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Molybdenum-enriched soybean seeds enhance N accumulation, seed yield, and seed protein content in Brazil

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ABSTRACT

High soybean yields require large amounts of nitrogen (N), which can be obtained mainly from symbiotic N_2 fixation. However, the efficiency of this biological process can be limited by micronutrient deficiencies, especially of molybdenum (Mo). In Brazil, soybean generally responds positively to fertilization with Mo in soils of low fertility and in fertile soils depleted of Mo due to long-term cropping. The micronutrient can be supplied by seed treatment, however toxicity of Mo sources to *Bradyrhizobium* strains applied to seed as inoculant has been observed, resulting in bacterial death and reductions in nodulation, N_2 fixation and grain yield. Therefore, use of seeds enriched in Mo could be a viable alternative to exterior seed treatment, allowing elite inoculant strains of *Bradyrhizobium* to sustain high rates of biological N_2 fixation. We demonstrated the feasibility of producing Mo-rich seeds of several soybean cultivars, by means of two foliar sprays of 400 g Mo ha^{-1} each, between the R3 and R5 stages, with a minimum interval of 10 days between sprays. As a result of this method, considerable increases in seed-Mo content were obtained, of as much as 3000%, in comparison to seeds obtained from plants which received no Mo. In field experiments performed in soils with low N content and without any N-fertilizer supply, inoculation of Mo-rich seeds produced plants with increased N and Mo contents in the grain and higher yields of total N and of grain. In most cases, Mo-rich soybean seeds did not require any further application of Mo-fertilizer.

1. Introduction

High soybean [*Glycine max* (L.) Merr.] yields require large amounts of nitrogen (N). The least expensive source of N for soybean is biological fixation of atmospheric N (N_2) (BNF) by the symbiotic association between the plant and bacteria belonging mainly to the genus *Bradyrhizobium*. The efficiency of the BNF process depends on many factors related to the plant, to the bacteria, to the symbiosis, and to the environment. Problems of low soil fertility and limited availability of macro and micronutrients are also among the most important constraints.

Molybdenum (Mo) is an essential micronutrient for BNF (Shah et al., 1984) and it has long been known that Mo deficiency frequently limits BNF under field conditions (Becking, 1961; Chatt et al., 1976; Brodrick and Giller, 1991; Campo et al., 2000; Albino and Campo, 2001). In N_2-fixing systems, Mo acts as a co-factor of the proteins responsible for electron transfer in the synthesis of the nitrogenase complex, responsible for the conversion of N_2 into ammonium (NH_3) (Shah et al., 1984; Martens and Westermann, 1991). Mo is also important for the metabolism of N (Price et al., 1972; Jacobson et al., 1986; Kaiser et al., 2005) and sulphur (S) (Kaiser et al., 2005). In addition, Mo deficiency renders plants more susceptible to stresses such as low temperature and flooding (Vunkova-Radeva et al., 1988).

Soils of high natural fertility usually can supply the soybean crop with the necessary micronutrients, but Mo deficiency may result from continuous cropping, soil erosion, reduction of soil organic matter, and acidification (Balík et al., 2006). Until recently, the need for fertilization with micronutrients in Brazil was restricted to the "Cerrados," where soils, mostly Oxisols and Ultisols (Adámoli et al., 1986), are infertile and highly acidic. The cultivation of soybean over many years has resulted in decreased availability of certain micronutrients in all Brazilian soils and it is now common to observe that the crop generally responds positively to fertilization with Mo (Lantmann et al., 1989; Campo and Lantmann, 1998). Sources of Mo for crops are scarce, but Mo can be supplied either in mixtures with fertilizers, as is the case of the water-insoluble MoO_3 or Mo frits, or as seed coating or foliar

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sprays of water-soluble Mo salts, mostly ammonium and sodium molybdates (Mortvedt, 1997). Molybdenized fertilizers are another source of Mo which have been successfully applied broadcast on pastures growing on Mo-deficient soils in Australia and New Zealand (Anderson, 1956; Mears and Barkus, 1970), but these fertilizers are not available in Brazil.

Seed treatment with Mo along with inoculation with *Bradyrhizobium* is recommended for soybean in Brazil because it is efficient, rather inexpensive, and convenient, but the toxic effects of Mo sources on rhizobia applied to seed in inoculants have long been known. Burton and Curley (1966) observed that seed pelleting with sodium molybdate affected bacterial survival on the seed, plant nodulation, and the efficiency of N_2 fixation, and reported that 99% of the inoculated bacteria died 4 days after seed treatment with inoculant and Mo. Data from the FAO (1985) confirmed the negative osmotic effects of salts used as sources of Mo on *Bradyrhizobium*, and similar results have been reported by other authors (Sedberry et al., 1973; Gault and Brockwell, 1980; Tong and Sadowsky, 1994; Campo et al., 2000; Albino and Campo, 2001).

Soybean response to Mo fertilization depends on various factors, including Mo content of the seed. Harris et al. (1965) observed that soybean plants grown from seed that came from Texas, in the U.S.A., did not respond to Mo fertilization and showed that soybean grains from those soils contained up to 22.4 μg Mo g^{-1}, sufficient to supply the crop's need. Brodrick et al. (1992) observed that common bean (*Phaseolus vulgaris* L.) plants grown from Mo-rich seed had higher nodule dry weight, accumulated more N in the shoots, and produced more seeds, which were also rich in Mo. Brodrick and Giller (1991) demonstrated that seed with good reserves of Mo gave plants that did not respond to Mo fertilization.

