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Herbaspirillum seropedicae inoculation and nitrogen fertilization on nitrogen use efficiency of different corn genotypes

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The biological N fixation provided by diazotrophic bacteria may be an alternative for corn production in more sustainable or low-input agricultural systems. Therefore, this study aimed to evaluate the interactive effect of seed inoculation with *Herbaspirillum seropedicae* and nitrogen fertilization on the nitrogen use efficiency of different corn genotypes, through the technique of isotopic dilution of ^{15}N. The experiment was carried out under controlled conditions in a greenhouse using a completely randomized design in a 3 × 2 × 2 factorial scheme with three corn hybrids: Maximus, P3646H and BRS3035, using inoculated and non-inoculated plants with two nitrogen doses (0.0 and 80 kg ha^{-1}) and four replicates. At 35 days after emergence plants were collected, divided into root and shoots and analyzed with regard to root and shoot dry matter production, accumulated N in plant, N percentage and content coming from the fertilizer and use efficiency of N applied as a fertilizer. Results showed that there is a difference between corn genotypes concerning shoot dry matter production and percentage of N shoot coming from the fertilizer. Shoot dry matter production is influenced by *H. seropedicae* inoculation. This inoculation coupled with nitrogen fertilization promotes shoot N increments of about 32 and 62% for the hybrids P3646H and BRS3035, respectively. These hybrids showed increases of 34.3 and 64.4%, respectively, in N use efficiency when inoculated with *H. seropedicae* without the addition of N, what allows inferring that the BNF behaves as an important source of N to the system.

Key words: *Zea mays* L., diazotrophic bacteria, ^{15}N, nitrogen fertilization.

INTRODUCTION

Corn is a crop that demands soil fertility especially nitrogen (N) whose deficiency may reduce grain yield from 10 to 22% (Subedi and Ma, 2009). Thus, inadequate management of nitrogen fertilization is still one of the obstacles for the increase in productivity. Currently nitrogen fertilizers represent more than 70% of corn

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fertilization which greatly influences the economic viability of the crop (Machado et al., 1998). The obtention of cultivars adapted to low-N soils and capable of associating with diazotrophic bacteria might represent an economically viable alternative for corn production in agricultural systems with low supply of inputs (Reis Junior et al., 2008).

The use of relatively high nitrogen doses during the growth stage of hybrid corn cultivars may lead to the selection of genotypes with low luxury consumption of this nutrient and/or requiring high nitrogen fertilization to express their productive potential (Carlone and Russel, 1987). On the other hand, low nitrogen doses may naturally contribute to the selection of genotypes efficient at associating with nitrogen-fixing bacteria which can provide reduction in the consumption of synthetic nitrogen fertilizers (Roesch et al., 2005) and selection and/or development of cultivars with higher N use efficiency (Carvalho et al., 2012).

The obtention of higher efficiency in N use has been the goal of both capitalized and low-input agriculture. This occurs due to the waste and scarcity of nitrogen which can cause economic, environmental, public health and food safety problems (Carvalho et al., 2012). However, the ways to obtain genotypes that are more efficient in N use are rather complex because nitrogen metabolism is influenced by various environmental factors (Hirel et al., 2001; Majerowicz et al., 2002).

The positive interaction between diazotrophic bacteria and corn has been reported by many authors (Dobbelaere et al., 2001) and a survey of various experiments carried out in many countries shows that the inoculation with bacteria from the genus *Herbaspirillum* resulted in most cases in increase of dry matter, productivity and nitrogen accumulation (Reis, 2007). Therefore, considering that the genetic range can influence the interaction between corn plants and endophytic diazotrophic bacteria, this study aimed to evaluate the interactive effect of seed inoculation with *Herbaspirillum seropedicae* and nitrogen fertilization on the N use efficiency of different corn genotypes determined through the ^{15}N-isotope dilution technique.

