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Effect of mineral N and N_2 on nitrogen nutrition in soybean plant

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Abstract

The biological nitrogen fixation is a sustainable option to nitrogen fertilization. The objective of this study was to evaluate the effect of mineral nitrogen and N_2 from the biological nitrogen fixation on nitrogen nutrition in soybean plants, six treatments resulting from combining three levels of mineral N: (0.0, 3.5 and 7 meq L^{-1} of NO_3^-) and inoculation with the CP-2 strain of *Bradhyrizobium japonicum* (with and without inoculation) were evaluated, each treatment was established in triplicate, in three-liter plastic pots and agrolite as substrate, transplanting six soybean seedlings per pot. Dry matter (DM) in plant organs, number and dry weight of nodules, fixation of N_2, soluble N (N-amino acid and N-ureido) and total N per organ were evaluated. The plants that received mineral N transported between 86 and 95% of the total soluble N as N amino acid, while in inoculated plants, the supply of mineral N negatively affected the number of nodules (29%) and the weight of nodules (64%), decreasing 55% the fixation of N_2, in these plants between 50 and 70% of the soluble N transported was as N ureido. At the R6 stage, it was estimated that 70% of the total N accumulated in the DM came from the fixed N_2, favoring the DM of leaves, stems, petioles and mainly the DM in pods. Therefore, in this study it is demonstrated that plants inoculated and supplied with mineral N obtain better effects for DM and total N.

Keywords: inoculation, N-amino acid, N-ureido.

Reception date: May 2022
Acceptance date: August 2022

Introduction

In agriculture, many plants of interest are legumes due to their high protein content (~40%), having a great nutritional value, however, it requires high nitrogen content, known to be a limiting factor, at the same time, a natural alternative implemented by plants to meet this need is through the BNF (Meena *et al*., 2017). However, some authors favor the implementation of inorganic fertilizers, but their excessive use and a bad practice leads to the alteration of ecosystems (Soumare *et al*., 2020). On the other hand, nitrogen fertilizers and BNF carried out by some microorganisms are the sources that allow nitrogen (N) to be incorporated into the soil; both processes use the N contained in the atmosphere.

The soybean plant, one of the most important legumes, establishes symbiosis with bacteria of the genus *Bradhyrizobium japonicum* and satisfies its needs for N through two assimilation pathways, one pathway is the BNF that is carried out in the bacteroids of the nodules formed in the root, where between 50 and 60% of the total N required by the plant is fixed, and the second pathway is the absorption by the root of the N-mineral (NH_4^+ and NO_3^-) present in the soil naturally or applied with chemical fertilizers (Ortez *et al*., 2017).

Worldwide, an estimated of ~2.5 × 1011 kg NH_3 is fixed annually with the BNF (Meena *et al*., 2017). Currently, the potential for symbiotic N fixation by legumes is underexploited because it only involves 13% of fertilization on farmland (Anglade *et al*., 2015). Since the concentration of N in soil organic matter is limiting and rapidly reduces with a few cultivation cycles and that the efficiency of nitrogen fertilizers turns out to be less than 50%, from an economic and environmental perspective, BNF is currently the most sustainable way to incorporate N into the soybean plant (Saturno *et al*., 2017).

Traditionally, the soybean plant has been used in controlled laboratory and field studies to understand the processes of absorption and metabolism of N-mineral and N_2 from BNF, as well as their distribution in plant organs (Ohyama *et al*., 2017; Senthilkumar *et al*., 2021). Studies with ^{15}N as NO_3^-, NH_4^+ or N_2, showed that the two sources of N are reduced to ammonium (NH_4^+) at the root (Yoneyama *et al*., 2020) and used in the synthesis of amino acids and proteins (Ertani *et al*., 2013); however, the source of N absorbed by the plant controls its distribution in the organs of the plant (Balta-Crisólogo *et al*., 2015).

In the soybean plant supplied with mineral N, the N is transported as N-amino acid and as N-ureido (allantoin and allantoic acid) the N from the BNF (Hartmann *et al*., 2012). Both forms of N supply foliage organs and reproductive structures (Kipp *et al*., 2020). N-amino acid supplies the vegetative apparatus and more specifically the stems (Yan *et al*., 2016), while N-ureido is used in developing organs and during the reproductive period are the pods, structures in which 52% of N from the BNF was determined (Gregg *et al*., 2015; Chen *et al*., 2018). However, the use of mineral N could increase yield in the soybean plant under very specific conditions, but this practice is rarely economically viable (Saturno *et al*., 2017).

