at 1 % in HCl 3 mol L^{-1} and 0.3 mL N-naphtyl-ethylenediamide at 0.02 %, and readings were performed at 540 nm wavelength. Nitrate reductase activity was expressed as μmol NO_2^- per g of fresh leaf weight per hour.

At harvest, shoots were cut at ground level and separated into leaves, stems and pods. In experiment

1, expanded trifoliates (including petioles) were detached and leaf area was measured photometrically (Li-Cor 3100 Area Meter). In experiment 2, abscised, fallen leaves from each pot were collected daily after 38 DAE, oven-dried and weighed.

In both experiments, the soil of each pot was placed in a plastic box, and the root system and detached nodules were carefully collected. Nitrogenase activity was measured in the root systems by the acetylene reduction assay (Hardy et al., 1973). Whole root systems were placed in 250 mL closed glass recipients and 30 mL of acetylene was injected by 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 (Perkin-Elmer with Flame Ionization Detector). Values of ethylene produced were converted to μmol h^{-1} C_2H_4 per plant, representing the nitrogenase activity. The specific nitrogenase activity was calculated as the ratio between nitrogenase activity and nodule dry mass.

Roots and nodules were washed and nodules detached and counted. The mass of one nodule was calculated as the ratio between nodule mass and number. In experiment 2, senescent leaves were collected from each pot. Leaves, senescent leaves, stems, pods, roots and nodules were oven-dried, weighed and finely ground using a roll-mill. Total N concentration in each plant portion was measured by the semi-micro Kjeldahl procedure. Accumulation of N was computed as the product of N concentration and dry mass. In experiment 2, pots with the control crops (non-nodulating bean, sorghum and sunflower) were harvested together with the common bean plants, 52 and 71 DAE. Shoots were cut at ground level and separated into stems and leaves, and each plant portion was dried, weighed and finely ground.

In experiment 2, ^{15}N isotope composition was initially measured (one replication per treatment) in leaves, stems and roots of beans at both harvests and also in pods at the second harvest, using a continuous-flow isotope-ratio mass spectrometer (Finnigan DeltaPlus, Finnigan MAT, Bremen, Germany) at Embrapa Agrobiologia. A t-test at 0.05 probability was used to check the similarity of ^{15}N enrichment among plant parts for each harvest. In the different bean plant parts, ^{15}N enrichment was similar (Table 1), as verified by Wolyn et al. (1991) in the best N_2-fixing bean lines and by Chagas et al. (2010) in a pot experiment. The leaves contained 67 and 40 % of the total N accumulated by inoculated plants 52 and 71 DAE, respectively, averaged across all treatments. Therefore, measurements of the N isotopic composition were only continued in leaves of inoculated bean plants, and in whole shoots of the non-fixing control crops, for all replications. The percentage of N derived from the atmosphere (%Ndfa) in leaves was estimated by the formula:

$$\%Ndfa = [1 - (\text{atom\% } ^{15}N \text{ excess of bean} / \text{atom\% } ^{15}N \text{ excess of non-fixing})] \times 100$$

Table 1. Enrichment of ^{15}N (in atom% ^{15}N excess) of different plant tissues of common bean and of shoots of non-fixing crops in experiment 2; means of four replicates for non-fixing crops and eight replicates for inoculated bean plants originating from seeds with low or high Mo concentration

Plant tissue	Harvest 1	Harvest 2
Leaves	0.0248	0.0230
Stems	0.0238	0.0237
Roots	0.0246	0.0271
Pods	-	0.0182
Shoot of non-fixing crops		
Non-nodulating bean	0.0344	0.0330
Sorghum	0.0274	0.0684
Sunflower	0.0417	0.0411

Values of different bean plant tissues or non-fixing crops for each harvest did not differ by the t test at 5 % probability.

Value of atom% ^{15}N excess of non-fixing crop was obtained by the average of the three control species, at each harvest time.

For both experiments, analysis of variance was performed for each harvest time in a two-factor design considering the combinations between seed Mo and N source, including data of days to flowering. Data of percentage of N derived from the atmosphere of inoculated plants of experiment 2 were analyzed in a two-factor design between seed Mo and harvest time. The least significant difference between treatments was estimated by the Tukey test at 0.05 probability.

RESULTS

Experiment 1

Averaged across all replications, common bean plants flowered 36.5 and 35.5 days after emergence (DAE) for plants grown from seeds with low or high Mo concentration, respectively, with no effect of N sources on days to flowering. Therefore, high-Mo seeds induced bean flowering one day earlier.

