Influence of $CaCl_2$ on the Foliar Biomass and Quality of Tobacco Leaves

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The aim of the present work was to determine the influence of different $CaCl_2$ dosages on foliar biomass and quality of tobacco plants (*Nicotiana tabacum* L. var. Tennesse 86). Plants were grown under controlled conditions and submitted to regular fertilization with macro- and micronutrients. The $CaCl_2$ was applied to the nutrient solution at 1.25 mM (T1), 2.5 mM (T2), and 5 mM (T3). The results indicated that, under the experimental conditions of this work, the application of 1.25 mM of $CaCl_2$ favored the growth and development of the leaves, this leading to improved biomass production in tobacco leaves. The increase in foliar biomass in treatment T1 could largely be a result of the stimulation of NO_3^- assimilation. However, this situation has negative consequences for tobacco quality, given that the T1 treatment augmented the NO_3^- concentration and the foliar concentration of nicotine (both effects being harmful for human consumption) and decreased the concentration of reducing sugars in leaves of tobacco plants compared with those of T2 and T3. Finally, a close and directly proportional relationship was found in our experiment between the parameters of foliar-biomass production, total N concentration, chlorophyll concentration, and decline in quality.

Keywords: *Ca^{2+}; Cl^-; alkaloids; biomass; quality; Nicotiana tabacum; nitrate reduction*

INTRODUCTION

Insufficient N substantially reduces the yield of tobacco, a conclusion recently confirmed by Balachandran et al. (*1*), who reported a sharp decline in foliar biomass and number of leaves, as well as the appearance of chlorosis, under N-deficient conditions. In fact, many reports show the close positive relationship in tobacco plants between yield and N fertilization as well as between yield and foliar N content (*2*−*7*). In addition, Mackown and Sutton (*6*) found the chlorophyll concentration in tobacco leaves to have a highly significant and positive relationship both with the foliar N content and with the final yield.

Tobacco quality, which is a complex combination of visual, physical, and chemical characteristics, is strongly influenced by the N fertilization (*8*). The tobacco leaves frequently show high NO_3^- concentrations derived from excessive fertilization with this form of N. Normally, in tobacco, NO_3^- accumulates when the absorption of this anion exceeds its capacity for reduction and assimilation (*2*). The NO_3^- levels in tobacco plants have a marked effect on chemical composition, because increased nitrous oxide, formation of volatile carcinogenic compounds, and presence of carcinogenic nitrosamines specific to tobacco are directly related to the content of this anion in these plants (*9, 10*). In short, with increased N fertilization the foliar levels of NO_3^- and nicotine rise while the sugar concentration falls, thus seriously diminishing the quality of the tobacco (*3, 11*).

Among the factors known to affect yield and quality in tobacco, the most striking appear to be the N source, the genotype used, and the availability of certain nutrients. Of these nutrients, Ca^{2+} appears to have a major impact in tobacco cultivation. For example, Sims et al. (*12*) observed that an incremental soil application of Ca^{2+} from 0 to 380 Kg/ha resulted in gradual increase of the foliar and total biomass, but application of 760 Kg/ha drastically diminished biomass production of the tobacco plants. Similar results have been found by Ruiz et al. (*13*) in tobacco plants, reporting decreased foliar biomass production after applications of high dosages of Ca^{2+} (5 mM). On the other hand, when the N source is in the form of NO_3^-, Ca^{2+} acts mainly to inhibit nitrate reductase (NR) activity (*13*−*15*). Furthermore, when Ca^{2+} is applied as $CaCl_2$, a reduction may occur in NO_3^- uptake and translocation toward the aerial part, together with a inhibition of NR activity, these effects being caused by the action of the Cl^- ion (*13, 16*−*18*).

In short, according to the results of the works cited above, it is logical to conclude that $CaCl_2$ can influence foliar biomass production as well as quality in tobacco, given the close relationship between $CaCl_2$, nitrogen metabolism, and tobacco performance. For this reason, the aim of the present work was to evaluate, under controlled cultivation conditions, the influence of different $CaCl_2$ dosages on yield and quality in tobacco plants (*Nicotiana tabacum* L. var. Tennesse 86).

