Revista
Brasileira de
Ciência do Solo

Division – Soil Use and Management | Commission – Soil Fertility and Plant Nutrition

Nitrate Reductase Activity and Nitrogen and Biomass Accumulation in Sugarcane under Molybdenum and Nitrogen Fertilization

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ABSTRACT: Prior research on sugarcane showed increment of productivity following molybdenum (Mo) fertilization and some indication of the potential role either on nitrate reductase activity (NRA). However, this increment can depend on sugarcane variety and nitrogen (N) fertilization. This study aimed to evaluate the effect of Mo fertilization on Mo uptake, on NRA, nitrate (NO_3^--N), and ammonium (NH_4^+-N) contents in leaves and roots of sugarcane, and the accumulation of N and biomass across two sugarcane varieties (RB 867515 and RB 92579). The varieties were subjected to two rates of N fertilization (without N fertilization and 60 kg ha^{-1} of N) and two rates of Mo fertilization (without Mo fertilization and 200 g ha^{-1} of Mo). At 70, 100, 130, 200, and 365 days after planting (DAP), the following parameters were determined: Mo, NO_3^--N and NH_4^+-N contents, NRA in the leaves and roots, and the accumulation of N, as well as shoot dry matter (SDM). Molybdenum fertilization increased N accumulation in 36 and 44 % in the varieties RB 867515 and RB 92579, respectively. This increase in N accumulation was associated with NRA stimulation of 13 % in the leaves and 42 % in the roots. The maximum NRA occurred around 100 DAP, and its contents in the leaves and roots of the RB 92579 were 0.83 and 0.46 μmol NO_2^- g^{-1} h^{-1} of fresh matter, respectively, and in RB 867515 these values were 0.61 and 0.43 μmol NO_2^- g^{-1} h^{-1} of fresh matter, respectively. The assimilation of NO_3^--N was increased with Mo application, both with and without N fertilization, suggesting that Mo fertilization is indicated in nutrition management of sugarcane. This study provides insight into how Mo fertilization interacts with N fertilization and sugarcane varieties, altering N accumulation and dry matter production.

Keywords: *Saccharum officinarum*, Mo content, N-Nitrate content, N assimilation, N-Ammonium content.

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Received: August 07, 2018
Approved: March 01, 2019

How to cite: Santos RL, Freire FJ, Oliveira ECA, Freire MBGS, West JB, Barbosa JA, Moura MJ, Bezerra PC. Nitrate reductase activity and nitrogen and biomass accumulation in sugarcane under molybdenum and nitrogen fertilization. Rev Bras Cienc Solo. 2019;43:e0180171.
https://doi.org/10.1590/18069657rbcs20180171

INTRODUCTION

Brazil is a traditional sugarcane (*Saccharum* spp) producer, responsible for 33 % of global sugarcane production (Jadoski et al., 2010). The N requirements of sugarcane are high, which can range from 150 to 325 kg ha^{-1} in many commercial varieties of sugarcane in the first crop cycle (Oliveira et al., 2010).

The high N requirement by the sugarcane, however, does not result in high rates of retention of applied mineral fertilizers. In typical sugarcane production, there are significant losses in the soil-plant-atmosphere system that cause the recommended doses of N fertilizers to be high (Franco et al., 2011). On average, 60 to 100, 150 to 400, and about 160 kg ha^{-1} of N per year are applied in Brazil, India, and Australia, respectively (Franco et al., 2011; Hirel et al., 2011). Franco et al. (2011) reported that the N plant derived from fertilizer (NDFF) contributed up to 40 % of the total N in the first crop cycle at initial stages of development. The magnitude of this contribution decreased during stages of maturity to approximately 10 % of total N at harvest. Nitrogen fertilizer use by sugarcane as low as 20 % have been reported (Franco et al., 2011). Strategies that could increase N fertilizer use efficiency by sugarcane, for example through higher N uptake and assimilation, would have important consequences for agricultural practices and the impacts of agriculture on surrounding ecosystems (Bell, 2014).

Both nitrate (NO_3^--N) and ammonium (NH_4^+-N) are accessed by sugarcane, with greater absorption of NH_4^+-N form, provided the plants are well nourished in N (Robinson et al., 2011); however, in aerated soils, NO_3^--N appears to be the most used mineral N form by mass concentration effect, or even, the sugarcane plants do not discriminate one or other mineral form, especially when the plants show low nutrition in N (Robinson et al., 2011; Liu et al., 2014). When NO_3^--N is absorbed, it needs to be reduced to NH_4^+-N in the root plastids or leaf chloroplasts, prior to assimilation and use in the formation of amino acids and proteins. The nitrate reductase enzyme activity is essential in this process, since this enzyme is responsible for the conversion of NO_3^--N to nitrite (NO_2^--N) (Hirel et al., 2011). Therefore, if nitrate reductase activity (NRA) is low, N assimilation would be expected to be reduced and the absorbed NO_3^--N normally is storage at the cell vacuoles (Wang et al., 2012).

Molybdenum is necessary for the Mo-molybdopterin cofactor in nitrate reductase that forms the active site of the enzyme (Mendel, 2007). Thus, when a plant is deficient in Mo, especially when NO_3^--N prevails in the soil, N assimilation can be reduced, with consequent reduction of plant growth and yield (Kaiser et al., 2005).

Early studies on micronutrients in sugarcane in Brazil, such as by Alvarez et al. (1979), indicated significant responses to Mo fertilization, leading to increases in stalk yield of about 19 Mg ha^{-1}. The effect of the interaction between N and Mo fertilization on sugarcane yield was evaluated by Oliveira (2012), who observed positive increments in stalk yield. These yield increases by Mo fertilization may be due to the potential role of Mo either on NRA and/or on enzyme nitrogenase activity of endophitic microorganisms in sugarcane that contribute to biological N fixation (BNF) (Taulé et al., 2012).

Although these results are promising, only a few studies have been developed to evaluate the NRA response to Mo fertilization, or the relationship between NRA to the accumulation of N and biomass. Shoot N accumulation in sugarcane was evaluated by Silveira and Crocomo (1990), who observed that the plants accumulated more N with the increase of the NRA. Li-Ping et al. (2007) studied the effects of Mo on NRA in sugarcane in a hydroponic system and observed that NRA in the leaves increased as Mo concentration increased.

We hypothesize that Mo fertilization by stimulating NRA contributes to reducing N fertilization, but depends on the variety of sugarcane. The behavior of the variety influences the accumulation of N and dry matter, but it depends on the interaction between N and Mo fertilizations. In this sense, this study aimed to evaluate NRA, Mo, and N (NO_3^--N and NH_4^+-N) content in the leaves and roots of two commercial varieties of sugarcane, and to quantify the accumulation of biomass and N as a function of the fertilization of Mo in plants fertilized and not with N, during the first crop cycle of the sugarcane (plant cane).

MATERIALS AND METHODS

Soil characterization

A field experiment was carried out at the Sugarcane Experimental Station of Carpina, in the municipality of Carpina, Pernambuco State, Brazil (7° 51’ 04” S; 35° 14’ 27” W; 184 m a.s.l.). According to Köppen’s classification system, the predominant climate in the experimental site is As’, tropical rainy with dry summers, and average annual temperature of 24.2 °C (Beltrão et al., 2005). The rainfall that occurred during the experiment was 1,359 mm. In addition, in the first two months after sugarcane planting, three water irrigations of 25 mm were applied.