For all treatments, the leaf area of bean plants increased until 45 DAE and decreased thereafter (Figure 1), illustrating the process of leaf senescence during mid-pod filling. Shoot mass increased continuously during the experiment. Inoculated plants grown from seeds with high Mo concentration had a greater leaf area 35, 45 and 55 DAE and greater shoot mass 45 and 55 DAE than inoculated plants raised from low-Mo seeds (Figure 1). For mineral-N-fertilized plants, seeds with high Mo concentration increased leaf area and shoot mass more than in plants grown from low-Mo seeds, but only 55 DAE. The intense shoot growth of inoculated plants originating from high-Mo

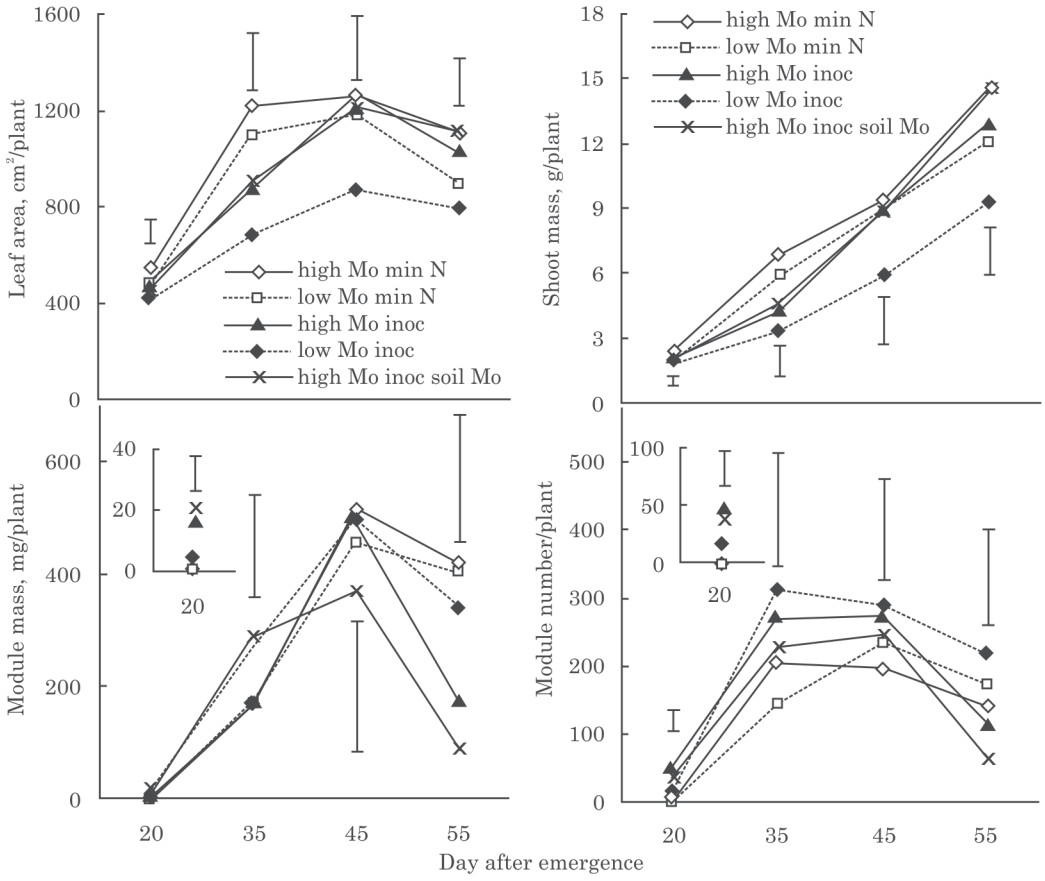

Figure 1. Leaf area, shoot dry mass, nodule dry mass and nodule number of common bean plants originating from seeds with low or high Mo concentration grown at two N sources (inoculated with rhizobia or mineral N) or with additional Mo applied to the soil, at four growth stages (Experiment 1); insets display values of 20 DAE. Vertical bars represent the least significant difference by Tukey's test at 0.05 probability and compare treatments on each evaluation day.

seeds between 35 and 45 DAE, in the early pod filling stage was noteworthy (Figure 1). The leaf area and shoot mass of inoculated plants originating from high-Mo seeds were not affected by additional Mo supplied to the soil during the whole experiment (Figure 1). In plants originating from high-Mo seeds, the pod mass was higher 55 DAE for both N sources than in plants from low-Mo seeds (Table 2). Root dry mass was higher for mineral-N-fertilized than inoculated plants during the whole experiment, without effects of seed Mo concentration (data not presented).