Studies on BNF in soybeans have also focused on understanding the complex molecular interactions that control a) the selectivity existing between the plant and *Bradhyrizobium japonicum*; b) the formation of the nodules (Bala and Giller, 2006); c) the factors that affect the efficiency of fixation, Freixas *et al*. (2011); d) the factors that influence the senescence of the nodules (Fernández-Luqueño and Espinosa-Victoria, 2008); and e) the methods to quantify the N from the BNF (Ortez *et al*., 2017).

In soybean-producing countries, genetic improvement programs have taken advantage of advances in the BNF, identifying lines highly efficient in the BNF (Cerezini *et al*., 2020), increasing yield and decreasing the use of nitrogen fertilizers (Fontanetto *et al.*, 2011). Studies conducted on genotypes commercially released in different decades (1980-2010) in the US and Argentina confirmed greater yield potential when receiving nitrogen fertilization, the genotypes released in 2010 had a higher yield between 15 and 34% than that recorded in materials released before 2010 in the US and Argentina, respectively.

Despite the aforementioned advances, the interaction between soybean genotypes and the nitrogen fertilizer response is a topic that requires further study, so the objective of the present study was to contribute to the knowledge of nitrogen nutrition and the dry matter production of the soybean plant.

Materials and methods

The research was carried out at the College of Postgraduates, Montecillo *Campus* (19° 28’ 4.26” north latitude, 98° 53’ 42.18” west longitude and an altitude of 2 250 m) in Texcoco, State of Mexico, under conditions of zenith-type greenhouse of metal structure covered with milky white plastic (gauge 720).

Seeds of soybean *cv* Santa Rosa were sown in polypropylene trays of 200 cavities, until the seedlings showed the first pair of true leaves. Subsequently, six seedlings were transplanted in a previously disinfected 5 L plastic pot (experimental unit), agrolite was used as a substrate in the pots. The treatments consisted of developing the soybean plant under six conditions, resulting from combining: a) three levels of mineral nitrogen 0, 3.5 and 7 meq L^{-1} of N, using reactive grade NH_4 NO_3 as a source of nitrogen; and b) two levels of inoculation (with and without inoculation); each treatment was applied in triplicate.

The experimental design used was completely randomized. On the third day after transplantation, the plants of the corresponding treatments were inoculated, applying at the base of the stem of each plant 1 ml of culture that contained 10^9 cells of *Bradyrizobium japonicum* bacteria from the CP2 strain of the microbiology collection of the CP. Irrigations, from germination to inoculation, were provided daily with distilled water and after inoculation with nitrogen-free nutrient solution (Tirado and Alcántar, 1989).

The three nitrogen treatments applied to the soybean plants were prepared with the nutrient solution, starting their application 15 days after inoculation, this nutrient solution was renewed every week.

Table 1. Composition of the nutrient solution without nitrogen used during the development of the study.

Salt	($meq\ L^{-1}$)
KH_2PO_4	2.4
$MgSO_4$	1.2
$CaCl_2$	1
K_2SO_4	1
	(ppm)
H_3BO_3	2
$MnSO_4\ 4H_2O$	1.8
$ZnSO_4$	0.2
$CuSO_4$	0.8
$NaMoO_4\ 2H_2O$	0.04
Fe-EDTA	16

During the development of the plant, four destructive samplings were carried out; vegetative stage (V7), flowering (R2), pod formation (R4) and maximum grain filling (R6), stages recorded at 28, 54, 79 and 103 days after sowing (DAS), respectively. The six plants per pot were taken in each evaluation, recording the wet (WW) and dry (DM) weight of leaves, stems (when present) and roots. In this last organ, in the first three phenological stages, the biological nitrogen fixation (BNF) was evaluated by the nitrogenase (N'asa) activity with the acetylene reduction method (ARA), the number and dry weight of nodules per plant.