The soil from experimental site are classified by the Brazilian Soil Classification System as *Argissolo Amarelo distrocoeso* (Santos et al., 2013), which corresponding to hardsetting Ultisol (Soil Survey Staff, 2014). Soil chemical characterization was performed for two soil layers: 0.00-0.20 and 0.20-0.40 m and 15 samples per depth were collected in the experimental area to form a composite sample (Table 1).

For soil chemical characterization, the following properties were determined: pH(H_2O), Ca^{2+}, Mg^{2+}, K^+, Na^+, Al^{3+}, (H+Al), P, Fe, Cu, Zn, Mn, Mo, NO_3^--N, NH_4^+-N, and TOC (total organic carbon). Exchangeable Ca, Mg, and Al were extracted using KCl 1.0 mol L^{-1}

Table 1. Soil chemical properties in different layers in the field experiment area at Sugarcane Experimental Station of Carpina, Brazil

Property	Layer	
	0.00-0.20 m	0.20-0.40 m
pH(H_2O)	5.20	4.80
Ca^{2+} ($cmol_c$ dm^{-3})	2.10	1.80
Mg^{2+} ($cmol_c$ dm^{-3})	0.50	0.60
K^+ ($cmol_c$ dm^{-3})	0.15	0.06
Na^+ ($cmol_c$ dm^{-3})	0.04	0.07
Al^{3+} ($cmol_c$ dm^{-3})	0.50	0.70
(H+Al) ($cmol_c$ dm^{-3})	5.50	7.50
P (mg dm^{-3})	19.00	4.00
Fe (mg dm^{-3})	55.40	41.20
Cu (mg dm^{-3})	0.10	0.30
Zn (mg dm^{-3})	2.50	2.40
Mn (mg dm^{-3})	6.60	5.00
Mo (mg dm^{-3})	0.14	0.15
TOC (g kg^{-1})	1.19	1.25
NO_3^--N (mg dm^{-3})	56.14	65.22
NH_4^+-N (mg dm^{-3})	20.02	20.64

pH in water at a ratio of 1:2.5 v/v; Ca^{2+}, Mg^{2+}, and Al^{3+} were extracted using KCl 1.0 mol L^{-1}; K^+, Na^+, P, Fe, Cu, Zn, Mn, and Mo were extracted using Mehlich-1; potential acidity was extracted with a $Ca(C_2H_3O_2)_2$ solution 0.5 mol L^{-1} and determined through titration; total organic carbon was determined by wet combustion with K dichromate and measured through titration; nitrate and NH_4^+-N were extracted with KCl 1.0 mol L^{-1} and determined through titration after steam distillation using the Kjeldahl method, according to Tedesco et al. (1995).

and determined through titration. Phosphorous, K^+, Na^+, Fe, Cu, Zn, Mn, and Mo were extracted using Mehlich-1; P and Mo (dosage by the $KI+H_2O_2$ method) determined through spectrophotometry UV-VIS, K^+, and Na^+ through flame photometry and Fe, Cu, Zn, and Mn through atomic absorption spectrophotometry. Potential acidity (H+Al) was extracted with a $Ca(C_2H_3O_2)_2$ solution 0.5 mol L^{-1} and determined through titration. Total organic carbon was determined by wet combustion with K dichromate and measured through titration. All analyses were performed according to the methodologies described by Donagema et al. (2011).

Nitate and NH_4^+-N were extracted with KCl 1.0 mol L^{-1} and determined through titration after steam distillation using the Kjeldahl method, according to Tedesco et al. (1995).

Experimental arrangement

The sugarcane varieties RB 92579 and RB 867515 were planted. These varieties were chosen because they are commonly cultivated in the Northeast region of Brazil (Chapola et al., 2013). The RB 92579 and RB 867515 are of medium and late maturity, respectively. The RB 867515 can be used in low fertility soils due to its high efficiency in nutrient use (Simões Neto et al., 2005). The RB 92579 shows low nutrient efficiency due to high nutritional requirement (Oliveira et al., 2010).

The experiment consisted of the application to the soil of two Mo soil levels (without application and fertilization with 200 g ha^{-1} of Mo) and two N soil levels (without application and fertilization with 60 kg ha^{-1} of N). The doses of N and Mo used were reported by Oliveira (2012) as recommended for yields equal to or greater than 100 Mg ha^{-1} of stems. Molybdenum and N forms applied to the soil were Na molybdate and urea, respectively. Before planting the sugarcane, the soil was plowed and fertilization with urea into the soil made possible that urea-N was rapidly transformed to NO_3^--N after its hydrolysis by action from soil urease enzymes. According to Lorensini et al. (2014), NO_3^--N availability increases after 38 days of application. The fertilizers were applied at sugarcane planting, as basal fertilization. Sodium molybdate was diluted in water and applied to the soil using a backpack sprayer to increase the efficiency of the low-dose application.

The experiment was composed of 32 plots. Each plot constituted of seven 10-m-long furrows, one meter apart between sugarcane rows, totaling 70 m^2. The plot useful area was formed by the five central furrows, disregarding one meter to each side (border), totaling 40 m^2. The two outer furrows of the useful area were used for destructive sampling and the three central ones for non-destructive sampling.

The sugarcane planting in the experimental site took place 40 days after a broadcast application of 2.2 Mg ha^{-1} of dolomitic limestone in the entire area and incorporation to a depth of 0.2 m. The need for liming was calculated using the base saturation method (Ribeiro et al., 1999), estimating an increase of base saturation to 60 %. Phosphorous and K were also applied as basal fertilization in all plots. Phosphorous fertilization was performed considering the available-P content and soil texture, or P-rem, according to Simões Neto et al. (2015), using 26.2 kg ha^{-1} of P corresponding to approximately 60 kg ha^{-1} of P_2O_5 as triple superphosphate (40 % of P_2O_5). For K, the recommendation was based on the expected production. Since yields above 100 Mg ha^{-1} were expected, an average K efficiency value of 1.2 kg Mg^{-1} of sugarcane was used (Oliveira et al., 2010), yielding, application rate of 120 kg ha^{-1} of K, corresponding to approximately 140 kg ha^{-1} of K_2O as K chloride (60 % of K_2O).

The sugarcane varieties were planted in furrows, approximately 0.2 m deep, where 14 Mg ha^{-1} of 7-month-old stalks were distributed in a double row arrangement so that the tip of one coincided with the base of another. The stalks were cut into sections called "setts", maintaining a density of 14 buds per meter. Then, fertilizers were applied over the seed stalks, and the furrows were covered with a thin layer of soil.

Plant sampling and measurements

The NRA and N and biomass accumulation were evaluated in the sugarcane varieties at 70, 100, 130, 200, and 365 days after planting (DAP), according to the phenological phases of growth of sugarcane. Molybdenum, NO_3^--N, and NH_4^+-N contents were determined in the first fully expanded leaf (leaf +1) [first leaf with a visible point of separation between the leaf blade and sheath called "dewlap", also called the TVD ("Top Visible Dewlap")] and roots. Molybdenum content was determined at 100 DAP and NO_3^--N and NH_4^+-N contents at 130 DAP. We chose to determine Mo content at 100 DAP because it was the period of greatest NRA. The NO_3^--N and NH_4^+-N content was determined at 130 DAP because between 100 and 130 DAP is the period of higher demand for N of sugarcane (tillering and elongation of the stem) (Jadoski et al., 2010).