Mass of nodules of bean plants increased from 20 to 45 DAE, whilst nodule number increased only until 35 DAE (Figure 1). Thus, the intense increase in nodule mass between 35 and 45 DAE resulted mainly from expanded individual nodule size, which almost doubled during this period (Table 2). The inoculated plants raised from high-Mo seeds had highest nodule mass and number 20 DAE (Figure 1). Between 35 and 45 DAE, nodulation was not significantly different among treatments. Inoculated plants raised from high-Mo seeds had lowest nodule number and mass 55 DAE, with and without soil-applied Mo, partially owing to a strong decrease in nodule mass 45 DAE (Figure 1). The variation in nodule size was complex: 20 DAE inoculated plants had larger nodules than plants under mineral N fertilization, regardless of seed Mo, whereas at the end of the experiment the inverse occurred, when plants fertilized with mineral N had larger nodules than the inoculated (Table 2).

Values of nitrate reductase activity in bean leaves were highest at the beginning of the experiment, decreased between 20 and 35 DAE and remained almost stable thereafter (Figure 2). Plants fertilized with mineral N showed higher nitrate reductase activity than inoculated plants at both seed Mo concentrations 35 DAE. Mineral-N-supplied plants grown from high-Mo seeds had higher nitrate reductase activity 45 DAE. In inoculated plants originating from high-Mo seeds, with or without soil-applied Mo, the nitrate reductase activity was higher 55 DAE than in plants from low-Mo seeds (Figure 2).

Nitrogenase activity in the root systems was highest 35 DAE, at flowering, whereas this activity was lower during the vegetative period and also during

Table 2. Mass of one nodule, pod mass, accumulation of N in pods, specific nitrogenase activity, and concentration of N in nodules, of common bean plants originating from seeds with low or high Mo concentration grown at two N sources (inoculated with rhizobia or mineral N) or with additional Mo applied to the soil, at four growth stages (in days after emergence - DAE), in experiment 1

Treatment	Mass of one nodule				Pod mass	N accumulation in pod	
	20 DAE	35 DAE	45 DAE	55 DAE	55 DAE	55 DAE	
	mg				g/plant	mg/plant	
Low Mo inoculated	0.31 b	0.91 a	1.83 a	1.71 b	2.2 b	59 b	
High Mo inoculated	0.40 ab	1.16 a	1.81 a	1.53 b	4.3 a	98 a	
Low Mo mineral N	0.04 c	1.34 a	2.25 a	2.50 a	2.4 b	49 b	
High Mo mineral N	0.07 c	1.36 a	2.66 a	3.00 a	4.5 a	100 a	
High Mo inoculated soil Mo	0.55 a	1.24 a	1.67 a	1.49 b	5.0 a	131 a	
	Specific nitrogenase activity				Concentration of N in nodules		
	μmol h^{-1} g^{-1} nodule				mg g^{-1} N		
Low Mo inoculated	62 a	85 a	8.2 a	8.1 a	43 a	44 ab	50 a
High Mo inoculated	32 a	68 a	10.6 a	7.4 a	48 a	56 a	45 ab
Low Mo mineral N	72 a	47 a	0.9 b	0.4 b	44 a	39 b	38 b
High Mo mineral N	10 a	13 a	1.0 b	1.1 b	41 a	44 ab	38 b
High Mo inoculated soil Mo	34 a	43 a	7.8 a	8.3 a	50 a	50 ab	50 a

Means followed by the same letter in columns do not differ by Tukey's test at 0.05 probability. Concentration of N in nodules was not measured 20 DAE due to little material available.

pod filling (Figure 2). Inoculated plants raised from high-Mo seeds had highest nitrogenase activity 20 DAE, with or without soil-applied Mo. Inoculated plants had highest nitrogenase activity 35 DAE for both seed Mo concentrations, and 45 DAE for high-Mo seeds (Figure 2). Specific nitrogenase activity decreased markedly after 35 DAE, indicating low nodule activity at pod filling (Table 2). Specific nitrogenase activity did not differ significantly among treatments 20 and 35 DAE, partially due to the high experimental error of this estimation whereas 45 and 55 DAE, inoculated plants had higher specific activity than plants under mineral N at both seed Mo concentrations (Table 2).