At each sampling stage, 0.22 g of fresh matter from petioles, 0.5 g for stems and leaves and 1 g in roots were taken, each sample was wrapped in aluminum foil and preserved in liquid nitrogen (-70 °C) until their use in the determination of N-soluble; the samples used for the determination of total nitrogen were dried in a forced-air oven for 72 h at 70 C. Samples of fresh plant material were ground with a mortar in 10 ml of the potassium phosphate buffer solution 0.1 M, pH 7.4, filtered through gauze to separate the thick residues. From this plant extract, the N-amino acid and the N-ureido were determined (Tirado and Alcántar, 1989). In dry plant material, the total N was determined by the Microkjeldahl method. The data obtained from the response variables were analyzed by analysis of variance and mean separation test using the Tukey test ($p\leq 0.05$).

Results and discussion

Number and activity of nodules

The number, dry weight of nodules and ARA values in the soybean plant, from the V7 stage to the R4 stage, were negatively affected by the mineral N supplied in the nutrient solution (Table 2). The supply of 3.5 and 7 meq L^{-1} of N in the nutrient solution caused a decrease of 9 and 25 nodules in the root of the soybean plant, respectively. Similarly, the dry weight of nodules per plant was mostly affected when the plant received 7 meq of N L^{-1} (Table 2).

Table 2. Average values of the number, dry weight and activity of nodules in the fixation of N_2 (ARA) recorded in three stages of evaluation due to the effect of the mineral N supplied in the nutrient solution.

Treatment	Nodules plant^{-1}		ARA
	Number	Dry weight (mg)	(nmol of C_2H_4 h^{-1} p^{-1})
0 meq L^{-1} of N + Inoc	85 (100)*	117.5 (100)	1.02 (100)
3.5 meq L^{-1} of N + Inoc	76 (89)	80.5 (69)	0.89 (87)
7 meq L^{-1} of N + Inoc	60 (71)	42.1 (36)	0.46 (45)

*= value in parentheses is % relative to nitrogen-free treatment.

N'asa activity showed variation with plant development and was also negatively affected by the application of mineral N. Due to the effect of the supply of N in the nutrient solution, the mean value of the N'asa activity estimated in three stages of the development of the plant (V7, R2 and R4) decreased by 13 and 55% when they received 3.5 and 7 meq L^{-1} of N in the nutrient solution, respectively.

The causes that originate the decrease in the N'asa activity could be due, according to Stal *et al*. (2017), to the effect of regulating compounds such as oxygen or temperatures, while Xia *et al*. (2017) mentioned that the application of NO_3^- reduced the sending of carbohydrates to the nodules and they attribute it to the formation of NO_2^-, which is toxic to nitrogenase and interferes with oxygen regulation and therefore in the fixation of N_2.

Soluble nitrogen (N amino acid and N ureido)

As the cycle progressed, the growth of vegetative and reproductive organs caused in the soybean plant an increase in the demand for N of the foliage, with significant increases ($p\leq 0.05$) in the concentration of soluble N transported from the V7 stage to the R4 stage. The exception of this trend was recorded in inoculated plants that did not receive mineral N and in non-inoculated plants developed with 7.5 meq L^{-1} of mineral N, both plants showed at R2 the maximum value in the concentration of soluble N transported and it decreased at the R4 stage (Table 3).

Particularly at the R2 stage, the plants that received mineral N in the nutrient solution (3.5 and 7 meq L^{-1} of N) had the highest concentration of soluble N transported, whose values decreased 20 and 7% when receiving inoculation; a decrease that could be caused by the competition between the development of nodules at the root and the growth of the foliage organs (Table 3). At the R4 stage, once the nodules in these same plants were established and developed, the activity of the nodules and the mineral N absorbed by the root from the nutrient solution caused an increase of 7 and 43% in the values in the concentration of soluble N transported in relation to the concentration of soluble N transported in non-inoculated plants supplied with 3.5 and 7 meq L^{-1} of mineral N in the nutrient solution, respectively (Table 3).

Table 3. Concentration of soluble N (µg of N gmf^{-1}) transported in the stem at different stages of development of the soybean plant.