The NRA was determined *in vivo*, according to the methodology suggested by Santos et al. (2018). Three leaves +1 and a portion of roots of each variety were randomly sampled. These samplings occurred always from 9:30 am to 12:30 pm, with 45 min reserved for the sampling of each block. The sampled plant parts were protected with aluminum foil, placed in insulated boxes with ice and then taken to the laboratory for NRA determination. In the light absence, 0.25 g of leaf tissue discs (middle third, removing the midrib) and 0.5 g of roots were incubated, in 5 mL of a solution composed of K_2HPO_4 0.1 mol L^{-1}, KNO_3 0.1 mol L^{-1}, n-propanol at 1 %, and an adhesive spreader at 0.01 %, for 1 h at 25 °C, after vacuum of 30 s. After incubation, 1-mL aliquots were collected, and then 5 mL of 0.5 % sulfanilamide solution were added to HCl 0.75 mol L^{-1} and 0.01 % N-ethylenediamine dihydrochloride (N-naphthyl) and 4 mL of deionized water. After 15 min of reaction, nitrite was determined in spectrophotometer at the wavelength of 540 nm and determining NRA in μmol NO_2^- g^{-1} h^{-1} of flesh matter.

For the determination of Mo, NO_3^--N, and NH_4^+-N contents in the leaf +1 and roots of the varieties, three additional leaves +1 and a portion of roots of each plot were randomly collected. For this, the collected plant tissues were dried in a forced-air oven at 65 °C and ground in a knife mill. For the Mo evaluation, plant tissues were digested in nitric-perchloric solution and Mo content determined in the spectrophotometer UV-VIS in 420 nm, according to the methodology proposed by Silva (2009). Molybdenum was dosed by the catalytic method ($KI+H_2O_2$). In this method, the iodide is oxidized to I_2 with H_2O_2 in acid medium. The amount of I_2 formed is proportional to the Mo concentration.

Nitrogen inorganic forms (NO_3^--N and NH_4^+-N) in the leaf +1 and roots were extracted with KCl 1.0 mol L^{-1} and determined by titration after Kjeldahl steam distillation, according to the procedure suggested by Tedesco et al. (1995).

For the determination of the N accumulation and production of shoot dry matter (SDM) in the pre-established periods, three plants (stalks+leaves) per useful plot area and 10 plants during end harvest (365 DAP) were sampled. The biomasses of the collected plant shoots were weighed and crushed into forage material, from which a subsample was taken for drying. The subsamples were dried in a forced-air oven at 65 °C, until constant mass. With the dry mass of the biomass subsamples, SDM production was estimated and expressed in grams per plant. For total-N content determination, the dried biomass subsample was subjected to sulfuric digestion and the extract to steam distillation, according to the methodology proposed by Silva (2009). Nitrogen accumulation was obtained from SDM and total-N content.

Statistical analyses

The experimental data were analyzed after checking their normal distribution and homoscedasticity of variance. An analysis of variance (ANOVA) was applied to the full factorial design, using an F test ($p<0.05$). When significant, the sugarcane varieties and the levels of Mo and N, and its interactions were compared by the Tukey test ($p<0.05$). The measurements repeated over time were subjected to regression analysis for each sugarcane varieties and level of Mo and N. Subsequently, the select models were those with significance on the coefficients of the parameters of highest degree by t test ($p<0.05$), concomitantly with largest coefficient of determination (R^2).

RESULTS

Molybdenum, NO_3^--N, and NH_4^+-N contents in the leaves +1 and roots

There is no relationship between Mo contents in roots and leaves +1 of sugarcane (Table 2). In general, Mo content in the roots were higher than in the leaves +1, increasing, in average, from 0.40 to 0.99 mg kg^{-1} of Mo for RB 867515 and from 0.59 to 0.90 mg kg^{-1} of Mo for RB 92579.

The Mo content of both leaves +1 and roots increased when Mo was applied, regardless of sugarcane variety and N fertilization (Table 2). The Mo content in leaves +1 was not influenced by the addition of N or by genotype. However, in the roots, Mo content was affected by N fertilization and depended on the sugarcane variety. Nitrogen fertilization increased Mo levels in the roots only in the variety RB 867515, which contained 30 % more Mo than the variety RB 92579 (Table 2).

As well as the results of NRA, there is no relationship between NO_3^--N in roots and leaves +1 of sugarcane (Table 3). In general, the NO_3^--N content in the leaves +1 of the sugarcane

Table 2. Molybdenum content in the leaves and roots of the sugarcane varieties RB 867515 and RB 92579, with and without Mo and N fertilization

Factor	RB 867515			RB 92579		
	Mo[1]		Average	Mo		Average
	Without	With		Without	With	
			mg kg^{-1}			
			Leaf			
Without N	0.37	0.43	0.40	0.37	0.44	0.40
With N	0.38	0.40	0.40	0.37	0.45	0.41
Average	0.37 b	0.40 a	0.41	0.37 b	0.44 a	
		F calculated			F calculated	
Mo		6.36 ($p<0.05$)			16.68 ($p<0.01$)	
N		0.32			0.01	
Mo*N		1.06			0.05	
CV (%)		8.92			8.31	
			Roots			
Without N	0.62	0.79	0.70 B	0.72	0.93	0.82
With N	1.10	1.44	0.89 A	0.89	1.05	0.97
Average	0.86 b	1.11 a		0.80	0.99	
		F calculated			F calculated	
Mo		21.10 ($p<0.01$)			2.48	
N		97.92 ($p<0.001$)			1.47	
Mo*N		2.05			0.10	
CV (%)		11.39			6.05	

(1) Digestion using nitric-perchloric solution. Values followed by different lowercase letters in line and uppercase in column are significantly different at $p<0.05$, according to the Tukey test.

Table 3. Nitrate and NH_4^+-N content in the leaves and roots of the sugarcane varieties RB 867515 and RB 92579, with and without Mo and N fertilization

Factor	**NO_3^--N content(1)**					
	RB 867515			**RB 92579**		
	Mo		**Average**	**Mo**		**Average**
	Without	**With**		**Without**	**With**	
	mg kg^{-1}					
			Leaf			
Without N	179.75 Ab	362.22 Aa	270.99	226.85 Aa	116.67 Ba	171.76
With N	112.63 Ab	210.00 Ba	161.32	190.56 Aa	303.33 Aa	246.95
Average	146.19	286.11	216.15	208.71	210.00	209.35
		F calculated			F calculated	
Mo		117.54 ($p<0.001$)			0.75	
N		64.92 ($p<0.001$)			7.29 ($p<0.05$)	
Mo*N		32.69 ($p<0.001$)			11.63 ($p<0.01$)	
CV (%)		3.17			6.29	
			Roots			
Without N	347.84	357.78	352.81 A	222.22 Aa	46.67 Bb	134.45
With N	236.93	210.00	223.47 B	70.00 Ba	93.33 Aa	81.67
Average	292.39	283.89	288.14	146.11	70.00	108.06
		F calculated			F calculated	
Mo		0.59			52.81 ($p<0.001$)	
N		35.76 ($p<0.001$)			7.23 ($p<0.05$)	
Mo*N		1.25			111.87 ($p<0.001$)	
CV (%)		2.67			3.80	
			NH_4^+-N content (mg kg^{-1})(1)			
Factor	RB 867515			RB 92579		
	Mo		Average	Mo		Average
	Without	With		Without	With	
			Leaf			
Without N	243.70	280.00	261.85	245.86	221.67	233.77
With N	251.05	326.67	288.86	216.48	221.67	219.07
Average	247.38	303.33	275.35	231.17	221.67	226.42
		F calculated			F calculated	
Mo		3.88			0.32	
N		0.17			0.42	
Mo*N		0.46			0.15	
CV (%)		4.18			4.55	
			Roots			
Without N	232.91	256.67	244.78	185.59	163.33	174.46 B
With N	257.39	295.56	276.47	327.31	291.67	309.49 A
Average	245.14	276.11	260.62	256.45 a	227.50 b	241.97
		F calculated			F calculated	
Mo		0.66			9.43 ($p<0.05$)	
N		0.27			196.95 ($p<0.001$)	
Mo*N		0.12			0.03	
CV (%)		5.4			1.41	

(1) Nitrogen inorganic forms (NO_3^--N and NH_4^+-N) were extracted with KCl 1.0 mol L^{-1} and determined by titration after Kjeldahl steam distillation, according to the procedure suggested by Tedesco et al. (1995). Values followed by different lowercase letters in line and uppercase in column are significantly different at $p<0.05$, according to the Tukey test.

varieties was higher than in the roots, increasing in average 9.7 % for RB 867515 and 93.7 % for RB 92579 (Table 3).