MATERIALS AND METHODS

Growth Conditions. Seeds of *Nicotiana tabacum* cv Tennesse 86 were sown in September 1999. The seedlings were grown in individual pots of peat in an experimental greenhouse in southern Spain (Granada) for 45 days and then transferred to a cultivation chamber under controlled environmental conditions with relative humidity of 60−80%, temperature 30/20 °C (day/night), and 16/8 h photoperiod at a PPFD of 350 μmol $m^{-2}s^{-1}$ (measured at the top of the plants with a 190 SB

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quantum sensor, LI-COR Inc., Lincoln, NE). The plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height) of 8 liters volume, filled with vermiculite. For one month (from day 45 until day 75 after sowing), before the experimental treatments, the plants received a nutrient solution of 6 mM KNO_3, 2 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1.5 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 5 μM H_3BO_3. The nutrient solution (pH 5.5 to 6.0) was renewed every 3 days.

Experimental Design. The treatments were started at day 75 after sowing by applying different levels of $CaCl_2$: considering the initial level 1.25 mM (T1), 2.5 mM (T2), and 5 mM (T3). The experimental design was a randomized complete block with three treatments, arranged in individual pots with six plants per treatment, and three replications.

Leaf Sampling. The plants were sampled beginning at the 14-leaf stage, just before the onset of flowering. From the same plants, leaves were subjected to two sampling dates: at the first one, day 105 after sowing, leaves were picked from the nodes 10 and 11. The second sampling, two weeks after, leaves from the nodes 12 and 13 were picked. All the sampled leaves were in the mature state with lengths of more than 10 cm. The material was rinsed three times in distilled water after disinfecting with nonionic detergent at 1% (*19*), then blotted on filter paper. The leaves from the nodes 10 and 12 were used fresh for the analysis of chlorophyll concentration, nitrate reductase activity, and carbohydrates concentration. Those picked from the nodes 11 and 13 were dried in a forced air oven at 70 °C for 24 h, and were used for the analysis of nitrates, total N, and alkaloids. Dry weight was recorded and expressed as g dry weight (dw)/leaf.

Plant Analysis. *Chlorophyll Determination*. To measure and express chlorophylls, we followed the procedure and used the equations described by Wellburn (*20*).

Detection of in vivo NR Activity. The basic method was an adaptation of the in vivo NR assay by Jaworski (*21*) and Mauriño et al. (*22*). Leaves were cut into 5-mm sections and the sample (0.5 g) was placed in 10 mL of incubation buffer (100 mM potassium phosphate buffer, pH 7.5) and 1% (v/v) propanol. The sample was infiltrated and the intracellular spaces of the tissues were flushed with buffer, using a vacuum of 0.8 bar. After 10 min, the vacuum was released and the samples were reevacuated. The samples were incubated at 30 °C in darkness for 1 h and placed in a bath of boiling water to stop the NR activity. The resulting NO_3^- was measured by the method of Snell and Snell (*23*), and the NR activity was expressed as μmol NO_3^- g^{-1} fresh weight (fw) h^{-1}.

Carbohydrates. Carbohdrates were measured following the method of Irigoyen et al. (*24*); appropriate adaptations were made for our plant material. A sample of 0.1 g of fresh matter was homogenized twice with 95% ethanol (v/v) and washed with 70% ethanol (v/v), and then centrifuged at 1500*g* for 15 min. Glucose, fructose, and sucrose were determined in the resulting supernatant by spectrophotometry at 650 nm, using the colorimetric assay with anthrone reagent. The concentrations of reducing sugars (glucose + fructose + sucrose) analyzed were expressed as mg g^{-1} fw.

Analysis of NO_3^--N and Cl^-. These were analyzed from an aqueous extraction of 0.2 g of dried (70 °C) and ground leaf material in 10 mL of filtered water (Millipore, Bedford, MA). For NO_3^- -N determination, a 100-μL aliquot was taken and added to 10% (w/v) salicylic acid in 96% sulfuric acid, and the NO_3^- -N concentration was measured by spectrophotometry as performed by Cataldo et al. (*25*). Cl^- was determined in an aqueous extraction (*25*), and was measured by titration with $AgNO_3$, according to the procedure of Koltoff and Kurada (*26*). The results were expressed as mg g^{-1} dw.

Total N and Ca Determination. A 0.1-g dw sub-sample was digested with sulfuric acid and H_2O_2 according to Wolf (*19*). After dilution with deionized water, a 1-mL aliquot of the digest was added to the reaction medium which contained buffer [5% potassium sodium tartrate, 100 μM sodium phosphate, and 5.4% (w/v) sodium hydroxide], 15%/0.03% (w/v) sodium salicylate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37 °C for 15 min and total N was measured by spectrophotometry according to Baethgen and Alley (*27*). Total Ca^{2+} was analyzed by atomic-absorption spectrophotometry (*28*). The results were expressed as mg g^{-1} dw.

