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## ***In vitro* multiplication of sugarcane with different nitrogen and sucrose concentrations**

### **Multiplicação *in vitro* de cana-de-açúcar com diferentes concentrações de nitrogênio e sacarose**

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#### **Abstract**

Sugarcane is of great economic importance worldwide. Hence, there is great interest in the development of efficient techniques for the production of good quality, homogeneous, disease- and pest-free seedlings. One way to eliminate viruses is through meristem culture. To guarantee success during meristem culture, it is necessary to optimize the components and concentrations of the culture medium. Sucrose is an essential component in the culture medium, serving as a source of carbon and energy for the seedlings. Nitrogen, in turn, is essential in plants because it is a component of biomolecules and countless enzymes. This work evaluates the best combination of nitrogen (75, 100, 125, and 150%) and sucrose (0, 20, 30, and 40 g L<sup>-1</sup>) concentrations in the *in vitro* multiplication and development of sugarcane cultivar RB036091. The design was completely randomized, in a factorial scheme with four replicates and five explants per experimental unit. The concentration of 20 g L<sup>-1</sup> sucrose with 100% nitrogen, from the original formulation of the MS culture medium, provided a better response in sugarcane multiplication.

**Additional keywords:** meristems; micropropagation; *Saccharum officinarum*; seedling production.

#### **Resumo**

A cana-de-açúcar apresenta grande importância econômica mundial e, portanto, há grande interesse no desenvolvimento de técnicas eficientes para a produção de mudas de boa qualidade, homogêneas e livres de doenças e pragas. Uma forma de eliminar as viroses é através da cultura de meristemas. Para garantir sucesso durante o cultivo de meristemas faz-se necessário otimizar os componentes e concentrações do meio de cultura. A sacarose é um componente essencial no meio de cultura servindo como fonte de carbono e energia para as plântulas. O nitrogênio é um componente essencial em plantas pois participa de biomoléculas e inúmeras enzimas. O objetivo deste trabalho foi avaliar a melhor combinação de concentrações de nitrogênio (75, 100, 125 e 150%) e sacarose (0, 20, 30 e 40 g L<sup>-1</sup>) na multiplicação e no desenvolvimento *in vitro* de cana-de-açúcar, cultivar RB036091. O delineamento foi inteiramente casualizado, em esquema fatorial, com quatro repetições e cinco explantes por unidade experimental. A concentração de 20 g L<sup>-1</sup> de sacarose com 100% de nitrogênio da formulação original do meio de cultura MS proporcionou melhor resposta na multiplicação de cana-de-açúcar.

**Palavras-chave adicionais:** meristemas; micropropagação; produção de mudas; *Saccharum officinarum*.

#### **Introduction**

Sugarcane (*Saccharum officinarum*) occupies an area of more than 27 million hectares in more than 130 countries (FAOSTAT, 2017). In the 2016/2017 harvest in Brazil, 9.1 million hectares were cultivated (CONAB, 2016), which demonstrates the great economic importance of this crop worldwide. Sugarcane is responsible for almost 70% of the world's sugar production, being also highlighted as a source for

bioalcohol production. In Brazil, the planted area of sugarcane exceeds 10 million hectares, with this country being the largest producer and exporter of sugarcane by-products (UNICA, 2015).

Due to its economic importance in Brazil and its worldwide prominence, many studies are directed at the development of sugarcane genetic breeding programs (Oliveira et al., 2016). Within this scenario, tissue culture techniques have been applied in plant propagation, allowing the production of good quality,

homogeneous, and disease- and pest-free seedlings (Kaur & Sandhu, 2015). Other advantages are the large-scale production of seedlings in a short period of time, using a smaller area compared to conventional cultivation, and the possible increase in yield and longevity of sugarcane plantations.

The absence of plant diseases and pests is of paramount importance for good quality seedlings. Therefore, meristem culture appears an alternative for the production of healthy sugarcane plants, further guaranteeing genetic stability and the production of a large number of seedlings throughout the year (Alcantara et al., 2014). In sugarcane, meristem culture has been shown to be a potential technique for the multiplication of cultivars developed by genetic breeding programs. Many factors are involved in the *in vitro* multiplication of seedlings, such as asepsis and control of luminosity and temperature. Nutrition of the culture medium is also of crucial importance.