Molybdenum fertilization increased the NO_3^--N content in the leaves +1 of the variety RB 867515, regardless fertilization or not with N. In the roots, Mo fertilization had no effect and N fertilization decrease NO_3^--N content (Table 3).

In the leaves +1 of the variety RB 92579, N fertilization only increased the NO_3^--N content when Mo was applied. In the roots, Mo fertilization reduced by 4.8 times the NO_3^--N content when there was no N fertilization (Table 3).

The effect of the N fertilization on the NO_3^--N content in the roots of the variety RB 92579 was strongly dependent on the presence of Mo. There was a large increase in NO_3^--N content in the roots with Mo fertilization for this variety (Table 3).

The NH_4^+-N content in the leaves +1 and roots of the two sugarcane varieties were not different (Table 3). Molybdenum fertilization reduced the NH_4^+-N content only in the roots of the RB 92579, while N fertilization increased the NH_4^+-N content in the same variety.

NRA in the leaves +1 and roots

The NRA in the leaves +1 and roots of the sugarcane varieties fit a sinusoidal model, so it is possible to select the period of the sugarcane plant cycle in which NRA reached maximum activity (Figures 1 and 2). The influence of Mo application in the sugarcane varieties with and without N fertilization was therefore evaluated in this period coinciding with maximum enzyme activity.

The NRA in the leaves +1 and roots of the sugarcane varieties increased at 50 DAP, reaching maximum activity around 100 DAP and then decreased, reaching stabilization around 130 DAP (Figures 1 and 2).

In general, maximum NRA in the leaves +1 was higher than in the roots, on average 0.72 μmol NO_2^- g^{-1} h^{-1} of flesh matter, compared to 0.44 μmol NO_2^- g^{-1} h^{-1} of flesh matter in the roots (Figures 1 and 2). Nitrogen fertilization reduced the NRA in the leaves +1 and roots and the reduction was greater in the roots of the variety RB 92579. However, in the roots of variety RB 867515, N fertilization increased NRA in the absence of Mo application (Figures 1 and 2).

Molybdenum fertilization stimulated NRA in the leaves +1, mainly in variety RB 867515 regardless of N fertilization (Figure 1). In the roots, this effect only occurred in the treatments without N fertilization. The Mo fertilization in the variety RB 867515 reduced NRA in roots when N was applied, and in the RB 92579, the N fertilization reduced NRA with and without Mo fertilization (Figure 2). Molybdenum fertilization reduced the time necessary for NRA to reach the peak of maximum activity in the leaves +1 for the variety RB 867515 with N fertilization and in the RB 92579 without N addition (Figure 1). However, in the roots of the variety RB 92579, the combined application of Mo and N significantly delayed the time for NRA reach the peak of its activity (Figure 2).

Nitrogen and SDM Accumulation

Nitrogen and SDM accumulation in the first crop cycle (plant cane) fit the sigmoid model for plant growth (Figure 3 and 4). In general, N accumulation in both sugarcane genotypes, without and with N, was higher with Mo fertilization (Figure 3). The increases in the N accumulation with Mo fertilization were higher in RB 867515 with N fertilization and in RB 92579 without N fertilization. Nitrogen fertilization increased N accumulation in RB 867515, but not in RB 92579. The highest increases of N in RB 867515 and RB 92579 through the Mo fertilization were, respectively, 47 and 60 %. This behavior was associated with the stimulation of NRA (Figures 1 and 2).

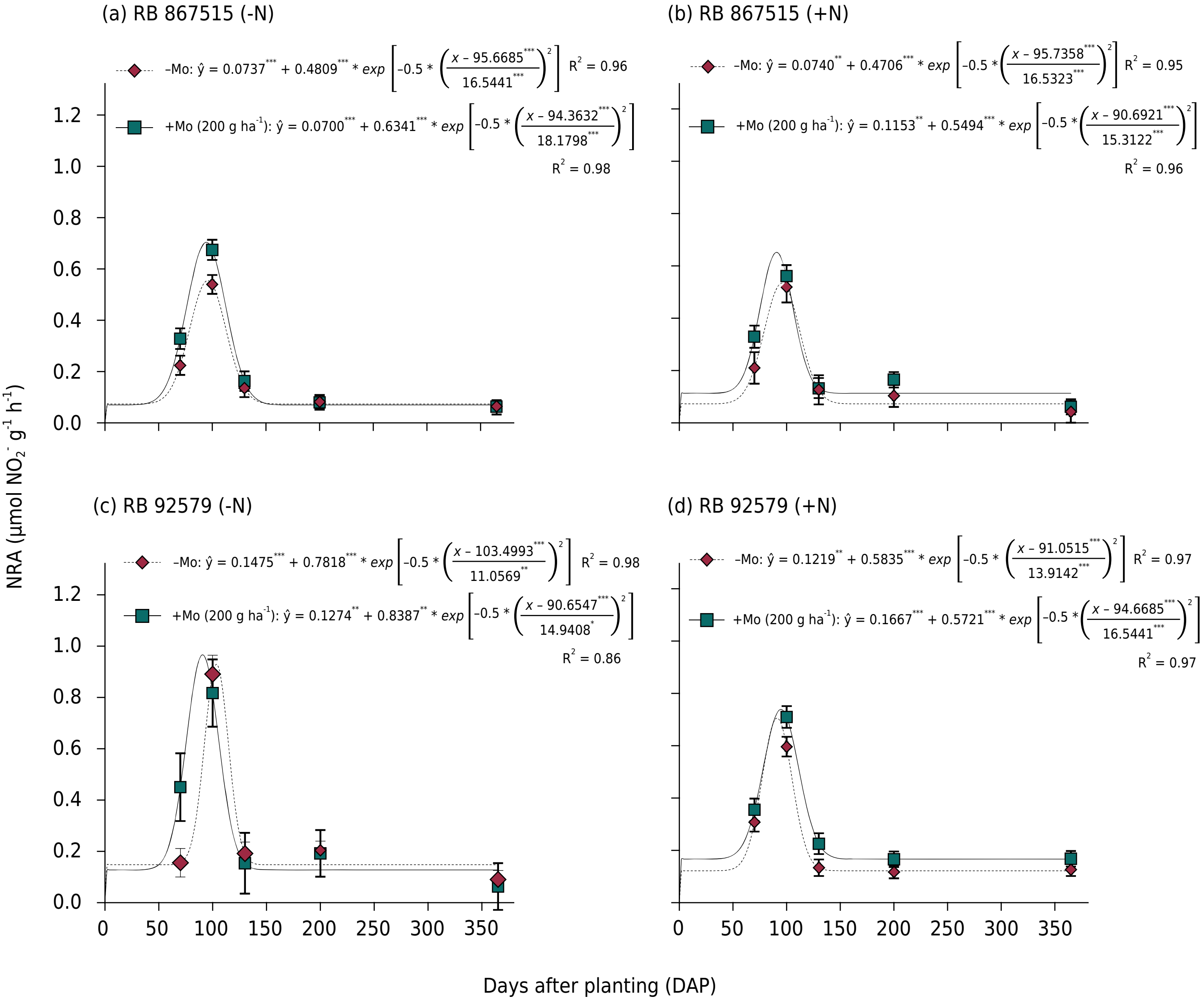

Figure 1. Nitrate reductase activity (NRA) in the leaves of sugarcane - Mo and +Mo (200 g ha^{-1}) fertilization for the variety RB 867515, -N and +N (60 kg ha^{-1}) fertilization (a and b, respectively), and for the variety RB 92579, -N and +N fertilization (c and d, respectively) at 70, 100, 130, 200, and 365 days after planting (DAP).