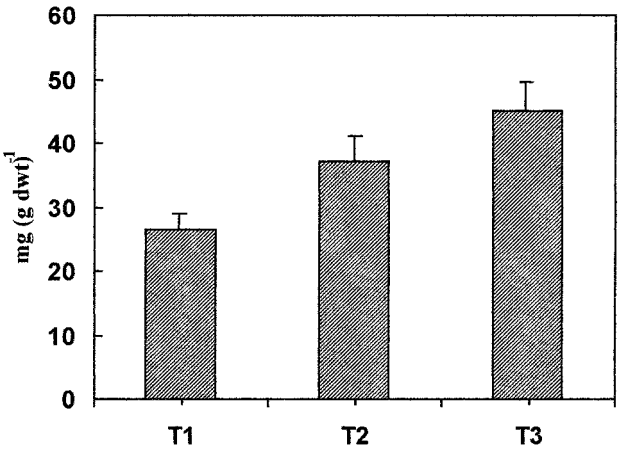

Figure 1. Effect of $CaCl_2$ treatments (T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$) as a mean of 6 tobacco plants on total Ca^{2+} in mg (g dwt)$^{-1}$ in leaves. SE is presented as T-bars.

Determination of Alkaloids. Nicotine, nornicotine, anatabine, and anabasine were analyzed by capillary electrophoresis, according to Yang et al. (*29*). After extraction of dry material (0.5 g) with an aqueous solution of 1% triethanolamine, the concentrations of the different alkaloids were determined in a solution that contained 100 mM SDS, 6 mM sodium phosphate, and 10 mM of sodium borate at pH 9.5, using wavelengths between 200 and 360 nm in a Perkin-Elmer 270A-HT capillary electrophoresis unit equipped with a UV detector (Beckman DV-6-UV−visible spectrophotometer). The results were expressed as μg g^{-1} dwt.

Statistical Analysis. Standard analysis of variance was used to assess the significance of treatment means. The data are presented as mean values ± standard error (SE). Differences between treatments means were compared using LSD at the 0.05 probability level. Levels of significance are represented by * at $P \leq 0.05$, ** at $P \leq 0.01$, *** at $P \leq 0.001$ and not significant (ns) at $P > 0.05$.

RESULTS AND DISCUSSION

Ca^{2+} and Cl^- Concentrations and Foliar Biomass Production. There were significant differences in Ca^{2+} content in leaves between treatments ($P \leq 0.001$; Figure 1). The Ca^{2+} content increased with increasing rates of supplied $CaCl_2$, T1 showed the lowest Ca^{2+} levels, and in T3 the Ca^{2+} content proved 1.7-fold higher than that for T1. The Cl^- levels in leaves (Figure 2), similar to Ca^{2+}, rose as the $CaCl_2$ application rate increased ($P \leq 0.001$), with treatment T3 leading to the highest foliar concentration.

In tobacco, as in other plants, Ca^{2+} and Cl^-, once taken up by the roots, are rapidly translocated toward the shoot, primarily toward the zones of highest transpiration rate, therefore accumulating in the leaves, as described in different studies (*15, 30*). Similar results in tobacco plants were also reported by Ruiz et al. (*13*) working with the variety Sevilla, suggesting that these two genotypes have a similar distributions of Ca^{2+} and Cl^- in the plant.

In different works on tobacco plants (*12, 13*) there has been observed an inversely proportional relationship between foliar biomass production and increased ap-

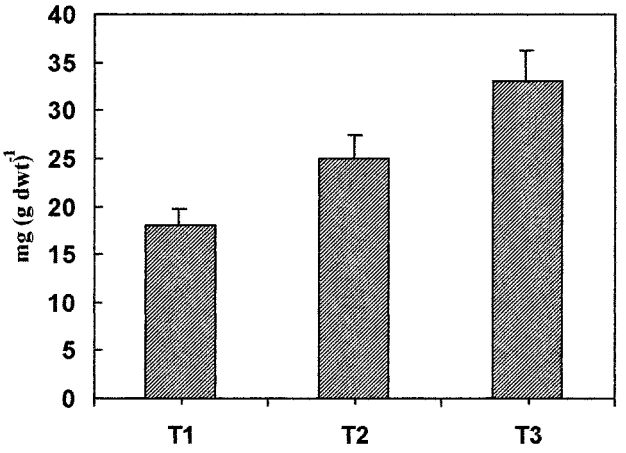

Figure 2. Effect of $CaCl_2$ treatments (T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$) as a mean of 6 tobacco plants on Cl^- in mg (g dwt)$^{-1}$ in leaves. SE is presented as T-bars.