The most widely used culture medium in *in vitro* propagation is that formulated by Murashige & Skoog (1962) (MS). Among the nutrients that make up this medium, nitrogen and carbon are the most supplied compounds. Sucrose is the carbon source, and potassium nitrate ( $\text{KNO}_3$ ) and ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) are the nitrogen sources. The amount of nitrogen in the culture medium needs to be optimized according to plant species and growth conditions (Leljak-Levanić et al., 2004). The role of nitrogen in plant growth and development is already recognized (Malik & Rengel, 2013), being essential in plant physiology and nutrition as an essential component of proteins, amino acids, nucleic acids, and other nitrogenous compounds that promote plant growth and development (Tripathi & Raghubanshi, 2014). The amount and source of nitrogen used also influence the growth of *in vitro* cultures, their chemical metabolism, and the formation and production of metabolites (Russowski & Nicoloso, 2003).

In sugarcane, nitrogen stimulates shoot meristem activity, which results in higher tillering rate and leaf area index. The demand for this nutrient varies according to the cultivar (Uribe et al., 2013). Nitrogen uptake depends on the availability of energy from carbohydrates. Sugars such as sucrose have the main function of providing energy for the metabolic reactions of plants, or providing carbon molecules for biosynthetic processes (Ferreira et al., 2016; Rocha et al., 2013).

In the traditional *in vitro* culture method, vitroplants do not receive adequate light for photosynthesis. Therefore, the nutrient medium must be supplemented with sucrose, the carbon source of culture media (Rocha et al., 2013). Being the carbon-nitrogen interaction responsible for plant development (Gabryszewska, 2015; Badr et al., 2015), it is important to determine which concentrations are ideal for *in vitro* plant development. Thus, the potential of these plants can be better exploited, ensuring a high multiplication rate and material savings with the use of lower con-

centrations of sucrose and, consequently, lower costs.

Cultivar RB036091 presents excellent sprouting and high tillering, as well as closure between rows. It is highly resistant to the main diseases, and agricultural production is highly stable. The cultivar stands out for its rapid initial growth and yield potential, being quite responsive to environment improvement (Daros et al., 2015).

In this way, the present work evaluates the best combination of sucrose and nitrogen concentrations in the *in vitro* multiplication and development of sugarcane (RB036091) isolated from meristems.

## Material and methods

Sugarcane plants of the cultivar RB036091, with one month of cultivation from billets, were cultivated in a greenhouse with daily irrigation and average temperature ranging from 18 to 25 °C. Immature leaves (approximately 2 x 10 cm cylinder) were disinfected by treating with 70% (v/v) ethanol for 1 minute, being then rinsed three times with autoclaved distilled water, incubated for 20 minutes in sodium hypochlorite solution with 2% active chlorine, and again rinsed three times with autoclaved distilled water. Transverse segments of the meristematic region were removed, and meristems were isolated with the aid of a stereomicroscope in a laminar flow chamber.

After isolation, meristems were inoculated in liquid MS medium (Murashige & Skoog, 1962) supplemented with 100 mg L<sup>-1</sup> myo-inositol, 0.1 mg L<sup>-1</sup> kinetin, 0.2 mg L<sup>-1</sup> 6-benzylaminopurine (BAP), 30 g L<sup>-1</sup> sucrose, and 6 g L<sup>-1</sup> agar. The medium was autoclaved at 121 °C, at 1.5 atm, with pH adjusted to 5.8 before autoclaving.

The multiplication phase was performed in MS medium supplemented with 100 mg L<sup>-1</sup> myo-inositol, 0.1 mg L<sup>-1</sup> kinetin, 0.2 mg L<sup>-1</sup> BAP, 30 g L<sup>-1</sup> sucrose, and pH 5.8 before autoclaving. Cultures were maintained at 25 ± 2 °C, in the dark, for 7 days. For sprout induction, cultures were maintained under cold white fluorescent light, with photon flux density of 30 mol<sup>-2</sup> s<sup>-1</sup> and 16 h photoperiod, for 60 days (corresponding to the other phases).