In general, SDM of both sugarcane varieties was higher when N was applied, with a more pronounced response by RB 92579 (Figure 4). At the end of the crop cycle, the gains of SDM by RB 867515 and RB 92579 through N fertilization were, in average, 10 and 53 %, respectively.

Molybdenum fertilization did not increase SDM in the variety RB 867515, either with or without N fertilization (Figure 4). However, Mo increased SDM production when there was no N fertilization at variety RB 92579. Shoot dry matter production was 25 % higher with Mo fertilization and without N fertilization.

DISCUSSION

Molybdenum, NO_3^--N, and NH_4^+-N content in the leaves +1 and roots

Gopal et al. (2016) studying the effect of Mo stress on growth, yield, and seed quality in black gram report that the values of Mo content of sufficiency and threshold of toxicity in leaves were 0.078 and 2.15 µg g^{-1} (dry matter). In our study, the content of Mo varied from

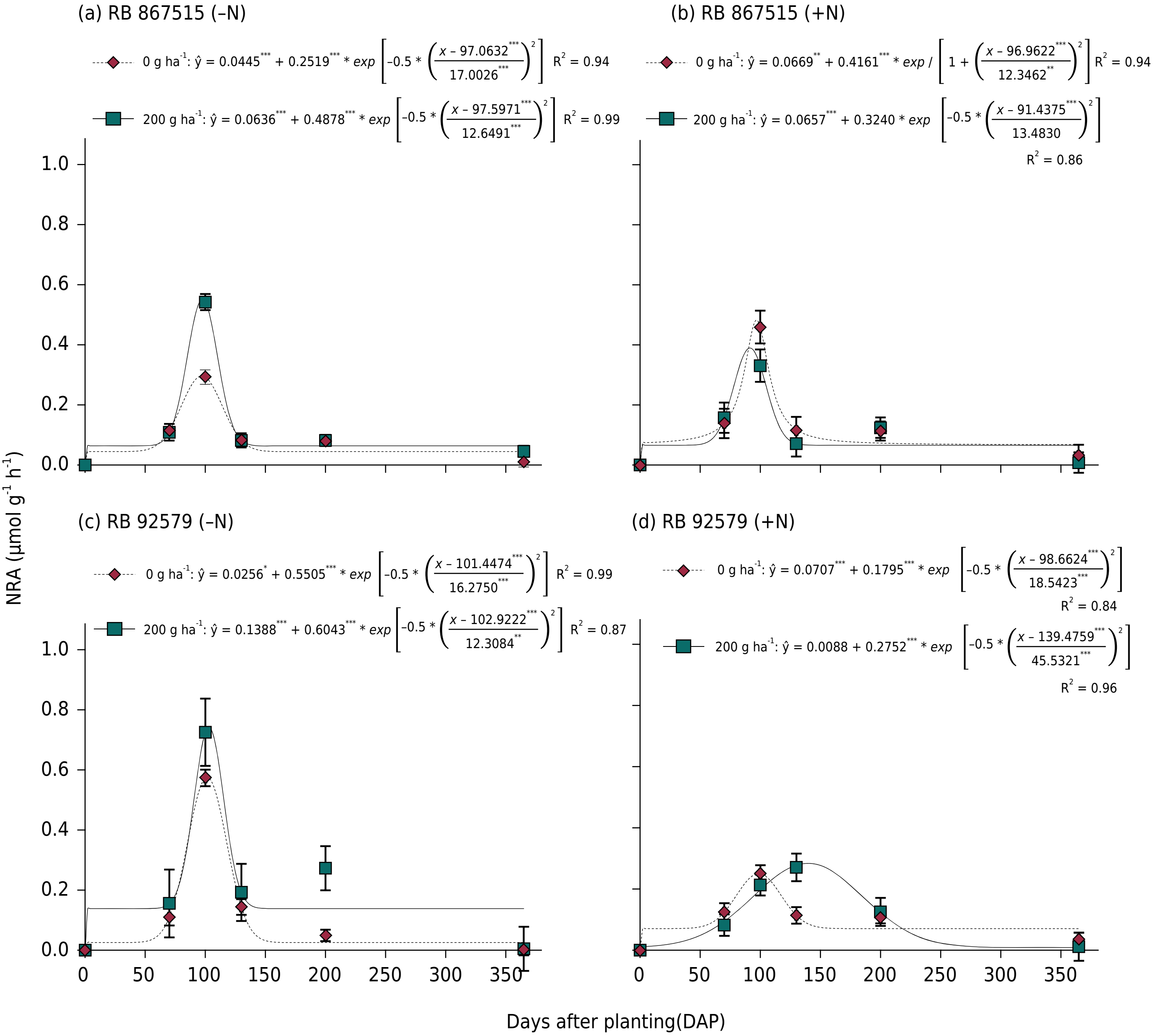

Figure 2. Nitrate reductase activity (NRA) in the roots of sugarcane - Mo and +Mo (200 g ha^{-1} of Mo) fertilization for the variety RB 867515, -N and +N (60 kg ha^{-1} of N) fertilization (a and b respectively), and for the variety RB 92579, -N and +N fertilization (c and d respectively) at 70, 100, 130, 200, and 365 days after planting (DAP).

0.36 to 0.67 mg kg^{-1} in leaves and from 0.63 to 1.43 mg kg^{-1} in roots. More Mo in roots than in leaves of corn was also found in a study conducted by Kovács et al. (2015). A Mo content variation of 0.07 to 0.27 mg kg^{-1} was found by Brennan and Bolland (2007) in a grain of wheat.

Molybdenum content in the leaves in both varieties increased in response to Mo addition, and there was no difference between varieties in this response, nor there was any effect of N fertilization on leaf Mo content. Root Mo content was highly responsive to Mo and N addition, and this responsiveness (at least to N addition) was dependent on variety. This suggests that sugarcane NRA is concentrated in the roots and that the availability of both Mo and N strongly modulate the uptake and subsequent reduction of NO_3^--N by roots in sugarcane (Mantelin and Tourani, 2004).