Treatment	Vegetative (V7)	Flowering (R2)	Reproductive (R4)
		(µg of N g mf^{-1})	
0 meq of N	77.5 c	104.5 f	128.8 e
3.5 meq of N	168.1 a	295.6 d	318.4 c
7 meq of N	111.3 b	384.7 a	284.3 d
0 meq L^{-1} of N + Inoc	83.7 c	329.3 c	296.5 d
3.5 meq L^{-1} of N + Inoc	110.6 b	235.2 e	341.5 b
7 meq L^{-1} of N + Inoc	120.2 b	359 b	406.9 a
HSD	17.2	9.6	15.8

Values with different letters in the same column are different (Tukey $p \leq 0.05$).

The total soluble N transported was formed by N-amino acid and N-ureido, their proportion in each evaluation depended on the stage of development and on the source of assimilated N (mineral N or N_2). In the initial stages of plant development (V7), N-amino acid predominated as the soluble N form regardless of the N form the plant received, Figure 2A. When the plants received exclusively mineral N, in the stages of R2 and R4, they presented between 86 and 95% of the soluble N in the form of N-amino acid, respectively (Figure 2B and 2C), values similar to those reported by Santachiara *et al*. (2017); Junior *et al*. (2020), who mentioned that, in soybean plants supplied with mineral N, between 77% and 90% of soluble N is transported as N amino acid.

The same Figures 2B and 2C show that when the soybean plant, in addition to the mineral N, also received inoculation, it had a decrease in the value of N amino acid transported with respect to the plants supplied only with mineral N. At the R2 stage the values of N amino acid fluctuated between 66 and 82%, Figure 2 B; while at the R4 stage, the mean value of the transported N amino acid decreased up to 35%, the decrease in the N amino acid was caused by the activity of N_2 fixation of the nodules that stimulated the production of N ureido, as mentioned by Lopes (2015); McCoy *et al*. (2018), results that agree with those reported by Tamagno *et al*. (2018), who mentioned that, in plants developed under these conditions, 60% to 81% of soluble N is transported for rhizodeposition pathways; that is, decomposition and deterioration of root nodules and cells (Fustec *et al*., 2010; Collier *et al*., 2012).

In the inoculated plants, the N-ureido transported showed the maximum values in the R4 stage, an inverse situation occurred in the values of ARA determined during this stage, the above can be attributed to the fact that both methods must be calibrated, as performed by Grageda *et al*. (2003), using non-destructive techniques, they reported that after R5 the soybean plant fixed 78% of N.

It is important to note that between the stages of R2 and R4, the plants supplied with 3.5 meq of N presented 79% of the soluble N transported as N ureido, observing that this form of N was reduced to 50% when the plant received 7 meq of N in the nutrient solution. Decreases in the amount of N-ureido due to the effect of external application of N had been reported by Ono *et al*. (2021), these researchers mentioned that, in nodulated plants, the N-ureido is relatively low, because it is converted to urea, actively occurring in the underground parts, especially in the roots, in their study, nitrate represented about 50% of N applying 5 mM of $NaNO_3$.