The toxicity of formulas containing Mo for the bacteria dictates the development of alternative strategies to accomplish simultaneous fertilization and inoculation. One approach is placement of the inoculant in the planting furrow with subsequent foliar-spray fertilization, both of which result in extra expenditure for the farmer (Hungria et al., 2007). As the need to supply Mo to soybean crops is now a general rule in Brazil in order to sustain high levels of BNF, grain protein content and yield, the utilization of Mo-enriched seeds could be an attractive and viable alternative to avoid toxic effects on *Bradyrhizobium*. This paper reports evaluation of methods to obtain soybean seeds enriched in Mo and their effects on parameters related to BNF and crop yield.

2. Materials and methods

The experiments were conducted at the experimental stations of Embrapa Soja (Soybean), in Londrina, and of Embrapa Transferência de Tecnologia (Escritório de Negócios), in Ponta Grossa, both in the State of Paraná, southern Brazil. The experiments were performed for five cropping seasons, from 1996/1997 to 2000/2001.

Soil characteristics at each location are presented in Table 1. Soil populations of bradyrhizobia were estimated at the 0–10 cm layer by the most probable number (MPN) method (Vincent, 1970) and the statistical tables of Andrade and Hamakawa (1994), with counts on soybean plants of the cultivar Embrapa 48.

In order to determine soil chemical properties, 20 sub-samples (0–20 cm) were taken from each location, dried (60 °C for 48 h) and finely ground (2-mm sieve). Soil analyses were performed according to Pavan et al. (1992). Soil pH was determined in 0.01 M $CaCl_2$ (1:2.5; soil:solution), after shaking for 1 h. Exchangeable Ca, Mg and Al were determined in the extract obtained with 1 N KCl (1:10; soil:solution) after shaking for 10 min. P and K contents were evaluated in the Mehlich-1 (0.05 M HCl + 0.0125 M H_2SO_4) extract (1:10; soil:solution) after shaking for 10 min. Aluminum was determined by titration with 0.015 N NaOH, with bromothymol blue as indicator. Concentrations of Ca and Mg were determined in an atomic absorption spectrophotometer; K in a flame photometer; P by colorimetry, using the Mo-blue method and ascorbic acid as reducing agent; C by the oxidation of dichromate; and N by the Kjeldahl method.

Before planting, soil pH and P and K fertility were corrected with lime and fertilizers according to the results of the analyses, in order to achieve the levels of fertility recommended for soybeans at the regions where the experiments were conducted (Embrapa, 1999). Plants in all plots received 20 g Co ha^{-1} in order to maximize the potential for BNF. Field plots measured 4.0 m (length) $\times$ 5.0 m (width), with 10 rows 0.5-m apart from one another, and were interspaced by small terraces of 1.0 m to prevent contamination by superficial run-off containing bacteria and fertilizer. Plant populations were around 300,000 plants ha^{-1}.

Eight soybean cultivars were used: BR 16, BR 37, Embrapa 48, BRS 133, BRS 153, BRS 156, BRS 183, and BRS 184. In the first year, Mo-poor seeds were obtained from areas where plants usually respond to Mo, but where no Mo had been applied. Seeds of medium Mo content were harvested from plants grown in areas where response to Mo fertilization is observed, and which received 20 g Mo ha^{-1} as seed treatment. Mo-rich seeds were harvested from greenhouse-grown plants, which received foliar applications of 800 g Mo ha^{-1}, at pod-filling stage. In the following years, all seeds were obtained from the field, and Mo-rich seeds came from plants grown from Mo-rich seeds and which received additional Mo complementation.

In all treatments except for the non-inoculated controls, seeds were treated with peat inoculant containing a mixture of strains SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7) of *Bradyrhizobium japonicum* and delivering 1.2 $\times$ 10^6 cells of *Bradyrhizobium* per seed. A 10% (w/v) sucrose solution was used to ensure adhesion of the inoculant, applied at 300 mL per 50 kg seeds. Seed inoculation took place at sowing and consisted of applying the sucrose solution to the seeds, followed by the peat inoculant. After mixing, seeds were allowed to air-dry in the shade for 15 min and sown within a maximum of 4 h. For the control treatments, seeds received only sucrose solution and, where applicable, the micronutrients.

In the experiments to evaluate the response of Mo-rich and Mo-poor seeds to Mo complementation, two control treatments, one

Table 1
Geographic coordinates, populations of soybean *Bradyrhizobium*, and chemical properties[a] of the soils at the locations where the experiments were carried out.

Location	Coordinates (latitude, longitude)	No. cells of *Bradyrhizobium* g^{-1} soil	pH in $CaCl_2$	Al	K	Ca	Mg	H + Al	BS[b] (%)	C (g dm^{-3})	P (mg dm^{-3})
				($cmol_c$ dm^{-3})							
Londrina	23°18′ S, 51°09′ W	1.6 $\times$ 10^6	4.64	0.00	0.85	5.71	1.79	4.64	64	16.7	12.8
Ponta Grossa	25°05′ S, 50°09′ W	<10	5.20	0.00	0.24	1.55	1.45	4.21	43	21.3	2.2

[a] Before the correction of pH with lime and P and K fertility with fertilizers, as recommended for the crop (Embrapa, 1999).
[b] Base saturation = (K + Ca + Mg)/T_{cec}) $\times$ 100, where T_{cec} = K + Ca + Mg + total acidity at pH 7.0 (H + Al).

without inoculation and another with 200 kg N ha^{-1} (urea, 50% at sowing and 50% 30 days after seedling emergence), were included.