MATERIALS AND METHODS

The experiment was carried out in a greenhouse (22°12' S; 54°56'W; 452 m) at the College of Agricultural Sciences of the Federal University of Grande Dourados, MS, Brazil, from December 2012 to January 2013. The climate in the region is Cwa according to Köppen's classification. The soils used in this study classified as Distroferric Red Latosol with very clayey texture (Embrapa, 2013) was collected in the layer of 0 to 20 cm. The chemical analysis of the soil before the experiment is as follows: pH ($CaCl_2$): 4.15; P: 26 mg dm^{-3}; K: 5.0 mmolc dm^{-3}; Ca: 9.0 mmolc dm^{-3}; Mg 2.0 mmolc dm^{-3}; Al: 3.3 mmolc dm^{-3}; H+Al: 41.6 mmolc dm^{-3}; Sum of Bases: 115.1 mmolc dm^{-3}; CEC: 531.1 mmolc dm^{-3} and Base Saturation: 21.7%. Granulometric analysis showed 225 g kg^{-1} of sand, 125 g kg^{-1} of silt and 650 g kg^{-1} of clay.

Soil pH correction was performed 30 days before sowing in order To increase base saturation to 50% using finely ground dolomitic limestone (RNV 100%) considering the results of soil chemical analysis. Due to the low soil fertility, base fertilization was also performed in order to guarantee the establishment of the crop. 100 kg ha^{-1} of P_2O_5 (270 mg dm^{-3}) and 60 kg ha^{-1} of K_2O (51 mg dm^{-3}) were applied (mixed to the soil) in the forms of single super phosphate and potassium chloride, respectively. Micronutrients were applied according to the crop demand in solution using deionized water and salts (p.a.) according to Epstein and Bloom (2006). Nitrogen fertilization was performed using 80 kg ha^{-1} of N (54 mg dm^{-3}) as urea (45%) is divided into two applications of 40 kg ha^{-1} of N (27 mg dm^{-3}). The first one was applied at sowing and the second in covering 15 days after plant emergence. The isotopic enrichment used was equal to 0.7% of ^{15}N atoms in excess for the dose of 80 kg ha^{-1}. In order to guarantee a uniform application, the nitrogen fertilizer was diluted in 50 mL of deionized water and applied using a pipet.

The adopted experimental design was completely randomized in a 3 × 2 × 2 factorial scheme with three corn hybrids: Maximus, P3646H and BRS3035 using inoculated and non-inoculated plants; with two nitrogen doses (0.0 and 80 kg ha^{-1}) and four replicates. The experimental units consisted of 10-dm^{-3} plastic pots daily irrigated with deionized water in a controlled way to replenish the water lost through evapotranspiration in order to maintain soils at 60% of the field capacity.

Corn seeds of the simple hybrid P3646H (Pioneer), double hybrid Maximus (Syngenta) and triple hybrid BRS3035 (Embrapa) were used in the sowing and previously inoculated with the Z-94 strain of *H. seropedicae* (inoculant cell concentration of about 10^9) in the peat-based formulation produced by Embrapa Agrobiology, Seropédica - RJ. The applied dose was 250 g of peat inoculant for each 10 kg of corn seeds. For inoculation, 60 mL of a sugary solution at 10% (m/v) were added for each 10 kg of seeds, aiming to increase the adhesion of the peat inoculant to the seeds. Seeds were left to germinate directly in the pots and eight days after emergence thinning was performed leaving only one plant per experimental unit.

At 35 days after emergence, plants were collected and divided into root and shoot. All collected material was sequentially washed in running water, 0.1 mol L^{-1} HCl solution and deionized water. Then, samples were accommodated in paper bags and dried in a forced-air oven at 65°C for 72 h. After drying the material, dry matter was weighed and ground in a Wiley-type grinder for analyses of N total content and N isotopic composition.

Total N in different parts of the plant (root and shoot) was determined through the Kjeldahl method according to the methodology described in Embrapa (2009). As for the ^{15}N isotopic composition analyses, samples were processed according to the method of Rittenberg (1946); from the final distillate obtained in the analysis of % of Total N, the extracts were again acidified with 0.5 mol L^{-1} H_2SO_4 and concentrated through evaporation; N-NH_4^+ was converted into N_2 through oxidation with lithium hypobromite (LiOBr) (Porter and O'Deen, 1977). The analyses of the ^{15}N isotopic composition were performed using a mass spectrophotometer Delta Plus from the John M. Day Stable Isotope Laboratory at Embrapa Agrobiology. With the results of nitrogen isotopic composition (% in ^{15}N atoms) of the samples was calculated as:

a) Total N content accumulated in the plant (TN, mg/plant)

$$TN = \frac{DMY \times N}{100}$$

Where DMY is the dry matter yield and N is the N content in the plant (g kg^{-1}).