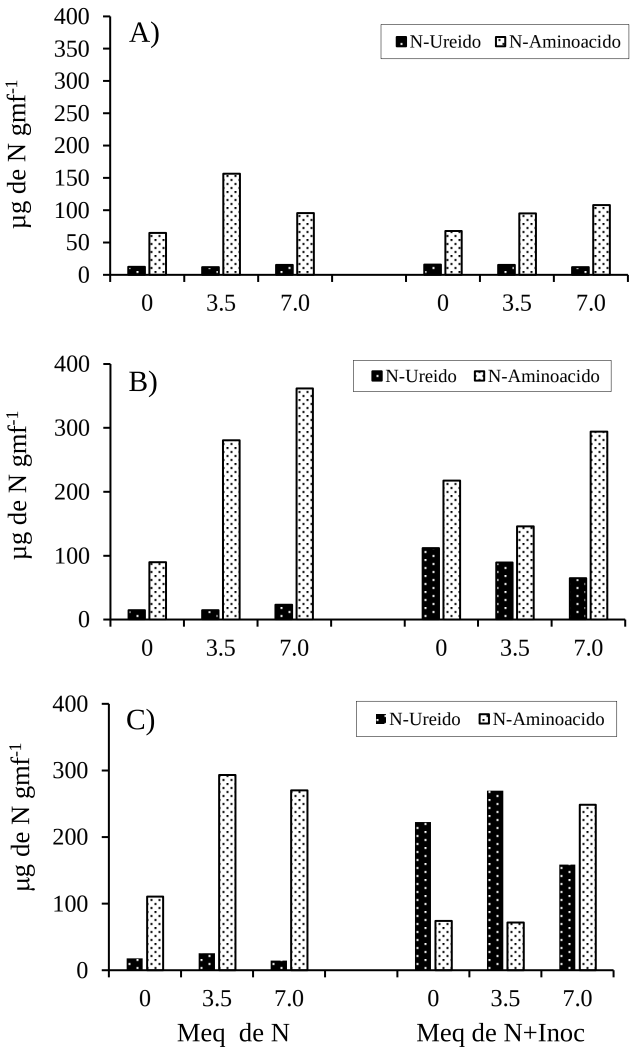

Figure 1. Concentration of N-amino acid and N-ureido (µg of N gmf^{-1}) transported in stems of soybean plants in three stages of development. A) vegetative; B) flowering; and C) pod formation.

Accumulation and distribution of N

The mineral N absorbed by the root or the N_2 fixed in the nodules, once assimilated and transported as soluble N, accumulated as total N in the DM of the organs of greatest demand at each stage of plant development. At the R2 stage, the plants that received 7 meq of N in the nutrient solution had the greatest accumulation of total N in their DM, this form of N favored the total N accumulated in the DM of the stems and petioles, whose differences due to the form of N received in the same organ were significant ($p \leq 0.05$).

The lowest value of total N occurred in the DM of plants that did not receive mineral N and inoculation, and represented 10% with respect to plants that accumulated the highest amount of total N. At this R2 stage, regardless of the source of N that the plant received, the total N recorded on average represented 72, 18 and 10% of the total N of the DM of leaves, stems and petioles, respectively.

The largest increase in total N in the DM of all plants was recorded between the stage of R2 and R6. When these were inoculated and received mineral N accumulated the largest amount of total N and on average it was 2.3 times more than the total N value recorded in the DM of plants that only received the mineral N, these differences were significant ($p \leq 0.05$). Particularly, in the soybean plant that received 3.5 meq of N L^{-1}, it had an accumulation of 158 mg of N $plant^{-1}$ in the DM, amount of total N that, considering a density of 437 plant ha^{-1}, allows estimating a total extraction of 72.2 kg of N ha^{-1}; this extraction of total N, due to the effect of the mineral N, represented 30% of the total N determined in the DM of plants supplied with the same dose of mineral N and which were also inoculated (Table 4). That is, in this plant, 160.5 kg of N ha^{-1} determined in the DM was obtained due to the effect of the activity of the nodules, deducing 70% of the N accumulated in the DM at the R6 stage came from the fixation of N_2; the above shows that both pathways of assimilation of N act in the plant in a complementary way, as previously mentioned by Lu *et al*. (2021).

Table 4. Amount of N accumulated (mg plant^{-1}) in the dry matter of the organs of the soybean plant during the reproductive stage (R6).

Treatment	Total	Leaf	Stem	Petiole	Pod
		(mg of N plant^{-1})			
0 meq of N	41.9 e	7.1 e	2.9 d	1 d	31 f
3.5 meq of N	158 c	36.7 c	4.6 c	2.2 d	114.5 d
7 meq of N	274.1 b	93.6 b	12.9 b	6.2 c	161.4 c
0 meq of N + Inoc	98.4 d	23.4 d	3.6 cd	2.2 d	69.2 e
3.5 meq of N + Inoc	509.2 a	94.6 b	21 a	13.9 a	379.7 a
7 meq of N + Inoc	487.1 a	128.9 a	22.2 a	10.3 b	325.7 b
HSD	26	4	1.7	0.9	7.1

Values with different letters in the same column are different (Tukey $p \leq 0.05$).

During this reproductive stage, the high demand for N of the pods caused that, in the plants supplied with mineral N exclusively and in the inoculated ones that did not receive mineral N, part of the total N determined at flowering in the organs of the foliage was mobilized towards the pods, in the inoculated plants that did not receive mineral N, 41.6% of the N determined in the pods came from the N stored in the foliage at R2, while in plants supplied exclusively with the highest dose of N, this percentage was 9.9% and 4.4% in those that received the intermediate dose.