Mo was applied as sodium molybdate ($Na_2MoO_4·2H_2O$), either via foliar spray or seed treatment. In the initial experiments to obtain Mo-rich seeds, foliar sprays of 400, 800, 1200, and 1600 g Mo ha^{-1} were used. In the experiments to study the response to complementation, Mo was applied as foliar sprays of 10 and 20 g Mo ha^{-1} and as seed treatment with 10 and 20 g Mo ha^{-1}. In the experiments designed to determine the best dose of Mo to obtain Mo-rich seeds, all were sprayed on the leaves at the R5 stage [beginning of pod filling (Fehr and Caviness, 1977)]. When the objective was to determine the best time of application to obtain Mo-rich seeds, foliar sprays were applied at 10, 20, and 30 days after flowering. In the experiments to evaluate the responses of Mo-poor and Mo-rich seeds to complementary Mo fertilization, foliar sprays were applied at V4 (four nodes on the main stem with fully developed leaves from the unifoliolated node), R1 (beginning of flowering – one open flower), R3 (beginning of pod formation) or R5 stages, when a single spray was applied, or at R3 and R5, when Mo complementation was split in two applications. For the foliar sprays, the appropriate amount of $Na_2MoO_4·2H_2O$ was dissolved in water to a final volume of 200 L ha^{-1}. Foliar sprays were accomplished by means of a coastal pump fitted with a CO_2 tank to increase pressure and, therefore, reduce the volume of the droplets, in order to obtain better coverage. When Mo was applied to seed, the appropriate weight of $Na_2MoO_4·2H_2O$ was added after the 10% sucrose solution and before inoculation.

Insects were controlled with biological and chemical insecticides and weeds with herbicides, according to the annual technical recommendation for the crop (Embrapa, 1999). None of the experiments was irrigated and, therefore, growth was conditioned by rainfall. All other cultural practices followed the recommendations for the crop (Embrapa, 1999).

Grain yields were determined from the six central rows of each plot (6 m × 6 m area) at physiological maturity, and data were corrected for 13% moisture content, after determining humidity level with a grain moisture tester (Vurroughf 700). Total N in the grains was estimated after Kjeldahl digestion and determination of N in a Tecator (Kjeltec Auto Sampler System 1035 Analyzer) automatic N analyzer. Protein contents were calculated from the total N in the grains. The Mo content of the seeds was estimated after the nitric acid digestion of seed tissues and determination with an inductively coupled plasma emission automated system (PerkinElmer Otima 3300DV), according to Halvin and Soltanpour (1980).

The experiments which tested doses of Mo or timing of application of Mo for seed enrichment had a completely randomized block design with six blocks as replicates (Cochran and Cox, 1957). In the case of experiments to test the response of Mo-rich and Mo-poor seed to Mo complementation, a split-plot design with six replicates was adopted, with the Mo content of the seeds assigned to the plots and the Mo complementation to the sub-plots (Cochran and Cox, 1957).

The data were analyzed using the SAS for PC statistical package (SAS Institute, 2001). All assumptions required by the analysis of variance (ANOVA) were verified. The error normality, according to the experimental model design, was evaluated by Shapiro-Wilk's test (Shapiro and Wilk, 1965), the variance of homogeneity by Burr-Foster's test (Burr and Foster, 1972), and the non-additivity of the model by Tukey's method (Tukey, 1949). Coefficient of skewness and kurtosis were also checked. Data from all experiments were first submitted to the tests of normality of the variables and of homogeneity of variances, and then to the ANOVA; when significant differences were detected by the ANOVA, means were compared by the Duncan test ($p \leq 0.05$) (SAS Institute, 2001).

Table 2
Mean Mo content ($\mu g\ g^{-1}$) in the seeds of four soybean cultivars in response to increasing doses of Mo applied as a single foliar spray at pod filling (R5) (Londrina, 1999/2000).

Amount of Mo applied (g ha^{-1})	Soybean cultivar				
	BR 16	BR 37	BRS 133	Embrapa 48	Mean[a]
0	2	2	2	4	2.5d
400	9	10	9	10	9.5c
800	18	16	18	21	18.2b
1600	28	32	27	32	29.8a

[a] Means ($n = 4$) followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).

3. Results

3.1. Seed enrichment with molybdenum

In the 1999/2000 season in Londrina, increasing contents of Mo in the seeds were observed in response to the foliar spray with increasing doses (of up to 1600 g Mo ha^{-1}) of sodium molybdate at R5 for all four cultivars tested (Table 2). In Ponta Grossa, when the application was made in two doses at R3 and R5, the same trend was observed for all cultivars, but the plateau accumulation was observed with 1200 g Mo ha^{-1} (Table 3). On average, Mo seed content was increased by 1090% with the application of 1600 g Mo ha^{-1} in Londrina (Table 2), and by 659% with the application of 1200 g Mo ha^{-1} in Ponta Grossa (Table 3).

An economic analysis of the cost of sodium molybdate and the effect on seed enrichment indicated that the best benefit was obtained with 800 g Mo ha^{-1}, which was then chosen for the other experiments to evaluate the possibility of obtaining Mo-enriched seeds.

In another experiment carried out in Londrina, in 1999/2000, sodium molybdate was applied at 800 g Mo ha^{-1} as a single foliar spray at various stages of plant growth. No differences were observed in grain yield, but there were significant increases in N yield and in N and Mo contents of the grains, with foliar spraying at 20 days after flowering (Table 4): N and Mo contents in the grains were increased by 9.3% and 3300%, respectively, in comparison to the no-Mo control.