b) Percentage of N in the plant coming from the fertilizer (%PNF)

$$\%PNF = \left(\frac{\%\text{ in 15N atoms excess plant test}}{\%\text{ in 15N excess fertilizer}}\right) x100$$

Table 1. Analysis of variance of shoot dry matter (SDM), root dry matter (RDM), shoot nitrogen percentage (%SN) and total shoot nitrogen (TSN) of three corn hybrids subjected to different nitrogen levels and inoculated with *Herbaspirillum seropedicae*. Dourados, MS, Brazil (2013).

Source of variation	DF	Mean Square			
		SDM	RDM	%SN	TSN
Hybrid (H)	2	51.30*	3.94	0.58*	8357.16*
Nitrogen (N)	1	224.42*	41.45*	8.50*	193080.79*
Inoculation (I)	1	33.41*	12.19	0.01	11041.70*
H*N	2	7.23	0.48	0.07	6130.75*
H*I	2	2.55	12.53	0.32*	2703.31
N*I	1	4.30	3.49	0.15*	1692.00
H*N*I	2	0.77	0.72	0.41*	5262.89*
Residue	36	3.33	5.01	0.02	916.63
CV (%)		24.07	9.18	11.32	25.95

* – significant by Tukey test to 5% probability. CV- coefficient of variation.

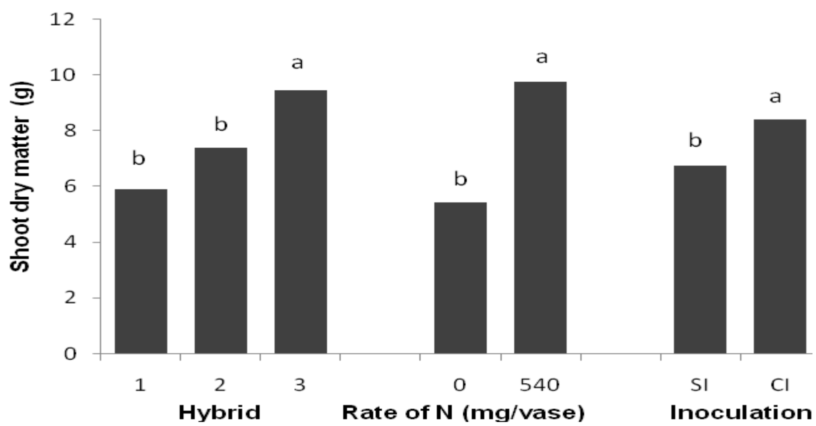

Figure 1. Shoot dry matter (SDM) of three corn genotypes inoculated or not with *Herbaspirillum seropedicae* under different nitrogen levels. Hybrid 1 (Maximus), Hybrid 2 (P3646H) and Hybrid 3 (BRS 3035); NI (no inoculation); WI (with inoculation).

c) N in the plant coming from the fertilizer (PNF)

$$\mathrm{PNF} = \frac{\%\mathrm{PNF\ x\ DMY}}{100}$$

d) Use efficiency of N applied as a fertilizer as a function of the applied quantity (NAQ)

$$\mathrm{NUE} = \frac{\mathrm{PNF}}{\mathrm{NAQ}} \mathrm{x}\ 100$$

The obtained results were subjected to the analysis of variance and the means were compared by Tukey test at 5% of probability using the statistical software Sisvar (Ferreira, 2000).

RESULTS AND DISCUSSION

Significant negative effects ($p \leq 0.05$) were observed in the interaction hybrid x inoculation x nitrogen for shoot N percentage (%SN) and total shoot N (TSN). Shoot and root dry matters (SDM and RDM) did not show significant effect of the interaction and are independently shown for each hybrid, inoculation and nitrogen in Table 1.

The corn hybrids significantly differed ($p \leq 0.05$) with respect to SDM (Table 1) which was 60.5% higher for the BRS 3035 compared to Maximus and 28.3% compared to P3646H (Figure 1). In 2008, Reis Junior and coworkers also observed difference in dry matter accumulation between the studied corn hybrids.