Replicates were performed in laminar flow chamber, every 15 days, in a total of four subcultures. In the fifth subculture, the shoots were transferred to liquid MS medium with changes in nitrogen (75, 100, 125, and 150%) and sucrose (20, 30 and, 40 g L<sup>-1</sup>) concentrations. The concentrations corresponded to N: 75% - 1237.5 mg L<sup>-1</sup>  $\text{NH}_4\text{NO}_3$  and 1435.0 mg L<sup>-1</sup>  $\text{KNO}_3$ ; N: 100% - 1650.0 mg L<sup>-1</sup>  $\text{NH}_4\text{NO}_3$  and 1900.0 mg L<sup>-1</sup>  $\text{KNO}_3$ ; N: 125% - 2062.5 mg L<sup>-1</sup>  $\text{NH}_4\text{NO}_3$  and 2375.0 mg L<sup>-1</sup>  $\text{KNO}_3$ ; N: 150% - 2475.0 mg L<sup>-1</sup>  $\text{NH}_4\text{NO}_3$  and 2650.0 mg L<sup>-1</sup>  $\text{KNO}_3$ .

The experiment was conducted in a completely randomized design, in a 4 x 4 factorial scheme (4 nitrogen concentrations and 4 sucrose concentrations), consisting of 16 treatments with four replicates, each replicate consisting of five explants of approxi-

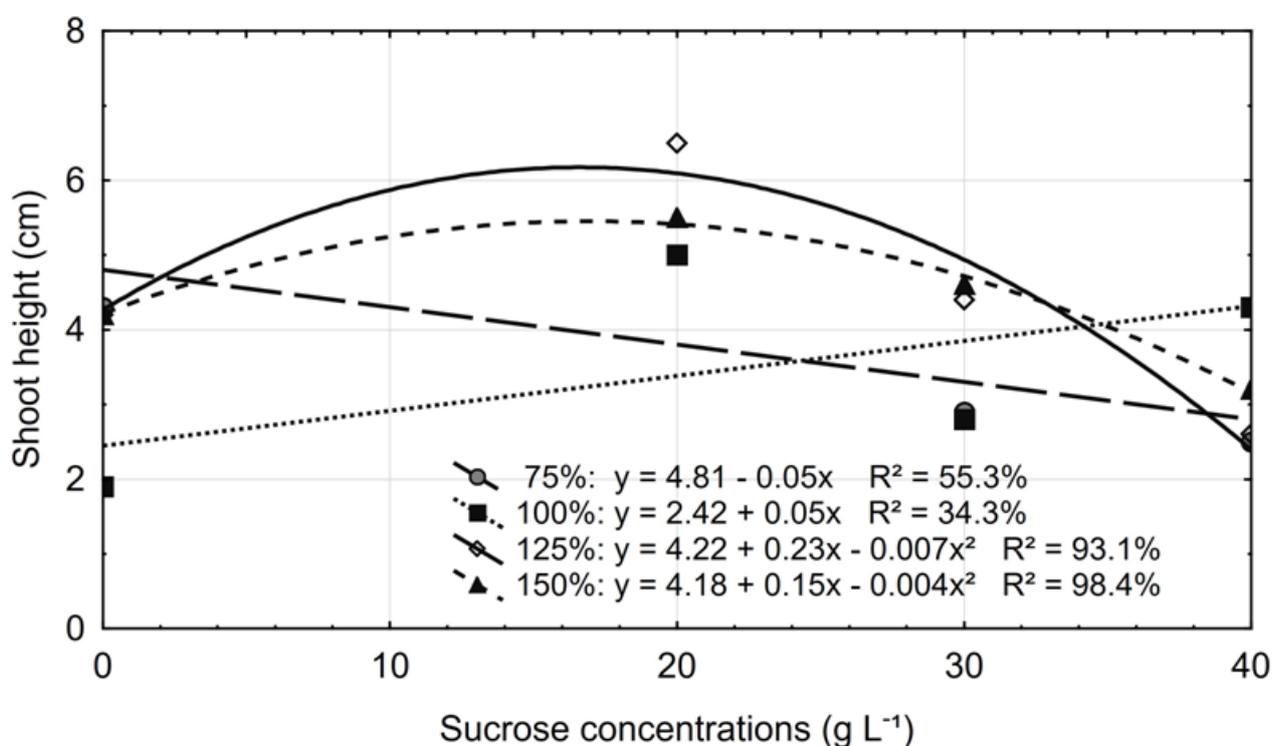
mately 3.0 cm. The treatments were formed by combinations of four nitrogen concentrations (75, 100, 125, and 150%) and three sucrose concentrations (20, 30, and 40 g L<sup>-1</sup>) plus the control (without sucrose). After 15 days, the following variables were evaluated: shoot height (cm), number of leaves, number of shoots, and fresh weight (g). Data were submitted to ANOVA and regression analysis was performed with the Sisvar<sup>®</sup> program (Ferreira, 2011).