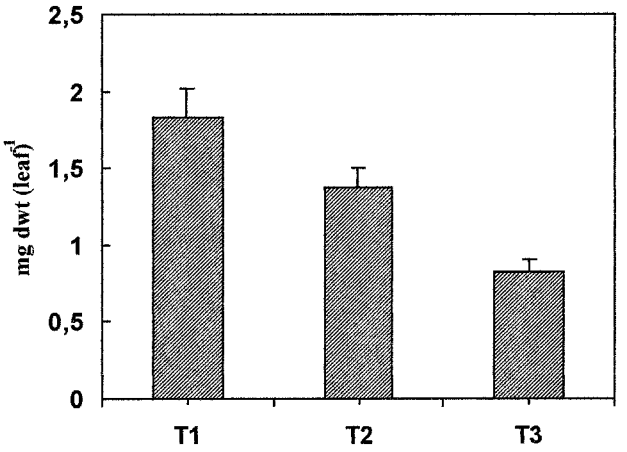

Figure 3. Effect of $CaCl_2$ treatments (T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$) as a mean of 6 tobacco plants on foliar biomass in mg (g dwt)$^{-1}$. SE is presented as T-bars.

plications/foliar concentrations of Ca^{2+} and Cl^-. In our experiment, the highest foliar biomass production was recorded in the T1 treatment, some 123% higher than the lowest value, which was in T3 ($P \leq 0.001$; Figure 3). Therefore, considering our results, we conclude that in the tobacco crop within the levels of $CaCl_2$ tested here, the significant increase in the Ca^{2+} and Cl^- concentrations depressed foliar biomass production (Ca^{2+} concentration − foliar biomass, $r = -0.97$ ***; Cl^- concentration − foliar biomass, $r = -0.96$***), and this could lead to a considerable fall in commercial yield of this crop. The negative effect of the $CaCl_2$ treatments on the biomass production may possibly be due more to the toxicity of the Cl^-, as this element is well-known to be a key factor in the injurious effects of salinity in plants than is the effect of Ca^{2+}.

NO_3^- Assimilation and Chlorophyll Concentration. As indicated in the Introduction, numerous studies have shown close positive relationships in tobacco plants between yield and N fertilizer as well as between yield and foliar-N concentration (*2*−*7*).

When the N fertilizer is applied in the form of NO_3^-, one of the major limitations of NO_3^- assimilation is NR activity (*13, 31*−*35*). In our experiment, the highest in vivo assay values of NR were found at T1, and they wer some 300% higher than the lowest values, which were recorded at T3 (Table 1). One of the principal factors regulating de novo NR synthesis and activity is the presence of NO_3^- (*36, 37*). The NO_3^- concentrations (Table 1) were positively correlated to those of NR activity, with the highest foliar concentrations at T1 and the lowest at T3, thus presenting a directly proportional relationship between the two parameters (r= 0.98***), suggesting the stimulation of NR activity by the change in the NO_3^- concentration, as observed in other works (*38, 39*).

Table 1. Effect of $CaCl_2$ Treatments as Mean ± SE of 6 Tobacco Plants on Nitrates, in Vivo NR Activity, and Total N in Leaves

treatment[a]	nitrate mg g^{-1} of dw	in vivo NR activity μM NO_2^- formed g^{-1} fw h^{-1}	total N mg g^{-1} of dw
T1	6.22 ± 0.43	2.41 ± 0.19	36.8 ± 3.17
T2	4.38 ± 0.29	1.72 ± 0.15	27.3 ± 2.82
T3	2.17 ± 0.24	0.69 ± 0.08	19.5 ± 2.11
significance	***	***	***

[a] T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$.

Both Ca^{2+} and Cl^- may be involved actively in the reduction of NR activity and NO_3^- concentration (in the T3 treatment). First, the NR activity may be inhibited by high concentrations of Ca^{2+} (5−10 mM) and Cl^- (*15, 18*). These two conditions are found in the leaves of the plants treated at the T3 level. Second, in relation to the concentration of NO_3^-, various studies have reported that Ca^{2+} (>5 mM) can inhibit the uptake of NO_3^- in the roots (*13, 17*). In addition, NO_3^- both in the uptake by the root and in the transport within the plant may be influenced significantly by Cl^-, given the antagonism between the two ions (*16, 40*). As reported by others, when the concentration of Cl^- applied to the growth medium is 20 mM, the entry of NO_3^- reduces significantly; on the contrary, at lower Cl^- concentrations (<20 mM), the interaction with NO_3^- occurs principally at the level of translocation toward the aerial part, accumulating anions in the roots (*13, 40*), thereby explaining the lowest foliar concentration of NO_3^- in T3 (Table 1).