The accumulation of NO_3^--N in the leaves increased with Mo fertilization, and the NRA did not metabolize the accumulated amounts of NO_3^--N. It is possible that the

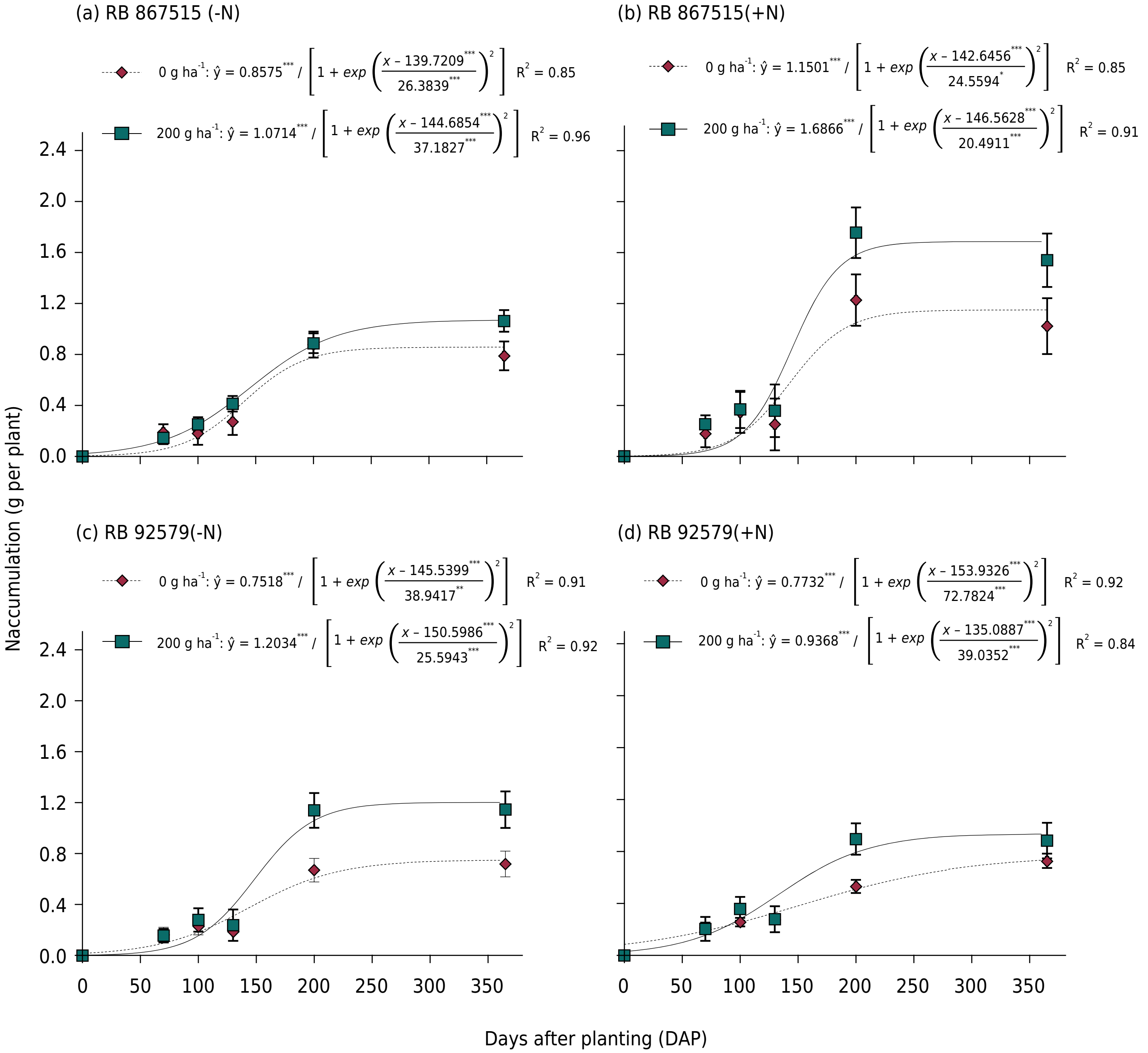

Figure 3. Nitrogen accumulation in the shoots of sugarcane -Mo and +Mo (200 g ha^{-1}) fertilization for the variety RB 867515, -N and +N (60 kg ha^{-1}) fertilization (a and b, respectively), and for the variety RB 92579, -N and +N fertilization (c and d, respectively) at 70, 100, 130, 200, and 365 days after planting (DAP).

rate of Mo used was not enough to potentiate the NRA in the leaves, influencing its activity (Li-Ping et al., 2007). In fact, Mo content in the leaves was lower than in the roots. The low Mo content did not stimulate the NRA because Mo is necessary for the Mo-molybdopterin cofactor in nitrate reductase (Mendel, 2007). In addition, the metabolism of N assimilation in the roots is lower (Oaks and Hirel, 1985) and the NRA may to metabolize all the NO_3^--N of this compartment. However, greater accumulation of NO_3^--N may not necessarily mean lower NRA.

The increase of the NO_3^--N content caused by N fertilization, which occurred only in the variety RB 92579 in the presence of Mo, must have been due to the increase of the N uptake in the NO_3^--N form. In fact, even with Mo stimulating NRA (Kaiser et al., 2005), it was not enough to metabolize the amount of NO_3^--N absorbed (Biscaro et al., 2011).