In this study, nitrogen fertilization promoted increase of 79.5% in shoot dry matter production of corn plants compared to the control treatment which was not fertilized (Figure 1). Similar results were found by Gava et al. (2010); these authors found that the increase in the dose of N fertilizer caused increase of dry matter and dry matter production rate in corn. Root dry matter in the fertilized treatment was 7.92% higher than in the non-

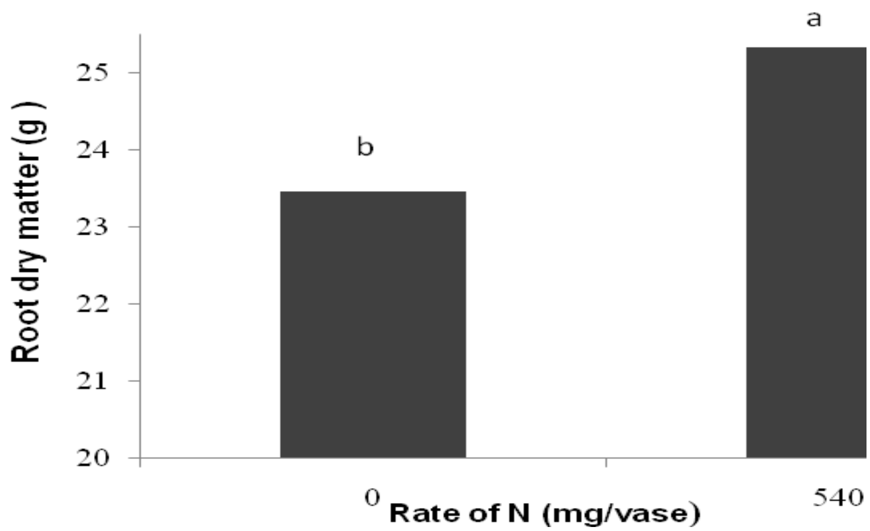

Figure 2. Root dry matter (RDM) as a function of nitrogen fertilization.

fertilized (Figure 2) corroborating the results found by Taylor and Arkin (1981). Glass (1990) reported change in root growth as a result of soil fertility. Moreover, nitrogen favors root system growth providing plants with the conditions for greater water and nutrient absorption (Rao et al., 1992).

It is observed that the inoculation with the Z-94 strain of *H. seropedicae* promoted an increase of 24.74% in the SDM production compared to the control. The values ranged from 6.75 g/plant in the control to 8.52 g/plant in the inoculated treatment (Figure 1). This greater SDM production in inoculated plants may have been favored by the production of growth-promoting substances by bacteria. Ferreira et al. (2011), Ferreira et al. (2010) and Guimarães et al. (2010) also observed significant effect of the inoculation with *H. seropedicae* on the SDM accumulation of rice plants.

For %SN of corn plants, there was significant interaction between corn genotypes, seed inoculation with *H. seropedicae* and nitrogen doses (Table 2). There was no significant difference between corn genotypes without inoculation and nitrogen fertilization (Table 2). In the presence of inoculation with *H. seropedicae* and no nitrogen fertilization, there was lower %SN in the hybrid BRS 3035 compared to the others (Table 2). In the study of Sala et al. (2007), for one of the two tested wheat genotypes without the addition of N fertilizer, plants not inoculated with diazotrophic bacteria had higher N accumulation than those in treatments with inoculation. Araujo et al. (2013) reported that, in the absence of nitrogen fertilizer, inoculation may reduce shoot dry matter of plants. This negative effect may also occur as a result of an increase of the root system at the expense of the shoot development. An explanation for this is that one of the benefits provided by diazotrophic bacteria for grass crops is the capacity to produce phytohormones (Dobbelaere et al., 2001, 2002; Mendonça et al., 2006; Sala et al., 2007).

In the presence of nitrogen fertilization and inoculation with *H. seropedicae*, increases of 26.8% and 36.2% in %SN of the hybrids P3646H and BRS 3035 was verified, respectively, when fertilized and inoculated with *H. seropedicae* (Table 2). However, opposite results were obtained for the Maximus hybrid for both %SN and TSN (Table 2).