## Results and discussion

The results of the analysis of variance showed homogeneous variances for the variables analyzed in the treatments. The analysis also revealed that the

interaction between sucrose and nitrogen concentration was statistically significant, indicating the dependence of the factors.

For 75% nitrogen, shoot height showed a linear decrease with the increase of sucrose concentration, with the highest height being reached with 20 g L<sup>-1</sup> sucrose. For 100% nitrogen, the concentration of 20 g L<sup>-1</sup> sucrose was also the one that showed the highest shoot height, with a subsequent decrease in height with 30 g L<sup>-1</sup> and a slight increase with 40 g L<sup>-1</sup> sucrose. For the concentrations of 125 and 150% nitrogen, regression curves showed a quadratic behavior, with higher shoot heights in the concentration of 20 g L<sup>-1</sup> sucrose (Figure 1).

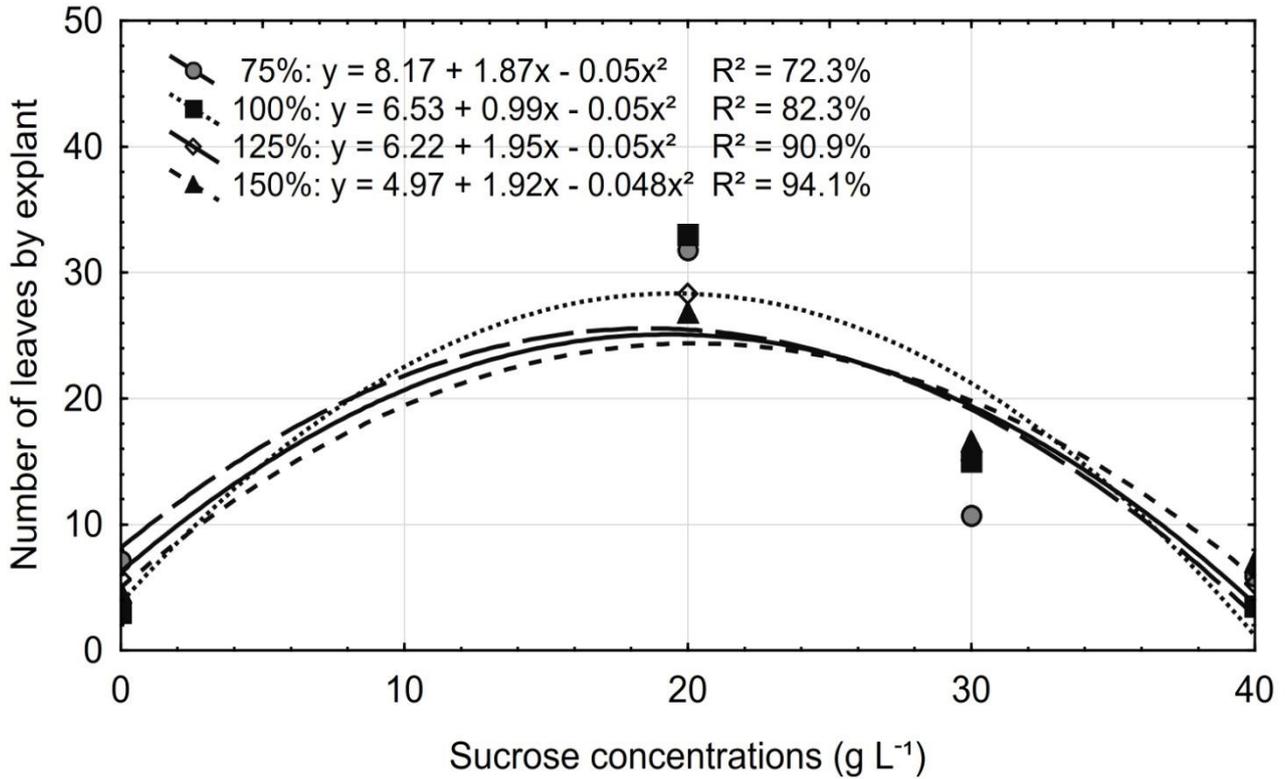


**Figure 1** - Shoot height (cm) of *Saccharum officinarum* 'RB 036091' as function of nitrogen and sucrose concentrations.