Accumulation and distribution of dry matter (DM) per plant

The lowest amount of DM was recorded in the plant that did not receive mineral N and neither inoculation, at the R2 stage, in this plant the accumulated DM represented 46% of the DM produced in the plant that received only inoculation. At this stage, the plant that received the highest dose of N and inoculation was surpassed 0.5 g of DM $plant^{-1}$ by the registered DM that received the highest dose of N (Table 5), a situation that can be associated with the competition that generated the formation of nodules in the root.

Table 5. Dry matter production in the soybean plant in two phenological stages.

Treatment	Flowering (R2)	Maximum grain filling (R6)	Increase (R2 to R6)
		(g plant^{-1})	
0 meq L^{-1} of N	0.6 d	1.4 f	0.8
3.5 meq L^{-1} of N	2 b	6.2 d	4.2
7 meq L^{-1} of N	2.8 a	8.1 c	5.3
0 meq L^{-1} of N + Inoc	1.5 c	3.3 e	1.8
3.5 meq L^{-1} of N + Inoc	2 b	11.8 b	9.8
7 meq L^{-1} of N + Inoc	2.3 b	13.3 a	11
HSD	0.4	0.8	

Values with different letters in the same column are different (Tukey $p \leq 0.05$).

The highest production of DM in the plant occurred between the stage of R2 and R6, regardless of the dose and form of N the plant received for nutrition. Plants supplied with N-mineral and consistently inoculated produced more DM, the mean value of DM recorded at R6 in these plants exceeded 1.7 times the DM of plants that only received N-mineral (3 and 7 meq L^{-1}) and 9.3 times the DM of plants developed without mineral N and without inoculation (Table 5).

Regardless of the nitrogen nutrition that the plant received during its development, of the total value of the DM produced at the R6 stage, on average 59% was recorded in pods, 26% in leaves and 15% in stems + petioles (Figure 1). The inoculated plants that received mineral N produced more amount of DM in their organs, in pods the value was 2.2 times significantly higher ($p \leq 0.05$) than the value of DM recorded in the pods of plants that only received mineral N; in stems plus petioles and leaves, this value was 1.4 and 1.3, respectively. These results are consistent with those recorded by Tirado *et al*. (1990), demonstrating that the two pathways of assimilation of N in the soybean plant are necessary to obtain high yields of DM.

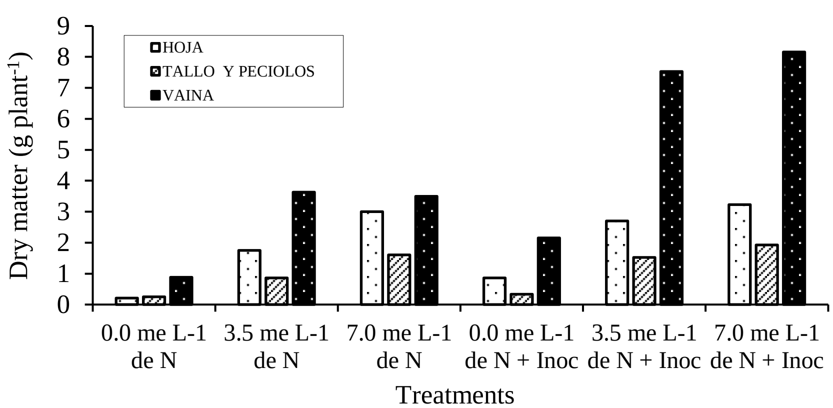

Figure 2. Dry matter (g plant^{-1}) accumulated in the organs of the soybean plant of the Santa Rosa variety, during the grain filling stage (R6).

These results indicate that, in nodulated soybean plants, the accumulation of DM in the pods is favored by the activity of the nodules and that the foliage is an important source of N, being able to conclude that the fixed N (N-ureido) is destined primarily to the supply of grains, as mentioned by (Anglade *et al*., 2015; Zhou *et al*., 2016; Ono *et al*., 2021).

Conclusions

The application of mineral N affected the number of nodules per plant, the dry weight of nodules and the nitrogenase activity. The greatest accumulation of dry matter and total nitrogen was in plants inoculated and supplied with mineral N. In nodulated plants, between 39 and 79% of the soluble N transported in the stem after flowering was recorded in the form of N-ureido. Of the total N determined in the pods, between 50 and 70% was assimilated via N_2 fixation.

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