The TSN did not differ statistically between corn hybrids for the treatment without nitrogen fertilization with and without inoculation. Nevertheless, in the presence of nitrogen fertilization, the inoculation with *H. seropedicae* caused an increase of approximately 32% in the TSN of P3646H and 62% of BRS 3035 compared to the treatment with fertilization and without inoculation with *H. seropedicae* (Table 2). Some reports reinforce that variable responses of inoculation with endophytic diazotrophic bacteria are common in grass crops which has justified investments to improve this technology (Sala et al., 2007). Even in controlled environments as in the greenhouse, discrepant responses of the association between diazotrophic bacteria and corn cultivars have been frequent which has probably limited the consolidation of commercial inoculants (Dobbelaere et al., 2002).

The percentage of nitrogen in the shoot coming from the fertilizer (%SNF) differed only between corn hybrids while the shoot nitrogen content coming from the fertilizer (SNF) and shoot N use efficiency (SNUE) were influenced by the interaction hybrid x inoculation with *H. seropedicae* (Table 3).

The lowercase letters match the hybrid effect, capital letters compared the effect of inoculation and the letters in italic compared the effect of nitrogen. Same letters do not differ by Tukey test at 5% probability. Hybrid 1

Table 2. Shoot nitrogen percentage (%SN) and total shoot nitrogen (TSN) of three corn genotypes inoculated or not with *Herbaspirillum seropedicae* under different nitrogen levels. Dourados, MS, Brazil (2013).

Nitrogen levels	%SN												
		H1				H2				H3			
N_0	SI	1.10	a	A	*b*	1.14	a	A	*b*	0.90	a	A	*b*
	CI	1.07	a	A	*b*	1.09	a	A	*b*	0.76	b	A	*b*
N_{540}	SI	2.09	a	A	*a*	1.86	a	B	*a*	1.38	b	B	*a*
	CI	1.54	c	B	*a*	2.36	a	A	*a*	1.88	b	A	*a*
	TSN (mg plant^{-1})												
		H1				H2				H3			
N_0	SI	33.76	a	A	*b*	48.20	a	A	*b*	50.12	a	A	*b*
	CI	64.78	a	A	*b*	60.19	a	A	*b*	62.49	a	A	*b*
N_{540}	SI	144.60	a	A	*a*	181.40	a	B	*a*	151.00	a	B	*a*
	CI	118.54	b	A	*a*	238.59	a	A	*a*	246.55	a	A	*a*

Table 3. Analysis of variance of shoot nitrogen percentage coming from the fertilizer (%SNF), shoot nitrogen coming from the fertilizer (SNF) and shoot N use efficiency (SNUE) of three corn hybrids subjected to different nitrogen levels and inoculated with *Herbaspirillum seropedicae.* Dourados, MS, Brazil (2013).

Source of variation	GL	Mean square		
		%SNF	SNF	SNUE
Hybrid (H)	2	18.32*	12129.35*	415.95*
Inoculation (I)	1	3.81	9100.66	312.09*
H*I	2	1.67	6157.04*	211.14*
Residue	18	2.44	1350.96	46.32
CV (%)		1.77	23.11	23.11

* – significant by Tukey test to 5% probability. CV- coefficient of variation.

(Maximus), Hybrid 2 (P3646H) and Hybrid 3 (BRS 3035); SI (without inoculation) CI (inoculation).

The %SNF was higher for P3646H with percent increases of 2.8 and 3.1% compared to the hybrids Maximus and BRS3035, respectively (Figure 3). In average, 89.8% of total N accumulated in the shoot of P3646H comes from the fertilizer. On the other hand, SNF differed between corn hybrids and in the presence and absence of inoculation with *H. seropedicae*. There was no significant difference ($p \leq 0.05$) between corn hybrids without inoculation with *H. seropedicae* while the hybrids P3646H and BRS3035 showed higher SNF in the presence of inoculation with *H. seropedicae*, both differing from the treatment without inoculation (Table 4). This increase in SNF when inoculated with *H. seropedicae* may have occurred due to the production of auxins by the bacteria which stimulates root growth and increases the explored volume of soil contributing to the increase in the amount of nutrient absorbed.