In a third experiment performed in 1999/2000 in Londrina, seed enrichment was evaluated as a response to foliar sprays of increasing doses of Mo, split in two applications. The Mo contents of the seeds of three cultivars increased with dose of Mo, and the highest concentrations where obtained with the highest dose, 1600 g Mo ha^{-1}, split in two applications (Table 5); averages for the three cultivars showed an impressive increase in seed Mo content, from 3.7 to 75 μg Mo g^{-1}.

The results of these experiments clearly demonstrate that it is possible to obtain Mo-enriched soybean seed by spraying the leaves with sodium molybdate after flowering, especially when the treatment is made in two applications. It is also clear that Mo

Table 3
Mean Mo content ($\mu g\ g^{-1}$) in the seeds of four soybean cultivars in response to increasing split doses of Mo applied as foliar sprays at the beginning of pod formation (R3) and pod filling (R5), Ponta Grossa, 1999/2000.

Amount of Mo applied (g ha^{-1})	Soybean cultivar				
	BRS 133	BRS 153	BRS 183	BRS 184	Mean[a]
0	7	14	11	12	11b
400 + 400	71	71	81	65	72a
600 + 600	91	87	83	73	83a
800 + 800	84	71	87	78	80a

[a] Means ($n = 4$) followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).

Table 4
Mean grain yield, N content (NG) and total N in the grains (TNG), and Mo content in the grains (MoG) of soybean cultivar BR 16 in response to the application of 800 g Mo ha^{-1} at different times at and after flowering, Londrina, 1999/2000.[a]

Treatment	Yield (kg ha^{-1})	NG (g kg^{-1})	TNG (kg ha^{-1})	MoG (μg g^{-1})
No Mo applied	3441[n.s.]	58.4bc	194c	1d
At flowering	3530	55.8c	197c	30b
10 days after flowering[b]	3590	58.5b	210bc	20c
20 days after flowering	3504	65.6a	230a	34a
30 days after flowering	3495	63.5a	222a	33a
CV (%)	8.5	5.1	16.8	2.3

[a] Means ($n = 6$) from a same column followed by different letters are significantly different ($p \leq 0.05$, Duncan's test). n.s., statistically non-significant.
[b] The low value for this time of application may be attributed to wash-off of the applied Mo due to a 48.6 mm rainfall on the night following foliar spray.

application to obtain Mo-enriched seeds resulted in greater accumulation of N in the plants and seeds, most likely as a result of greater N_2 fixation.

3.2. Grain yield and N content of plants grown from Mo-rich seeds using BNF as the main source of N

Mo-enriched seeds of soybean cultivars obtained from 1996/1997 to 1999/2000 were planted in experiments to evaluate parameters related to BNF and grain yield in several cropping seasons, with or without complementary supplies of 10 and 20 g Mo ha^{-1}, from 1997/1998 to 2000/2001. Increasing contents of Mo in the seeds were obtained in each new cropping season, as a result of the previous enrichment (Table 6).

In 1997/1998, the utilization of Mo-enriched (7.6 μg Mo g^{-1} -seed) seeds of cultivar BR 16, in comparison to Mo-poor (content undetected by the method) seeds, resulted in yield increases of 22% without any supply of Mo, and of 32%, with a further application of 20 g Mo ha^{-1} (Table 6). In the following season, Mo-enriched seeds (13.3 μg Mo g^{-1} seed) of the same cultivar resulted in yield increases of 56% without Mo complementation and of 68% with the extra application of 20 g Mo ha^{-1}, when compared to Mo-poor (0.73 μg Mo g^{-1} seed) seeds. In 1999/2000, the occurrence of a dry spell during pod filling decreased the magnitude of the responses, but, even so, when Mo-rich and very rich seeds (15.6 and 31.6 μg Mo g^{-1} seed, respectively) were used, average yield increases of 6.8% and 15%, respectively, with no further supply of Mo were observed (Table 6).

Mo-rich and Mo-poor seeds of cultivars BR 37 and Embrapa 48 were complemented or not with additional Mo applied to the seeds (20 g Mo ha^{-1}) or via foliar sprays (10 and 20 g Mo ha^{-1}) and were inoculated and field-evaluated in Londrina, in 1999/2000. Both cultivars responded to Mo complementation, especially when raised from Mo-poor seeds (Table 7). On average, grain-protein content increased from 33.9% to 35.1% in cultivar BR 37, and from 33.0% to 34.6% in cultivar Embrapa 48, when Mo-rich seeds were compared with Mo-poor seeds. For grain yield, gains obtained with

Table 5
Mean Mo content (μg g^{-1}) in the seeds of three soybean cultivars in response to increasing doses of Mo applied as single or split foliar sprays at the beginning of pod formation (R3) and beginning of pod filling (R5), Londrina, 1999/2000.[a]

Amount of Mo applied (g ha^{-1})	Soybean cultivar		
	Embrapa 48	BRS 133	BRS 156
0	3g	4f	4f
400 at R3	23e	22e	19e
400 at R5	17f	18e	20e
400 (200 at R3 and 200 at R5)	31d	35c	35d
800 at R3	36c	33cd	33d
800 at R5	25e	26d	35d
800 (400 at R3 and 400 at R5)	43b	50b	56b
1600 at R3	39c	50b	52b
1600 at R5	39c	39c	43c
1600 (800 at R3 and 800 at R5)	61a	82a	81a
CV (%)	8.8	18.7	13.2

[a] Means ($n = 6$) from a same column followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).