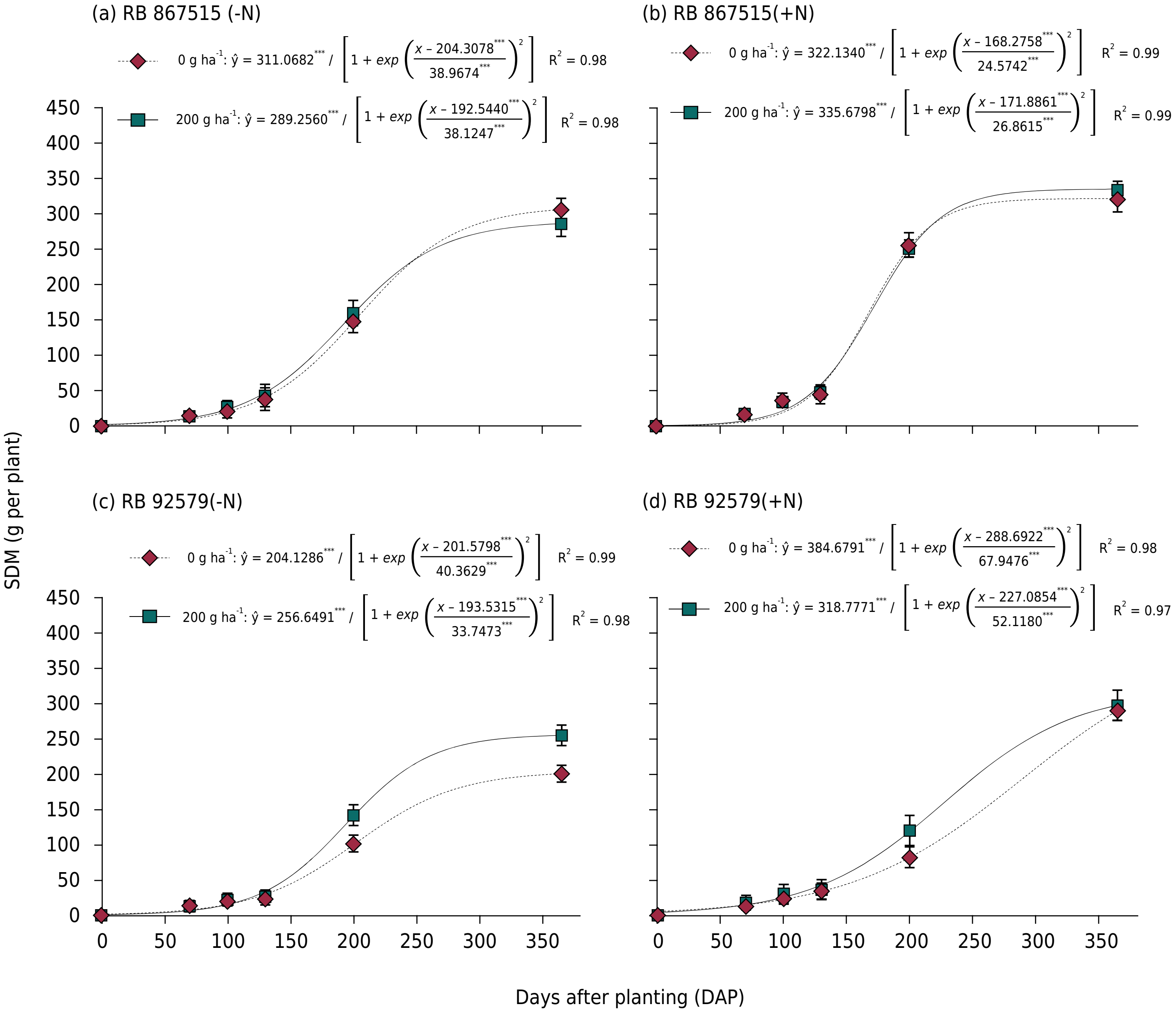

Figure 4. Shoot dry matter (SDM) of sugarcane - Mo and +Mo (200 g ha^{-1} of Mo) fertilization for the variety RB 867515, -N and +N (60 kg ha^{-1} of N) fertilization (a and b, respectively), and for the variety RB 92579, -N and + N fertilization (c and d, respectively) at 70, 100, 130, 200, and 365 days after planting (DAP).

The N fertilization was performed with urea, which can increase NH_4^+-N content in soil in its first days after N fertilization, but tends to oxidize into NO_3^--N over time, increasing its content (Lorensini et al., 2014). Evaluating the transformations of N fertilizers, Gargantini and Catani (1957) observed that 10 days after application, 77 and 9 % of urea had been converted into NH_4^+-N and NO_3^--N, respectively; at 40 days, they already represented 80 and 20 %, respectively. Therefore, this indicates that there may be significant NO_3^--N increases with urea application throughout the sugarcane cycle.

More intense nitrification of N from urea was observed by Lorensini et al. (2014). According to these authors, the highest NH_4^+-N content occurred from the zero time to 38 days after incubation and then decreased. The NO_3^--N content increased from 9 to 150 mg kg^{-1} at 38 days after incubation, i.e., in this period, NO_3^--N availability was increased in 16.7 times, corresponding to 30 times the value of NH_4^+-N. The rapid hydrolysis of urea was attributed to this behavior, releasing NH_4^+-N, which then was oxidized into NO_2^--N by the bacteria of the genus *Nitrobacter*, and into NO_3^--N by bacteria of the genus *Nitrosomonas* (Lorensini et al., 2014).

Nitrogen fertilization without Mo application did not interfere significantly with NO_3^--N content, both in the leaves and in the roots at RB 867515. When Mo was added, it reduced the NO_3^--N content only in the leaves of the RB 867515. The rapid increase in soil NH_4^+-N content when urea was applied in the sugarcane (Gargantini and Catani, 1957) can have been sufficient for a higher NH_4^+-N uptake by RB 867515, compared with NO_3^--N. In this case, the reduction of NO_3^--N was possibly more due to the higher NH_4^+-N uptake than to a possible higher NRA. Therefore, the RB 867515 managed to absorb NH_4^+-N in the short period in which this N form prevailed in the soil, suggesting high capacity to absorb NH_4^+-N of the variety (Oliveira et al., 2010).

The difference in NH_4^+-N content between the varieties allows suggesting that the preferential metabolic route for N assimilation by RB 867515 is ammoniacal, which would explain the lower NH_4^+-N accumulation in relation to NO_3^--N found in this variety. For RB 92579, the preferential metabolic route seems to be the nitric, evidenced by a significant higher NRA, which would explain the higher NH_4^+-N accumulation in relation to NO_3^--N in this variety. Weigelt et al. (2005), studying preferential uptake of soil N forms by grassland plant species, reported that species-specific differences in direct uptake of different N forms combined with total N acquisition could explain changes in competitive dominance of species in soils of differing fertility.

The Mo addition did not interfere on the NH_4^+-N content in the leaves of the varieties. Molybdenum does not interfere with N assimilation when NH_4^+-N is the form incorporated into amino acids and proteins (Masclaux-Daubresse et al., 2010). Li-Ping et al. (2007) also observed no changes in NH_4^+-N content in the leaves of the varieties RB 72454 and RB 867575 with Mo application.

Nitrogen fertilization did not change NH_4^+-N content in plant tissues. Even for a short period when N addition has caused higher NH_4^+-N availability, instead of NO_3^--N, there is a subsequent natural predominance of NO_3^--N in soil (Lorensini et al., 2014), which can explain the non-interference of N fertilization in NH_4^+-N content of the shoots.

NRA in the leaves +1 and roots

The NRA was higher in the leaf +1 than in the roots, despite the higher Mo content observed in the roots. We expected a direct relationship between Mo content and NRA (Li-Ping et al., 2007), but our results suggest a more complex relationship between NRA and Mo contents in sugarcane plant tissues. This difference in Mo content between leaves and roots may have been influenced by the higher biomass production from leaves than from roots, effectively diluting Mo content in the leaves. Our results are similar to those observed by Kaiser et al. (2005) in chardonnay grapes. Otto et al. (2009) in another research work with plant cane evaluated shoot and dry root phytomass produced by sugarcane. According to the authors, the average shoot dry mass production was 57 Mg ha^{-1} and, in the roots, it was 2.5 Mg ha^{-1}, i.e., shoot phytomass was about 23 times higher than root phytomass.

We observed that the peak of the NRA occurred around 100 DAP and stabilization at a relatively low rate by around 130 DAP across roots and leaves and for all N and Mo treatments. Silveira and Crocomo (1990) reported peak NRA at 120 DAP and decreasing during the rest of the crop cycle. These results suggest strong coordination between NRA and phenological stages. In the period of tillering and initial growth, there is a high demand for N (Jadoski et al., 2010) for assimilation into nitrogenous compounds. Another aspect that made NRA different in the leaves and in the roots was the time for NRA to reach its maximum expression. While in the leaves, this peak was reached on average with 94.5 days, in the roots it only occurred around 103 days, i.e., approximately 10 days after.

On average, the maximum NRA in the leaves and roots for RB 92579 was higher than for RB 867515. Maximum NRA in the leaves and roots for RB 92579 was 0.83 and 0.46 μmol NO_2^- g^{-1} h^{-1} of flesh matter, respectively. For RB 867515, the maximum NRA in the leaves and roots was 0.61 and 0.43 μmol NO_2^- g^{-1} h^{-1} of flesh matter, respectively. Prior reports have shown that NRA can vary with sugarcane genotypes (Hemaprabha et al., 2013). In addition, greater contents of NO_3^--N in plant tissues tend to be associated with higher enzyme activity in the plant (Robinson et al., 2011; Wang et al., 2011). Traditionally, the uptake of NO_3^--N by plants may be greater when there are favorable soil conditions for nitrification (Wang et al., 2012). However, there are reports of greater uptake of NH_4^+-N in sugarcane, with potentially large differences among varieties (Armas et al., 1992; Robinson et al., 2011). According to the study of Armas et al. (1992), for example, when the Cuban sugarcane variety Ja605 received nutrient solution for 45 days with NH_4^+-N, the fresh biomass was 27 % higher compared with sugarcane receiving the NO_3^--N.