Shoot N use efficiency (SNUE) which refers to the use of N fertilizer in relation to the amount of N applied as a fertilizer differed between corn hybrids and in the presence and absence of inoculation with *H. seropedicae* (Table 4). There was no significant difference ($p \leq 0.05$) for SNUE between corn hybrids without inoculation with *H. seropedicae* while the hybrids P3646H and BRS 3035 showed higher SNUE in the presence of inoculation with *H. seropedicae*, with percentage increases of about 34.3 and 64.4% compared to the treatment without inoculation (Table 4). Opposite results were found for the hybrid Maximus regarding SNF and SNUE. This hybrid showed decrease of 22.11% in SNF and SNUE when compared to the treatment inoculated with *H. seropedicae* (Table 4). Most studies show that there is great variation in the use of N fertilizers by plants, rarely exceeding 50% of the applied. In this study, the relatively high use by the corn hybrids compared to the treatments with and without inoculation with *H. seropedicae* is probably related to the conditions in the pot, where the root system remains confined, exploring the entire volume of soil and also

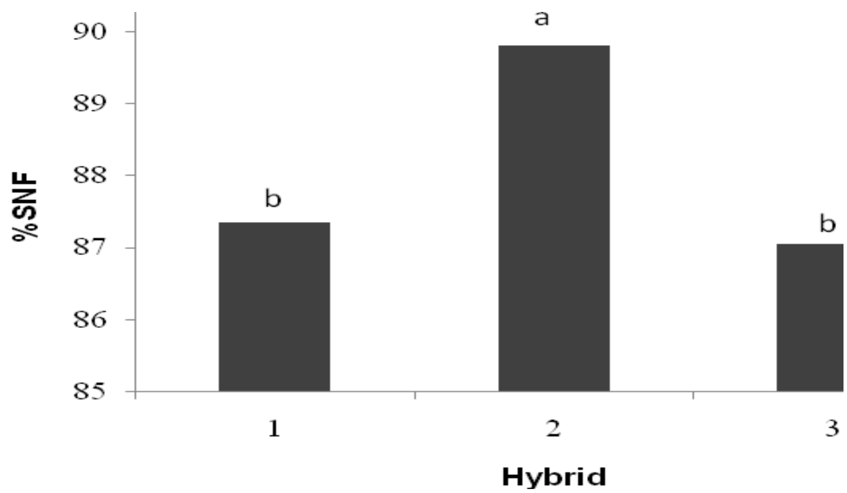

Figure 3. Shoot nitrogen percentage coming from the fertilizer (%SNF) of three corn genotypes. Hybrid 1(Maximus), Hybrid 2 (P3646H) and Hybrid 3 (BRS 3035).

Table 4. Shoot nitrogen coming from the fertilizer (SNF) and shoot nitrogen use efficiency (SNUE) of three corn genotypes inoculated or not with *Herbaspirillum seropedicae*. Dourados, MS, Brazil (2013).

	SNF (mg plant^{-1})						SNUE (%)					
	H1		H2		H3		H1		H2		H3	
SI	126.46	aA	161.25	aB	131.03	aB	23.41	aA	29.86	aB	24.26	aB
CI	103.57	bA	216.59	aA	215.41	aA	19.17	bA	40.11	aA	39.89	aA

without N leaching losses to other layers out of roots' reach. Similar results were obtained by Brito et al. (2011) evaluating the contribution of nitrogen biological fixation, nitrogen fertilizer and soil nitrogen to the development of cowpea and common beans.

The lowercase letters match the hybrid effect and capital letters compared the effect of inoculation. Same letters do not differ by Tukey test at 5% probability. Hybrid 1 (Maximus), Hybrid 2 (P3646H) and Hybrid 3 (BRS 3035); SI (without inoculation) CI (inoculation).

Conclusions

There is a distinction between corn genotypes regarding shoot dry matter production and shoot nitrogen percentage coming from the fertilizer. The shoot dry matter production is influenced by the inoculation with *H. seropedicae*. The inoculation with *H. seropedicae* in the presence of nitrogen fertilization promotes increases of approximately 32% in the amount of nitrogen in the shoot of the hybrid P3646H and 62% of the hybrid BRS 3035.
The hybrids P3646H and BRS3035 showed increases of 34.3 and 64.4%, respectively in nitrogen use efficiency when inoculated with *H. seropedicae* with no addition of nitrogen, what allows to infer is that the BNF behaves as an important source of N to the system.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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