Table 6
Mean grain yield (kg ha^{-1}) of soybean cultivar BR 16 grown from seeds with low (poor), medium, high (rich), and very high (very rich) Mo contents, in response to complementation with 0, 10 or 20 g Mo ha^{-1} applied to the seeds at sowing, Londrina, 1997/1998, 1998/1999, 1999/2000.[a]

Seed[b]	Mo complementation		
	0	10	20
1997/1998			
Poor (0.0)	2766cB	3075bA	3020bA
Medium (0.3)	3049bA	3217bA	3045bA
Rich (7.6)	3378aB	3508aAB	3641aA
1998/1999			
Poor (0.73)	2314bB	2645bA	2793bA
Medium (7.5)	3167aB	3794aA	3790aA
Rich (13.3)	3602aA	3892aA	3823aA
1999/2000			
Poor (2.4)	2398bB	2684aA	2699aA
Medium (9.8)	2592abA	2596aA	2603aA
Rich (15.6)	2561abA	2670aA	2630aA
Very rich (31.6)	2750aA	2701aA	2753aA

[a] Means ($n = 6$) from a same column (lowercase) or line (uppercase) followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).
[b] Numbers in parentheses denote Mo content in the seeds in μg Mo g^{-1} seed. Mo contents of poor, medium or enriched seeds varied from one cropping season to the next due to progressive enrichment of the seeds.

Table 7
Mean protein content in the grains and grain yield of soybean grown from Mo-poor (P) and Mo-rich (R) seeds of cultivars BR 37[a] and Embrapa 48[b], in response to complementation with Mo applied to the seeds at planting or as foliar sprays at the beginning of pod formation (R3), Londrina, 1999/2000.[c]

Mo complementation	BR 37		Embrapa 48	
	P	R	P	R
Protein content in the grains (%)				
None	32.4Bb	34.1Ab	31.7Bb	33.7Ac
20 g ha^{-1} on the seeds	34.2Ba	35.8Aa	32.8Ba	34.9Aab
10 g ha^{-1} foliar spray	34.4a	34.9ab	33.9a	34.3bc
20 g ha^{-1} foliar spray	34.8a	35.5a	33.8ba	35.7Aa
Mean	33.9B	35.1A	33.0B	34.6A
CV (%)	3.2		2.7	
Grain yield (kg ha^{-1})				
None	3096Bb	3574Aa	2521Bb	3174Aa
20 g ha^{-1} on the seeds	3416Bab	3632Aa	3028a	3088a
10 g ha^{-1} foliar spray	3607a	3836a	2854ab	3139a
20 g ha^{-1} foliar spray	3550a	3825a	3028a	3143a
Mean	3417B	3717A	2858B	3136A
CV (%)	7.1		8.8	

[a] Mo-poor seeds of cultivar BR 37 had 0.1 μg Mo g^{-1} seed; Mo-rich seeds had 33 μg Mo g^{-1} seed. Mo contents of poor, medium or enriched seeds varied from one cropping season to the next due to progressive enrichment of the seeds.
[b] Mo-poor seeds of cultivar Embrapa 48 had 0.1 μg Mo g^{-1} seed; Mo-rich seeds had 26 μg Mo g^{-1} seed. Mo contents of poor, medium or enriched seeds varied from one cropping season to the next due to progressive enrichment of the seeds.
[c] Means ($n = 6$) from a same column (lowercase) or line (uppercase) followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).

Table 8
Mean total N in the grain (TNG, kg N ha^{-1}) and grain yield (kg ha^{-1}) of soybean cultivars Embrapa 48 and BRS 133 grown from Mo-poor (P)[a] or Mo-rich (R)[b] seeds, in response to the complementary application of 20 g Mo ha^{-1} on the seeds, at sowing, or as foliar spray, at the beginning of pod filling (R5), Londrina, 2000/2001.[c]

Treatment	Embrapa 48		BRS 133	
	TNG	Yield	TNG	Yield
P, non-inoculated	168c	2988de	227a	3671ab
P, inoculated + 200 kg N ha^{-1}	209a	3315bc	242ab	3853a
P, inoculated[d]	173bc	3029d	220b	3493b
P, inoculated, Mo on the seed	184b	3252c	237a	3670ab
P, inoculated, Mo as foliar spray	151d	2771e	221b	3615ab
R, inoculated	185b	3179c	241ab	3775ab
R, inoculated, Mo on the seed	213a	3594a	248a	3846ab
R, inoculated, Mo as foliar spray	214a	3506ab	241ab	3915a
CV (%)	10.0	8.3	11.4	11.5

[a] Mo-poor seeds of cultivars Embrapa 48 and BRS 133 had 4.6 and 2.2 μg Mo g seed^{-1}, respectively. Mo contents of poor, medium or enriched seeds varied from one cropping season to the next due to progressive enrichment of the seeds.
[b] Mo-rich seeds of cultivars Embrapa 48 and BRS 133 had 31.6 and 27.2 μg Mo g seed^{-1}, respectively. Mo contents of poor, medium or enriched seeds varied from one cropping season to the next due to progressive enrichment of the seeds.
[c] Means (n = 6) from a same column followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).
[d] Inoculation with 500 g peat inoculant 50 kg^{-1} of seeds.

Mo complementation of both cultivars were more marked for Mo-poor seeds and, on average, Mo-rich seeds yielded 8.7% (BR 37) and 9.7% (Embrapa 48) more than Mo-poor seeds (Table 7).

Mo-rich and Mo-poor seeds of cultivars Embrapa 48 and BRS 133 were sown in 2000/2001, in one experiment with or without Mo complementation (20 g Mo ha^{-1}) applied either by seed treatment or foliar spray, and N fertilization (200 kg N ha^{-1}). Both cultivars responded to Mo application, and, in all treatments with Mo-rich seeds, grain yields and total grain N were equivalent to results obtained with heavy N fertilization (Table 8). These responses are consistent with the argument that Mo is important for BNF.