Accumulation of N in shoots increased continuously during the experiment, except for plants raised from low-Mo seeds under mineral N after 45 DAE (Figure 2). Accumulation of N increased rapidly between 35 and 45 DAE, i.e. beginning of pod filling, particularly in inoculated plants originating from high-Mo seeds. Seeds with high Mo concentration improved N accumulation in shoots at 35, 45 and 55 DAE, both in plants under inoculation or mineral N (Figure 2). Mo- enriched seeds doubled the amount of N accumulated in pods 55 DAE from both N sources (Table 2). The nodule N concentration of inoculated was higher than of non-inoculated plants 45 and 55 DAE (Table 2).

Experiment 2

Seeds enriched with Mo advanced bean flowering by one day: plants raised from seeds with low or high Mo concentration flowered 35.8 and 34.9 DAE, respectively, with no effect of N source. Shoot growth was not significantly affected by seed Mo concentration 38 DAE, whereas 51 DAE high-Mo seeds increased shoot and pod mass with both N sources (Table 3). Addition of mineral N increased root mass 51 DAE, whereas root growth was not affected by seed Mo. The different treatments did not affect the mass of senescent leaves accumulated between 38 and 51 DAE (Table 3). High-Mo seeds enhanced nodule mass of inoculated plants 38 DAE but did not significantly affect nodule number with either N source. Plants originating from high-Mo seeds had less nodules for both N sources 51 DAE, but their greater nodule size resulted in similar nodule mass of plants from low-Mo seeds (Table 3). Inoculated plants had higher nodule mass than plants under mineral N 51 DAE, at both seed Mo concentrations (Table 3).

Activities of nitrate reductase in leaves and of nitrogenase in root systems decreased as plants aged, although the nitrogenase decay was much more intense, and specific nitrogenase activity decreased by two orders of magnitude between 38 and 51 DAE (Table 4). Nitrate reductase activity was not affected by treatments 38 DAE, whereas 51 DAE plants raised from high-Mo seeds had higher nitrate reductase with both N sources (Table 4). Nitrogenase activity was highest 38 DAE in inoculated plants regardless of seed Mo, whereas 51 DAE plants originating from low-Mo seeds under mineral N fertilization had the lowest nitrogenase activity and specific nitrogenase activity (Table 4). Seeds enriched with Mo increased N