Finally, another possible explanation of the low foliar concentrations of NO_3^- in T3 (Table 1) could be by virtue of its reduction to NO_2^- by NR; however, as we confirmed, the NR activity in T3 was the lowest (Table 1), reflecting the negative effect of the $CaCl_2$ dosage on the foliar concentration on NO_3^- (Ca^{2+} concentration − NO_3^- concentration, r = −0.94***; Cl^- concentration − NO_3^- concentration, r = −0.97***).

In general, an increase in NO_3^- assimilation also implies a significant rise in the N content (*41*). Our results confirm this, because the highest total N concentration occurred at T1 and the lowest occurred at T3 (Table 1; NR activity − total N, r= 0.98 ***).

In terms of N metabolism, the plants treated with T1 had the greatest uptake, translocation, and assimilation of NO_3^-, reflecting the highest foliar concentrations of this anion, the highest NR activity, and the greatest foliar concentrations of total N (Table 1). These trends appear to correspond to improved efficiency in the utilization of nitrogenous fertilizers applied in the form of NO_3^-. Meanwhile, the opposite trend characterized the plants treated with T3 (Table 1). Similar results were reported by Ruiz et al. (*13*) working with *Nicotiana tabacum* L. var. Sevilla plants. These authors found that 5 mM of $CaCl_2$ inhibited NO_3^- assimilation and decreased the N concentration as well as foliar biomass. However, the var. Sevilla under similar cultivation

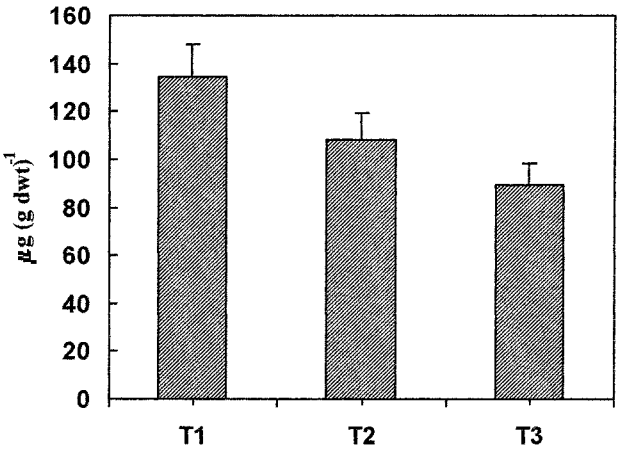

Figure 4. Effect of $CaCl_2$ treatments (T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$) as a mean of 6 tobacco plants on total chlorophyll in μg cm^{-2} in leaves. SE is presented as T-bars.

conditions and with the same $CaCl_2$ treatments as here presented highest efficiency in N utilization under the T2 treatment (2.5 mM $CaCl_2$). These results reaffirm the importance of the genotype in this metabolic process (*42*).

As indicated above, previous works such as that by Mackown and Sutton (*6* have demonstrated that the chlorophyll concentration in tobacco leaves shows a good relationship with the foliar-N concentration and therefore with yield, in this crop. In our experiment, the highest concentrations of total chlorophyll were presented by T1, and the lowest were presented by T3 plants, with the latter falling 33% below the former ($P \leq 0.001$; Figure 4). Therefore, our results support the conclusions by Mackown and Sutton (*6*) as in the present work the relationships found between the concentration of total chlorophyll and the parameters foliar-N concentration and foliar biomass were respectively $r = 0.91$ *** and $r = 0.96$ ****.

Tobacco Quality (Alkaloids and Reducing Sugars). The quality of tobacco is a complex combination of visual, physical, and chemical characteristics (*43*). Notable among the chemical characteristics is NO_3^- concentration and the concentration of alkaloids, principally nicotine (*3*).

The NO_3^- levels in tobacco have a marked effect on the chemical composition of this plant: considering increased nitrous oxide, the formation of volatile carcinogenic compounds, and the presence of specific carcinogenic nitrosamines of tobacco proved to be closely related to the NO_3^- content of the plant (*9*, *10*).