4. Discussion

Soybean can obtain most of its N from BNF and in Brazil rates of BNF of up to 300 kg N ha^{-1} have been reported, supplying up to 94% of the crop's needs (Hungria et al., 2005, 2006a,b, 2007). However, many factors can reduce the efficiency of BNF, especially edaphic constraints such as soil acidity, high temperatures, drought, and low soil fertility (Hungria and Vargas, 2000).

In Brazil, intensification of soybean cultivation has resulted in depletion of this micronutrient even in otherwise fertile soils. Responses to fertilization with Mo have become frequent in all types of soils (Lantmann et al., 1989; Campo and Lantmann, 1998). Soybean fertilization with Mo can then be accomplished either by seed treatment at the time of inoculation, or by foliar spray at certain stages of crop development. With the first method, toxicity of the Mo source to the N_2-fixing bacteria applied as inoculant to the seeds can reduce nodulation and N_2 fixation.

In order to obtain effective nodulation from inoculant strains and maximum efficiency of BNF, it is recommended that the seed surface hold at least 1000 times more rhizobia than is present in the soil population (Weaver and Frederick, 1974). Therefore, agricultural practices designed to guarantee the adequate levels of fertility to sustain BNF must not affect the survival of the inoculant bacteria. Assuring an adequate number of the inoculant strain on the seed is even more critical in soils that have never received inoculant before, or where the crop has not been grown for a long time. In such cases, since there is no naturalized population of *Bradyrhizobium* in the soil, the death of the inoculated bacteria due to the toxic effects of the sources of micronutrients and other products applied to the seeds, such as pesticides, cannot be compensated for the soil bacteria, and nodulation and BNF may completely fail. Therefore, it is important to develop strategies to supply the crop with the necessary amounts of nutrients, without harming the inoculant *Bradyrhizobium*. Our results have demonstrated that it is possible to increase the amounts of N in the grains of plants grown on BNF as the main N source by almost 40 kg N ha^{-1} with the foliar spray of sodium molybdate 20 days after flowering, thus suggesting an adequate strategy to meet the crop's Mo requirements without affecting the inoculated bacteria.

Harris et al. (1965) demonstrated that soybean seeds rich in Mo may not respond to Mo fertilization and the same has been observed for common beans by Brodrick and Giller (1991) and Brodrick et al. (1992), although Ferreira et al. (2003) did not observe any effects of the Mo content of seeds of the black bean cultivar Meia-Noite, which consistently responded to Mo fertilization. These findings may be explained by the observations of Franco and Munns (1981), who detected differences among common bean genetic backgrounds with respect to Mo accumulation in the seeds. In our experiments, enrichment of seeds with Mo was observed for all cultivars tested, which may alleviate Mo deficiency of the subsequent crop. Seed enrichment with Mo has been suggested as a means of circumventing problems of toxicity of the micronutrient sources to inoculated N_2-fixing bacteria (Campo and Hungria, 2002). When Mo-enriched seeds of soybean cultivar Embrapa 48 were grown on BNF as the primary N source and received an extra dose of Mo either on the seed or as foliar spray, total N in the grains were equivalent to what could be obtained with 200 kg N ha^{-1} from N fertilizer. These findings strongly point to a benefit for BNF from the utilization of Mo-enriched seeds. Furthermore, micronutrient-rich seeds are viewed as a strategy to increase crop yields and maintain adequate micronutrient levels when crops are grown in micronutrient-poor soils (Welch and Graham, 2002; Welch, 2005).

Another important aspect is related to the protein content of soybean grains. On a global basis, soybean is the chief source of protein in animal feeds, and it plays an increasing role in human nutrition. However, genetic breeding of soybean for increased oil production generally results in decreased protein content (Johnson et al., 1955; Burton, 1984). An evaluation of several improved soybean cultivars developed in Brazil between 1991 and 1996 confirmed that increased seed-oil content is accompanied by decreased protein content (Bonato et al., 2000). In our study, the utilization of Mo-rich seeds resulted in higher protein content that can contribute to improved animal and human nutrition.

5. Conclusions

Results obtained in our research, from experiments performed over five cropping seasons in the State of Paraná, Brazil, have demonstrated that it is possible to produce Mo-rich soybean seeds by means of the application of 800 g Mo ha^{-1}, split in two foliar sprays of 400 g Mo ha^{-1} each, between the R3 and R5 stages, with a minimum interval of 10 days between sprays. Foliar applications increased Mo content in soybean grains, by as much as 3000%, in relation to regular seeds produced without any supply of Mo. Higher doses of Mo, which may result in further enrichment of the seeds in Mo, were not economically viable in our conditions. The production of Mo-enriched soybean seeds may be very interesting to seed growers in Brazil. When planted in soils deficient in N without any N-fertilizer supply and, therefore, having to rely on BNF as the main N source, inoculation of Mo-rich seeds gave plants that were apparently able to sustain higher rates of BNF and resulted in higher grain and protein yields. In most cases, soybean plants grown from Mo-rich seeds did not require any further supply of Mo-fertilizer. Due to the increasing prices of N fertilizers, the strategy of planting Mo-enriched seeds which can give rise to plants that fully benefit from BNF is an attractive economic alternative for soybean growers in Brazil.