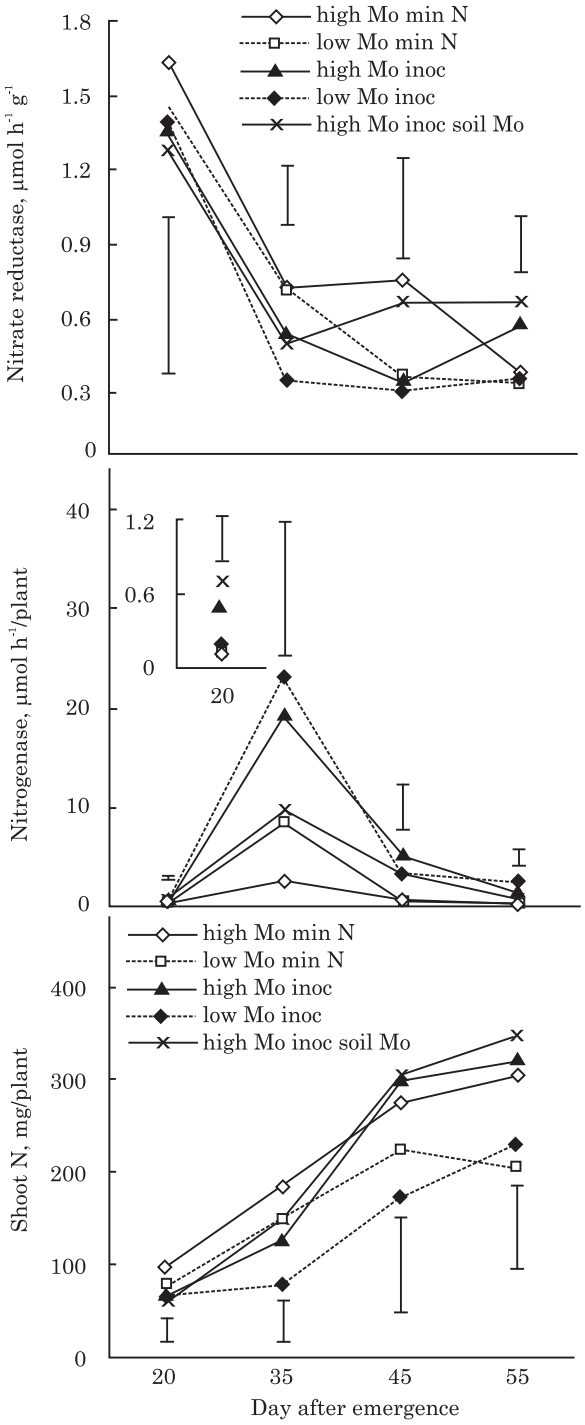

Figure 2. Nitrate reductase activity in leaves, nitrogenase activity in root systems, and N accumulated in shoots, of common bean plants originating from seeds with low or high Mo concentration grown at two N sources (inoculated with rhizobia or mineral N) or with additional Mo applied to the soil, at four growth stages (Experiment 1); inset displays values of 20 DAE. Vertical bars represent the least significant difference by Tukey's test at 0.05 probability and compare treatments on each evaluation day.

concentration in leaves 38 and 51 DAE, and N concentration in nodules 38 DAE, with both N sources. Plants grown under mineral N presented lowest N concentration in nodules 38 and 51 DAE at both seed Mo concentrations (Table 4). Plants raised from seeds with high Mo concentration accumulated most N in shoots 38 DAE when inoculated, and 51 DAE with both N sources (Table 4).

Plants originating from seeds with high Mo concentration showed a higher contribution of biological N_2 fixation in leaves at both evaluation times, but the stimulus of Mo-enriched seeds was more pronounced 51 DAE (Table 5). The contribution of N_2 fixation increased between 38 and 51 DAE at both seed Mo concentrations (Table 5).

DISCUSSION

Two experiments evaluated the effects of sowing seeds with low or high Mo concentrations on common bean plants fertilized by either symbiotic N or mineral N. The evaluations covered most of the reproductive development of the cultivar Carioca, with a growth cycle of nearly 85 days in the field. Considering that a low starter N fertilization may stimulate plant growth and N_2 fixation (Rennie & Kemp, 1984), inoculated plants received 30 mg kg^{-1} N soil at sowing, whereas non-inoculated plants were fertilized with 60 mg kg^{-1} N at sowing plus two cover applications of 300 mg N per pot. The very similar results of the two experiments make our findings more meaningful. Additionally, the bean plant development was strong in both experiments, yielding 10 g of shoots at pod setting (Figure 1, Table 3).

Plant growth and nodulation

Sowing seeds with high Mo concentration increased shoot mass of inoculated plants by 41 and 26 % at the end of experiments 1 and 2, respectively (Figure 1, Table 3). These yield increases were greater than those reported by Brodrick et al. (1992) and Kubota et al. (2008), who evaluated only plants grown under symbiotic N. Furthermore, the shoot mass of mineral-N-fertilized plants from Mo-enriched seeds increased by 20 % at the pod-filling stage (Figure 1, Table 3). It should be stressed that the seeds with different Mo concentrations tested by Brodrick et al. (1992) had been produced under different growth conditions, i.e. on fertile soil or perlite, and these seeds were likely to have different physiological properties.

The additional Mo applied to the soil, a treatment included in experiment 1 only for inoculated plants raised from high-Mo seeds, had no significant effect on plant growth and nodulation, enzyme activities or N accumulation, at any growth stage (Figures 1 and 2). This confirmed that Mo-enriched seeds, which

Table 3. Mass of shoots, roots, senescent leaves, pods and nodules, number of nodules, and mass of one nodule, of common bean plants originating from seeds with low or high Mo concentration grown at two N sources (inoculated with rhizobia or mineral N), at two growth stages (in days after emergence - DAE), in experiment 2

Treatment	Shoot mass		Root mass		Senescent leaf mass	Pod mass
	38 DAE	51 DAE	38 DAE	51 DAE	38-51 DAE	51 DAE
	g/plant					
Low Mo inoculated	9.1 c	11.6 c	1.3 a	1.5 b	1.3 a	1.8 c
High Mo inoculated	10.2 bc	14.6 b	1.3 a	1.5 b	1.0 a	3.8 b
Low Mo mineral N	12.1 ab	16.3 b	1.6 a	1.9 a	1.3 a	2.9 b
High Mo mineral N	12.7 a	20.2 a	1.5 a	1.9 a	1.4 a	5.0 a
	Nodule mass		Number of nodules		Mass of one nodule	
	mg/plant		per plant		mg	
Low Mo inoculated	336 b	1145 a	311 ab	427 a	1.14 a	2.73 b
High Mo inoculated	525 a	1123 a	361 a	342 b	1.50 a	3.63 a
Low Mo mineral N	267 b	702 b	222 b	396 a	1.24 a	1.82 c
High Mo mineral N	371 b	813 b	258 ab	243 c	1.55 a	3.40 a

Means followed by the same letter in columns do not differ by Tukey's test at 0.05 probability.