In a study using a hydroponic system, with ^{15}N-enriched fertilizers ($^{15}NH_4NO_3$ and/or $NH_4{}^{15}NO_3$), Robinson et al. (2011) observed that when the sugarcane varieties (Q138, Q157, and Q179) had contact with the solution containing high concentration of N with similar proportions of NO_3^--N and NH_4^+-N for 24 h, a higher proportion of NH_4^+-N was taken up, when the sugarcane plants showed a good nutrition with N. According to these authors, the greater incorporation of NH_4^+-N, instead of NO_3^--N, was about five times higher in the roots and from four to ten times higher in the tillers. The greater uptake NH_4^+-N by sugarcane varieties was also shown in field experiments conducted by Robinson et al. (2011). After soil pit opening and in situ incubation of the root system for 30 and 120 min, in nutrient solution containing ^{15}N-enriched fertilizer, the authors observed that, after 30 min there were not any difference between sugarcane that received or not N fertilization. After 120 min only roots of sugarcane plants that received N fertilizer shown greater incorporation of NH_4^+-N.

Robinson et al. (2011) reported that the greater absorption of NH_4^+-N in sugarcane occurs only in plants with adequate N nutrition, even coexisting both mineral-N forms in the soil. It is necessary, however, using other methodologies, to advance in these indications, which can be very important to the N nutrition of these varieties, largely cultivated in Northeast of Brazil.

In an experiment conducted by Armas et al. (1992), it was observed that when sugarcane was subjected to an environment with only NH_4^+-N, the NRA in the leaf of the plants was reduced, representing only 45 % of the activity in plants cultivated with NO_3^--N. In this experiment, NRA evaluations were performed in six-month-old plants that received a nutrient solution of N in either cationic or anionic form at each 45 days, in a substrate composed of vermiculite and peat. This is consistent with our observation here that NRA is responsive to soil N availability, particularly when the added N is not likely to increase the soil NO_3^--N pool.

The effect of Mo application on NRA in the leaves of the variety RB 867515 without N addition was higher than when was added N. This was possibly due to a higher N uptake in the form of NO_3^--N without N fertilization, i.e., in soils not fertilized with N, the effect of Mo on NRA was more pronounced. The higher the soil NO_3^--N content and its uptake, which is more common in unfertilized soils (Wang et al., 2011), the higher the enzyme activity in sugarcane tissues, as observed.

Nitrogen and SDM accumulation

We observed greater N accumulation in both varieties and in both N-fertilized and unfertilized conditions with the addition of Mo. This suggests that Mo availability limits the ability of sugarcane to assimilate N. Glass et al. (2012) reported that Mo availability can limit NO_3^--N assimilation when NH_4^+-N is scarce and showed that this limitation manifests in suppressed enzyme activity and protein content. Further, the increase in N accumulation from Mo application was due directly to its function as an activator of NRA (Mendel, 2007). Therefore, these results confirm the importance of the adoption of Mo fertilization practices to increase the efficiency of the N assimilation by sugarcane. Higher NRA causing higher N accumulation in sugarcane was also observed by Silveira and Crocomo (1990).

The results from Oliveira (2012) also corroborate our observations. According to the author, the components of the shoots of the sugarcane showed a tendency to accumulate N with Mo fertilization. The highest increments in the accumulation of de N were observed when RB 92579 was cultivated with 200 g ha^{-1} of Mo, having accumulated 135 % more N than plants that were not fertilized with Mo. Molybdenum fertilization also increased N accumulation in RB 72454 in another previous study (Polidoro, 2001) where the application of up to 200 g ha^{-1} of Mo increased N accumulation in the shoots of sugarcane across N fertilization levels (including unfertilized).

Although dry matter accumulation in function of N and Mo application has not been commonly reported for grasses like sugarcane or corn, there are some reports showing enhancement in the literature. Santos et al. (2010) evaluated the effect of different periods for N and Mo fertilization in corn. According to those authors, dry matter production was not affected by the application of 90 g ha^{-1} of Mo, regardless of the N availability. However, Brennan and Bolland (2007) observed that wheat yield was increased with Mo application in acid soils in Australia. The increase in grain yield was obtained with double the recommended dose of Mo, without the correction of soil pH. Oliveira (2012) also reported positive increments in sugarcane stalk yield when applied 60 kg ha^{-1} of N combined with 200 g ha^{-1} of Mo.

The difference of increase in the SDM production as an effect of Mo application in the sugarcane genotypes indicates varietal specificity (Weigelt et al., 2005), especially when there is low N availability in soil. It is possible that RB 92579 absorbs more of the Mo available in the soil when fertilized with N, because of a larger root system allowing exploration of a larger soil volume (Otto et al., 2009). In a condition of low soil availability of N, and RB 92579 having absorbed more Mo, NRA was stimulated, leading to higher N assimilation by the sugarcane and, consequently, to a higher dry matter production (Kaiser et al., 2005). Interactions between biomass allocation (e.g., above versus below ground) and Mo-linked nitrate reductase activities should be explored further, particularly with respect to potentially large differences between sugarcane varieties.

Our results suggest that RB 92579 has higher Mo demand since its nutritional demand was apparently not met by the pre-existing content in soil. This variety responded positively to Mo fertilization, accumulating more SDM, which did not occur for RB 867515 and showed larger increases in N accumulation in response to Mo addition. This higher demand can be due to the higher potential for biomass production of RB 92579 compared with RB 867515. This can be evidenced in the study of Oliveira et al. (2011), which evaluated these two genotypes cultivated in two water regimes. According to these authors, the RB 92579 was more productive than RB 867515, both in rainfed and irrigation conditions, with a difference of about 80 Mg ha^{-1} of stalks with higher water availability. Our results, however, suggest that this high potential productivity is strongly limited by the availability of soil nutrients, since under Mo- and N-limiting conditions, RB 92579 accumulated only 65 % of the dry matter accumulated by RB 867515 in the same conditions.

CONCLUSION

Molybdenum application increased Mo content in the leaves and roots of sugarcane and stimulated nitrate reductase activity (NRA). The maximum NRA occurred around 100 days after planting (DAP) of sugarcane, proving to be the best period for nutritional studies on N. The maximum NRA was higher in RB 92579 than in RB 867515. Molybdenum application increased N accumulation in the RB 867515, regardless of N fertilization and in the RB 92579 when no added N. Molybdenum fertilization is indicated as a technical procedure in the crop nutrition management. Shoot dry matter (SDM) accumulation by the sugarcane varieties had different responses to the Mo application in the absence of N fertilization, with RB 92579 producing more dry matter in the presence of Mo. Nitrogen fertilization increased the accumulation and production of SDM of the sugarcane varieties, especially when RB 92579 was not fertilized with Mo. Interactions between biomass allocation and Mo-linked nitrate reductase activities should be explored further, particularly with respect to potentially large differences between sugarcane varieties. This study provides insight into how Mo fertilization interacts with N fertilization and sugarcane varieties, altering N accumulation and dry matter production.

ACKNOWLEDGMENTS

The authors would like to thank the National Council for Scientific and Technological Development (CNPq), for granting the scholarships, and the scholars and volunteers, for their help in conducting the experiment and collecting the data. This study was supported by CNPq Project No. 471575/2009-7 entitled “Evaluation of the availability of molybdenum to sugarcane and its contribution to nitrogen fertilization and nitrogen fixation”.

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