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References

Adámoli, J., Macêdo, J., Azevedo, L.G de, Madeira Netto, J., 1986. Caracterização da região dos Cerrados. In: Goedert, W.J. (Ed.), Solos dos Cerrados: tecnologias e estratégias de manejo. Embrapa Cerrados, Brasília, Brasil, pp. 33–74. [in Portuguese]

Albino, U.B., Campo, R.J., 2001. Efeito de fontes e doses de molibdênio na sobrevivência do *Bradyrhizobium* e na fixação biológica de nitrogênio em soja. Pesq. Agropec. Bras. 36, 527–534 [in Portuguese, English abstract].

Anderson, A.J., 1956. Molybdenum as a fertilizer. Adv. Agron. 8, 163–202.

Andrade, D.S., Hamakawa, P.J., 1994. Estimativa do número de células de rizóbio no solo e inoculantes por infecção em planta. In: Hungria, M., Araujo, R.S. (Eds.), Manual de métodos empregados em estudos de microbiologia agrícola. Embrapa-SPI, Brasília, Brasil, [in Portuguese], pp. 63–94.

Balík, J., Pavlíková, D., Tlustoš, P., Sýkora, K., Černý, J., 2006. The fluctuation of molybdenum content in oilseed rape plants after the application of nitrogen and sulphur fertilizers. Plant Soil Environ. 52, 301–307.

Becking, J.H., 1961. A requirement of molybdenum for the symbiotic nitrogen fixation in alder (*Alnus glutinosa* Gaertn). Plant Soil 15, 217–227.

Bonato, E.R., Bertagnolli, P.F., Lange, C.E., Rubin, S. de A.L., 2000. Teor de óleo e proteína em genótipos de soja desenvolvidos após 1990. Pesq. Agropec. Bras. 35, 2391–2398 [in Portuguese, English abstract].

Brodrick, S.J., Giller, K.E., 1991. Genotypic difference in molybdenum accumulation affects N_2-fixation in tropical *Phaseolus vulgaris* L. J. Exp. Bot. 243, 1339–1343.

Brodrick, S.J., Sakala, M.K., Giller, K.E., 1992. Molybdenum reserves of seed, and growth and N_2 fixation by *Phaseolus vulgaris* L. Biol. Fertil. Soils 13, 39–44.

Burr, I.W., Foster, L.A., 1972. A test for equality of variances. University of Purdue, West Lafayette, 26 p (Mimeo series, 282).

Burton, J.C., Curley, R.L., 1966. Compatibility of *Rhizobium japonicum* and sodium molybdate when combined in a peat carrier medium. Agron. J. 58, 327–330.

Burton, J.W., 1984. Breeding soybeans for improved protein quantity and quality. In: Proceedings of the World Soybean Research Conference, 3, 1984, Ames, Iowa, Westview, Boulder, CO, pp. 361–367.

Campo, R.J., Hungria, M., 2002. Importância dos micronutrientes na fixação biológica do nitrogênio. In: Saraiva, O.F., Hoffman-Campo, C.B. (Eds.), Perspectivas do agronegócio da soja. Embrapa Soja, Londrina, Brazil, pp. 355–366. [in Portuguese]

Campo, R.J., Albino, U.B., Hungria, M., 2000. Importance of molybdenum and cobalt to the biological nitrogen fixation. In: Pedrosa, F.O., Hungria, M., Yates, G., Newton, W.E. (Eds.), Nitrogen Fixation: From Molecules to Crop Productivity. Springer, The Netherlands, pp. 597–598.

Campo, R.J., Lantmann, A.F., 1998. Efeitos de micronutrientes na fixação biológica do nitrogênio e produtividade da soja. Pesq. Agropec. Bras. 33, 1245–1253 [in Portuguese, English abstract].

Chatt, J., Dilworth, J.R., Richards, R.L., 1976. Recent advances in the chemistry of nitrogen fixation. Chem. Rev. 78, 589–625.

Cochran, W.G., Cox, G., 1957. Experimental Designs. John Wiley, New York, 611 p.

Embrapa, 1999. Recomendações técnicas para a cultura da soja no Paraná 1999/2000. Embrapa Soja, Londrina, Brasil [in Portuguese].

FAO – Organización de las Naciones Unidas para la Agricultura y la Alimentación. 1985. Inoculantes para leguminosas y su uso. FAO, Roma. [in Spanish]

Fehr, W.R., Caviness, C.E., 1977. Stages of soybean development. Iowa State University, Ames, IA (Special report, 80).

Ferreira, A.C., de, B., Araújo, G.A., de, A., Cardoso, A.A., Fontes, P.C.R., Vieira, C., 2003. Diagnose do estado nutricional molíbdico do feijoeiro em razão do molibdênio contido na semente e da sua aplicação foliar. R. Bras. Agrociência 9, 397–401 [in Portuguese, English abstract].

Franco, A.A., Munns, D.N., 1981. Response of *Phaseolus vulgaris* L. to molybdenum under acid conditions. Soil Sci. Soc. Am. J. 45, 1144–1148.

Gault, R.R., Brockwell, J., 1980. Studies on seed pelleting as an aid to legume inoculation. 5. Effects of incorporation of molybdenum compounds in the seed pellet on inoculant survival, seedlings nodulation and plant growth of lucerne and subterranean clover. Austr. J. Exp. Agric. Anim. Husb. 20, 63–71.

Halvin, J.L., Soltanpour, P.N., 1980. A nitric acid plant tissue digest method for use with inductively coupled plasma spectrometry. Commun. Soil Sci. Plant Anal. 11, 969–980.

Harris, H.B., Parker, M.B., Johnson, B.J., 1965. Influence of molybdenum content of the soybean seed and other factors associated with seed source on progeny response to applied molybdenum. Agron J. 57, 397–399.