Table 4. Nitrate reductase activity, nitrogenase activity, specific nitrogenase activity, concentration of N in leaves and nodules, and accumulation of N in shoots, of common bean plants originating from seeds with low or high Mo concentration grown at two N sources (inoculated with rhizobia or mineral N), at two growth stages (in days after emergence - DAE), in experiment 2

Treatment	Nitrate reductase activity		Nitrogenase activity		Specific nitrogenase activity	
	38 DAE	51 DAE	38 DAE	51 DAE	38 DAE	51 DAE
	µmol h^{-1} g^{-1} fresh wt		µmol h^{-1} per plant		µmol h^{-1} g^{-1} nodule	
Low Mo inoculated	0.48 a	0.22 b	30.4 a	0.84 a	92 a	0.74 a
High Mo inoculated	0.50 a	0.41 a	45.6 a	0.99 a	91 a	0.91 a
Low Mo mineral N	0.46 a	0.19 b	16.3 b	0.03 b	68 a	0.05 b
High Mo mineral N	0.65 a	0.48 a	20.2 b	0.76 a	60 a	0.92 a
	N concentration				N accumulation	
	Leaves		Nodules		Shoots	
	mg g^{-1}				mg/plant	
Low Mo inoculated	17 c	17 b	48 b	49 ab	131 b	166 c
High Mo inoculated	25 a	25 a	55 a	53 a	215 a	304 b
Low Mo mineral N	21 b	18 b	40 c	40 c	218 a	255 b
High Mo mineral N	24 a	22 a	47 b	42 bc	258 a	397 a

Means followed by the same letter in columns do not differ by Tukey's test at 0.05.

contained the same amount of 3.6 µg/seed proposed by Vieira et al. (2011) as sufficient for beans, provided enough Mo to achieve an adequate plant growth without supplemental Mo fertilization. Although the additional soil Mo was not tested in plants raised from high-Mo seeds receiving mineral N, the lack of response of these plants to soil-applied Mo was expected, since plants under mineral N usually require less Mo than plants relying on symbiosis (Parker & Harris, 1977).

Mo-enriched seeds accelerated the reproductive development of bean plants, be it under inoculation or mineral N, advancing flowering by one day and almost doubling pod mass at the end of the experiments (Tables 2 and 3). Under field conditions, Vieira et al. (2005) also verified that the growth cycle of bean plants originating from seeds with high Mo concentration was a few days shorter. This rapid development of plants originating from high-Mo seeds could be associated with a higher biosynthesis of abscisic acid due to increased activity of aldehyde oxidases (Mendel & Hänsch, 2002).

Our results provide insights into the complex

Table 5. Contribution of biological N_2 fixation (estimated by the ^{15}N isotope dilution technique) in leaves of common bean plants inoculated with rhizobia and originating from seeds with low or high Mo concentration, at two growth stages (in days after emergence - DAE), in experiment 2

Seed concentration	N derived from the atmosphere in leaves	
	38 DAE	51 DAE
	%	
Low Mo	6.8 Bb	17.2 Ab
High Mo	32.3 Ba	61.1 Aa

Means followed by the same letter, capital letters in rows and lowercase letter in columns, do not differ by Tukey's test at 0.05 probability.

ontogenetic development of nodulation of common bean. Nodule number and mass increased sharply in vegetative stage (20 - 35 DAE), whereas during early pod filling (35 - 45 DAE) nodule mass increased but not nodule number (Figure 1), mainly as a result of enlarged individual nodule size (Table 2). During mid-pod filling (45 - 55 DAE), nodulation was impaired as pods developed, evidencing the process of nodule senescence at late reproductive stages, which coincided with the onset of leaf senescence after 45 DAE (Figure 1). A deeper comprehension of the intricate ontogenetic variation of nodulation of bean plants interacting with the studied treatments requires plant evaluations in different growth stages, as proposed by Araújo & Teixeira (2000).