Tobacco leaves frequently show very high NO_3^- concentrations, as this ion normally accumulates when uptake exceeds reduction and assimilation (*2*). In our experiment, although the lowest $CaCl_2$ rates stimulated the NR activity in vivo and therefore the reduction and assimilation capacity of NO_3^-, the foliar concentration of this anion was significantly higher at T1 (Table 1). In short, the application of 1.25 mM of $CaCl_2$, although beneficial for foliar biomass production, could lower the quality of the tobacco because of the foliar accumulation of NO_3^-. The fact that in this treatment the quantity of Ca^{2+} and Cl^- applied via the nutrient solution at the lowest rate possibly favored the uptake of NO_3^-, overwhelming the reduction as well as assimilation capacity and resulting in accumulation, is in accordance with Mackown et al. (*2*).

Table 2. Effect of $CaCl_2$ Treatments as Mean ± SE of 6 Tobacco Plants on Content of Alkaloids in Leaves

treatment[a]	nicotine μg g^{-1} of dw	nornicotine μg g^{-1} of dw	anatabine μg g^{-1} of dw	anabasine μg g^{-1} of dw
T1	4522 ± 31	626 ± 19	442 ± 14	202 ± 8
T2	3350 ± 27	484 ± 17	377 ± 15	141 ± 8
T3	2753 ± 25	393 ± 18	255 ± 12	116 ± 5
significance	***	***	***	***

[a] T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$.

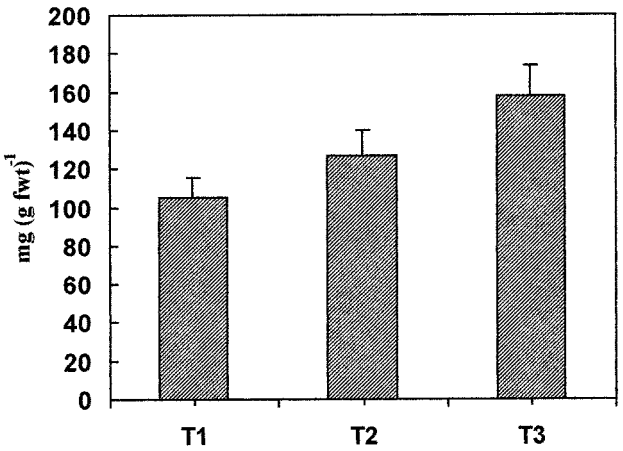

Figure 5. Effect of $CaCl_2$ treatments (T1, 1.25 mM $CaCl_2$; T2, 2.5 mM $CaCl_2$; and T3, 5 mM $CaCl_2$) as a mean of 6 tobacco plants on reducing sugars in mg (g dwt)$^{-1}$ in leaves. SE is presented as T-bars.

Quality of tobacco leaves is also determined by the concentrations of the various alkaloids. Tropane alkaloids are defined as secondary metabolites that contain a N atom in their molecules, given that these are formed initially of the two amino acids, ornithine and arginine, showing the close relationship between N metabolism and alkaloid synthesis (*44*). The most frequent alkaloids in tobacco include nicotine, nornicotine, anatabine, and anabasine, the first being the primary alkaloid, quantitatively as well as qualitatively (*45*). In our experiment, the studied alkaloids nicotine, nornicotine, anatabine, and anabasine had their highest concentrations at T1, respectively being 64, 46, 73, and 74% higher than the lowest concentrations at T3 (Table 2). Our results confirm the close relationship between NO_3^- assimilation and alkaloid concentration, because the regression analysis between NR activity and concentration of the different alkaloids reflects a directly proportional relationship (NR activity − nicotine, $r = 0.92$ ***; NR activity − nornicotine, $r = 0.89$ ***; NR activity − anatabine, $r = 0.89$ ***; NR activity − anabasine, $r = 0.94$ ***).

Finally, another chemical trait often used in judging tobacco quality is reducing sugars (RS), as a greater concentration of RS is regarded as an indication of better smoking quality (*46*). In our experiment, the highest concentrations of RS were found in T3, some 51% higher than the lowest values which were in T1 ($P \leq 0.001$; Figure 5). Our results are consistent with those of other works which have shown that excessive Ca^{2+} concentrations cause sugar accumulation, due primarily to the stimulation of enzymatic activities responsible for the synthesis of these compounds (*30*, *46*). This appears to explain the directly proportional relationship found between the two parameters ($r = 0.97$***). In addition, on the basis of these results, we conclude that the application of 1.25 mM of $CaCl_2$ reduces the quality of

tobacco leaves compared with that acheived at higher application values.

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