Hungria, M., Vargas, M.A.T., 2000. Environmental factors impacting N_2 fixation in legumes grown in the tropics, with an emphasis on Brazil. Field Crops Res. 65, 151–164.

Hungria, M., Campo, R.J., Mendes, I.C., Graham, P.H., 2006a. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: the success of soybean (*Glycine max* L. Merr.) in South America. In: Singh, R.P., Shankar, N., Jaiwal, P.K. (Eds.), Nitrogen nutrition and sustainable plant productivity. Studium Press LLC, Houston, TX, pp. 43–93.

Hungria, M., Campo, R.J., Mendes, I.C., 2007. A importância do processo de fixação biológica do nitrogênio para a cultura da soja: componente essencial para a competitividade do produto brasileiro. Embrapa Soja, Londrina, Brasil. 80 p (Embrapa Soja. Documentos, 283) [in Portuguese].

Hungria, M., Franchini, J.C., Campo, R.J., Crispino, C.C., Moraes, J.Z., Sibaldelli, R.N.R., Mendes, I.C., Arihara, J., 2006b. Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and of N fertilizer to grain yield. Can. J. Plant Sci. 86, 927–939.

Hungria, M., Franchini, J.C., Campo, R.J., Graham, P.H., 2005. The importance of nitrogen fixation to soybean cropping in South America. In: Werner, W., Newton, W.E. (Eds.), Nitrogen Fixation in Agriculture, Forestry, Ecology and the Environment. Springer, Dordrecht, Amsterdam, The Netherlands, pp. 25–42.

Jacobson, M.R., Premakumar, R., Bishop, P.E., 1986. Transcriptional regulation of nitrogen fixation by molybdenum in *Azotobacter vinelandii*. J. Bacteriol. 167, 480–486.

Johnson, H.W., Robinson, H.F., Comstock, R.E., 1955. Genotypic and phenotypic correlations in soybeans and these implications in selection. Agron J. 47, 477–483.

Kaiser, B.N., Gridley, K.L., Ngaire Brady, J., Phillips, T., Tyerman, S.D., 2005. The role of molybdenum in agricultural plant production. Ann. Bot. 96, 745–754.

Lantmann, A.F., Sfredo, G.J., Borkert, C.M., Oliveira, M.C.N., 1989. Resposta da soja a molibdênio em diferentes níveis de pH do solo. Rev. Bras. Ciênc. Solo 13, 45–49 [in Portuguese, English abstract].

Martens, D.C., Westermann, D.T., 1991. Fertilizers applications for correcting micronutrient deficiencies. In: Mortvedt, J.J., Cox, F.R., Shuman, L.M., Welch, R.M. (Eds.), Micronutrients in Agriculture. 2nd edition. Soil Science Society of America, Madison, WI, pp. 90–112.

Mears, P.T., Barkus, B., 1970. Response of *Glycine wightii* to molybdenized superphosphate on a krasnozem. Aust. J. Exp. Agric. Anim. Husb. 10, 415–425.

Mortvedt, J.J., 1997. Sources and methods for molybdenum fertilization of crops. In: Gupta, U.C. (Ed.), Molybdenum in Agriculture. Cambridge University Press, New York, NY, pp. 171–181.

Pavan, M.A., Bloch, M.F., Zempulski, H.D., Miyazawa, M., Zocoler, D.C., 1992. Manual de análise química do solo e controle de qualidade. Instituto Agronômico do Paraná, Londrina, Brasil. 40 p (Circular 76) [in Portuguese].

Price, C.A., Clark, H.E., Funkhouser, E.A., 1972. Functions of micronutrients in plants. In: Mortvedt, J.J., Giordano, P.M., Lindssay, W.L. (Eds.), Micronutrients in Agriculture; Zn, Fe, Mo, Cu, B, Mn. Soil Science Society of America, Madison, WI, pp. 231–242.

SAS Institute, 2001. Proprietary of software, Version 8.2. 6th edition. SAS Institute, Cary, NC, USA.

Sedberry, J.E., Sharmaputra, R.H., Brupbacher, S., Phillips, J.G., Marshall, J.G., Slvane, L.W., Melville, D.R., Ralb, J.I., Davis, J., 1973. Molybdenum investigations with soybeans in Louisiana. Louisiana Agricultural Experimental Station, LA. Bulletin no. 670.

Shah, V., Ugalde, R.A., Imperial, J., Brill, W.J., 1984. Molybdenum in nitrogenase. Annu. Rev. Biochem. 53, 231–257.

Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality. Biometrika 52, 591–611.

Tong, Z., Sadowsky, M.J., 1994. A selective medium for the isolation and quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkani* strains from soils and inoculants. Appl. Environ. Microbiol. 60, 581–586.

Tukey, J.W., 1949. One degree of freedom for non-additivity. Biometrics 5, 232–242.

Vincent, J.M., 1970. A manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Publications, Oxford, 119 p. (IBP handbook 15).

Vunkova-Radeva, R., Schiemann, J., Mendel, R.-R., Salcheva, G., Georgieva, D., 1988. Stress and activity of molybdenum-containing complex (molybdenum cofactor) in winter wheat seeds. Plant Physiol. 87, 533–535.

Weaver, R.W., Frederick, L.R., 1974. Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. I. Greenhouse studies. Agron. J. 66, 229–232.

Welch, R.M., 2005. Biotechnology, biofortification, and global health. Food Nutr. Bull. 26, 419–421.

Welch, R.M., Graham, R.D., 2002. Breeding for enhanced micronutrient content. Plant Soil 245, 205–214.