In both experiments, inoculated plants raised from Mo-enriched seeds had higher nodule mass but less nodules at mid-pod filling in the first evaluation (Figure 1, Table 3). Other results also indicated a somewhat deleterious effect of Mo on late nodulation of bean plants. Brodrick et al. (1992) observed that plants raised from seeds with a high Mo content produced lower nodule mass when the growth media was not supplemented with Mo. Kubota et al. (2008) verified reduced nodule number at pod filling of bean plants raised from Mo-enriched seeds, and Chagas et al. (2010) noticed that seeds with high Mo concentration reduced nodule mass of beans at high soil P availability. Additionally, Vieira et al. (1998b) reported that the foliar Mo application 25 DAE reduced nodule number of field-grown beans, although without affecting nodule mass.

Schulze (2004) reviewed the mechanisms associated with the regulation of N_2 fixation in legumes, concluding that the hypothesis of competition between growing pods and nodules for available assimilates being limiting for nitrogenase activity during pod filling seems to be clearly excluded for common bean, at least under non-stress conditions. Alternatively, it is proposed that a product of N fixation or assimilation could exert a feedback regulatory impact during pod filling (Schulze, 2004), and the remobilized N from lower leaves circulating within the plant during leaf senescence may be involved in causing the drop of N_2 fixation during pod-filling in common bean (Fischinger et al., 2006). The absence of treatment effect on the senescent leaf mass (Table 3) obscured a possible association between leaf senescence and nodulation. Nevertheless, the improved reproductive development of bean plants originating from high-Mo seeds, which resulted in advanced flowering by one day and doubled pod mass in both experiments (Tables 2 and 3), could be associated with an earlier N remobilization from leaves and the reduced nodule number at late growth stages.

Nitrogen metabolism

Although N acquisition by shoots increased continuously during plant development, the most intense shoot N accumulation of inoculated plants occurred between 35 and 45 DAE, i.e., at early pod filling (Figure 2). The maximal rate of N accumulation was observed between 30 and 48 days in field-grown bean lines (Kipe-Nolt & Giller, 1993), whereas the greatest amount of N_2 was fixed between early and late pod-filling (55 - 75 days) by a climbing cultivar (Kumarasinghe et al., 1992) or during pod filling (60 - 77 days) in a bush cultivar (Kimura et al., 2004). Based on evaluations of grain yield and nutrient accumulation at various growth stages in two field experiments with different cultivars, Araújo & Teixeira (2008) concluded that bean grain yield is not intrinsically associated with the vegetative vigor at flowering and that acquisition of N and P during pod filling can strongly influence the final crop yield. Therefore, early pod-filling is a critical stage in N acquisition of common bean.

This rapid accumulation of plant N at early pod filling coincided with the highest nitrogenase activity of the nodulated root systems (Figure 2). Since young reproductive organs require large amounts of N, the nitrogenase activity usually peaks during early pod filling in legumes, although in some species nitrogenase activity drops sharply at later growth stages (Schulze, 2004). Although the acetylene reduction assay is of limited use to measure N_2 fixation over whole growth periods, the technique can be effective to study the symbiosis at different points in time under defined experimental conditions (Unkovich & Pate, 2000).

The improved growth of bean plants from Mo-enriched seeds was associated with a greater shoot N accumulation, amounts of N in the double-sized pods, and higher leaf N concentration, both in inoculated and mineral-N-supplied plants (Figure 2, Table 4). Inoculated plants originating from high-Mo seeds showed an even more rapid N acquisition during the early pod filling (Figure 2). Larger N accumulation of inoculated bean plants originating from high-Mo seeds was also verified by Kubota et al. (2008) in pots with

soil and by Brodrick et al. (1992) in the field. Furthermore, the benefits of Mo-enriched seeds for N accumulation of bean plants receiving N fertilizer were also verified (Figure 2, Table 4).

Assessments of ontogenetic variations of the activities of nitrogenase and nitrate reductase in plants under symbiotic or mineral N contributes to elucidate the relevance of the mechanisms of N_2 fixation and N assimilation from the soil. Seeds with high Mo concentration increased nitrogenase activity of inoculated plants 20 DAE without significant effects at reproductive stages (Figure 2, Table 4). Kubota et al. (2008) also verified that plants of the cultivar Carioca raised from Mo-enriched seeds had higher nitrogenase activity 30 DAE but not 45 DAE, whereas Brodrick et al. (1992) found no effect of seed Mo concentration on nitrogenase activity of bean plants grown in nutrient solution for 38 days. Besides the effects on nitrogenase activity, the additional Mo supply given by enriched seeds may stimulate ureide synthesis via increased activity of xanthine dehydrogenase in the plant fraction of nodules of ureide-producing species such as common bean (Kaiser et al., 2005).

On the other hand, seeds with high Mo concentration increased the nitrate reductase activity in leaves mainly at the reproductive stages: 45 DAE in mineral N-fertilized plants and 55 DAE in inoculated plants in experiment 1 (Figure 2), and for both N sources 51 DAE in experiment 2 (Table 4). Vieira et al. (1998a) and Pessoa et al. (2001) verified that the foliar application of Mo 25 DAE in the field increased the nitrate reductase activity in bean leaves at reproductive stages, extending the period of high enzyme activity. In field-grown common bean, Franco et al. (1979) observed that nitrate reductase activity per leaf fresh weight peaked in the early stages of leaf development but per plant it peaked at early pod filling, indicating the relevance of N assimilation after flowering.

Nitrogenase activity was affected more strongly by N sources than by seed Mo concentrations. In both experiments, inoculated plants had higher nitrogenase activity near flowering than plants receiving mineral N, whereas after 45 DAE inoculated plants had higher specific nitrogenase activity, irrespective of seed Mo levels (Figure 1, Tables 2 and 4). Therefore, addition of mineral N during plant growth inhibited nitrogenase activity and reduced N concentration in nodules, which confirms the high sensitivity of bean symbiosis to soil nitrate (Leidi & Rodríguez-Navarro, 2000). Alternatively, plants receiving mineral N had higher nitrate reductase activity than inoculated plants, irrespective of seed Mo concentration, 45 DAE in experiment 1 and 55 DAE in experiment 2 (Figure 2, Table 4). Of the total nitrate reductase activity of common bean, approximately 95 % is localized in leaves, and this activity responded positively to increasing exogenous nitrate levels, indicating the presence of a nitrate-inducible form (Silveira et al., 2001).

Estimates of the percentage of N derived from the atmosphere in leaves of inoculated plants raised from high-Mo seeds increased from 32 % at pod setting to 61 % at mid-pod filling (Table 5). This confirmed that N_2 fixation during early pod filling is extremely important for common bean (Kumarasinghe et al., 1992; Kimura et al., 2004). Estimates of contribution of N_2 fixation, assessed by ^{15}N isotope dilution in bean plants grown in pots with soil of other authors were rather similar: 50 to 72 % (Rondon et al., 2007) or 54 to 79 % (Chagas et al., 2010). Using the ^{15}N natural abundance method, Mortimer et al. (2008) estimated contributions of biological fixation of 50 to 55 % in beans grown in nutrient solution. In plants originating from low Mo seeds the contribution of N_2 fixation was lower (Table 5), indicating that a limited Mo supply acutely impairs the symbiosis. This demonstrates the benefits of sowing Mo-enriched seeds to improve biological N_2 fixation of common bean.

Concluding remarks

The results clearly demonstrated the benefits of sowing Mo-enriched seeds to improve growth and N nutrition of common bean plants both when inoculated with rhizobia or receiving N fertilization. Mo-enriched seeds increased the nitrogenase activity in the vegetative stage, the nitrate reductase activity at reproductive stages, and the contribution of biological N_2 fixation. Therefore, plants originating from seeds with high Mo concentration, either inoculated or receiving mineral N, accumulated more biomass and N in pods and shoots at mid-pod filling. These Mo enriched seeds were harvested from crops treated with two foliar sprays of 120 g ha^{-1} Mo in the reproductive stages, which elevated the Mo amount per seed to 3.5 µg. These enriched seeds sown in soils with low Mo and N levels could enhance yields and improve the contribution of biological N_2 fixation to common bean. This technique could be used to produce Mo-enriched bean seeds for distribution to small family farms by government agencies, or such seeds could be produced by co-operatives and associations of farmers, which would raise the common bean yields in areas where chemical fertilizers are currently rather rare, or even to increase the efficiency of mineral N fertilizers.

CONCLUSIONS

1. Common bean plants raised from seeds with high Mo concentration had a greater leaf area, shoot mass and N accumulation than plants raised from seeds with low Mo concentration, both when inoculated with rhizobia or fertilized with mineral N.

2. Bean seeds enriched with Mo increased nitrogenase activity at the vegetative stage in plants inoculated with rhizobia, and nitrate reductase activity at late growth stages in plants inoculated with rhizobia or fertilized with mineral N.

3. The contribution of biological N_2 fixation was increased to 61 % in plants originating from high-Mo seeds from 17 % in plants grown from low-Mo seeds.

4. The results demonstrate the benefits of sowing Mo-enriched seeds on growth and N nutrition of bean plants both when inoculated with rhizobia or fertilized with mineral N.

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