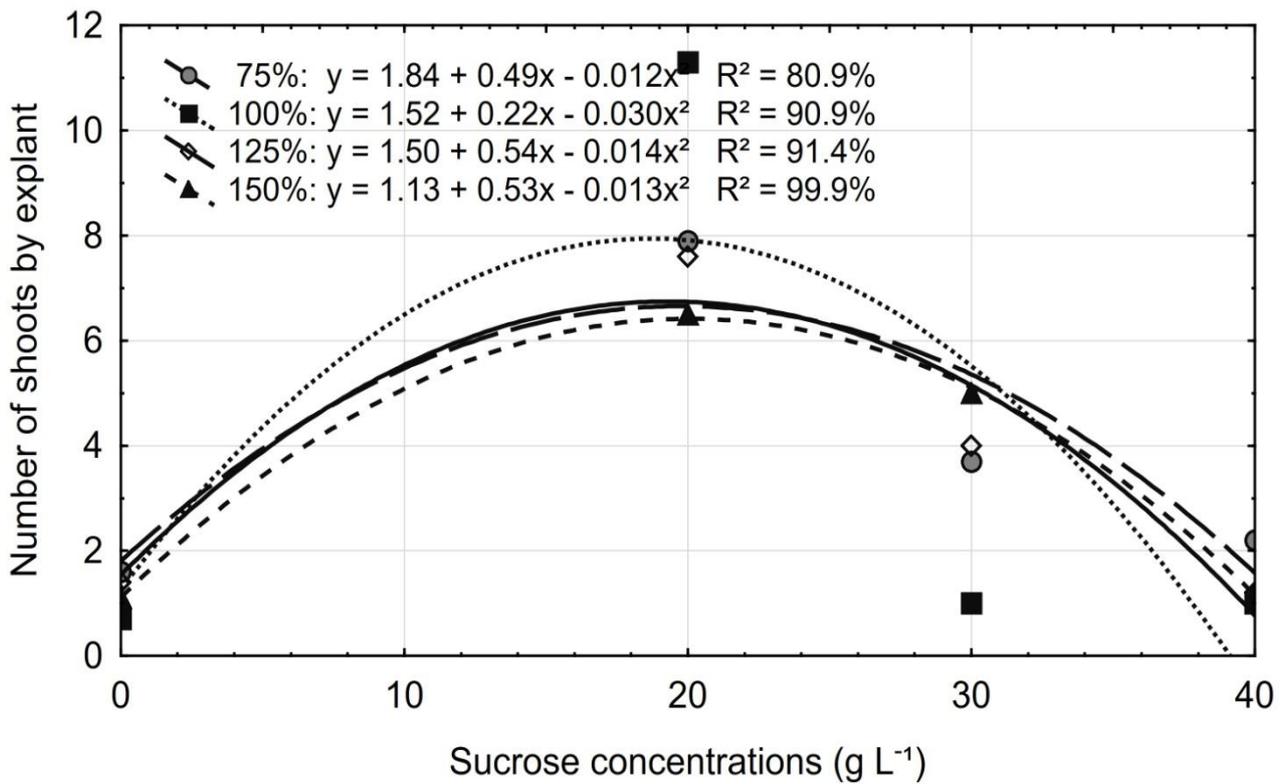
For the number of leaves per explant (Figure 2), number of shoots per explant (Figure 3), and shoot fresh weight (Figure 4), regression curves showed quadratic behavior, reaching higher responses with concentration close to 20 g L<sup>-1</sup> sucrose, for all nitrogen concentrations. For shoot growth and quality, the sucrose concentration (20 g L<sup>-1</sup>) showed better responses for all nitrogen concentrations tested. However, the concentration of 1650 mg L<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub> and 1900.0 mg L<sup>-1</sup> KNO<sub>3</sub>, which corresponds to 100% nitrogen, showed the best results.

A possible explanation for the fact that the sugarcane shoots formed in the present study did not present a positive effect with increased nitrogen concentration may be related to the fact that the plants are

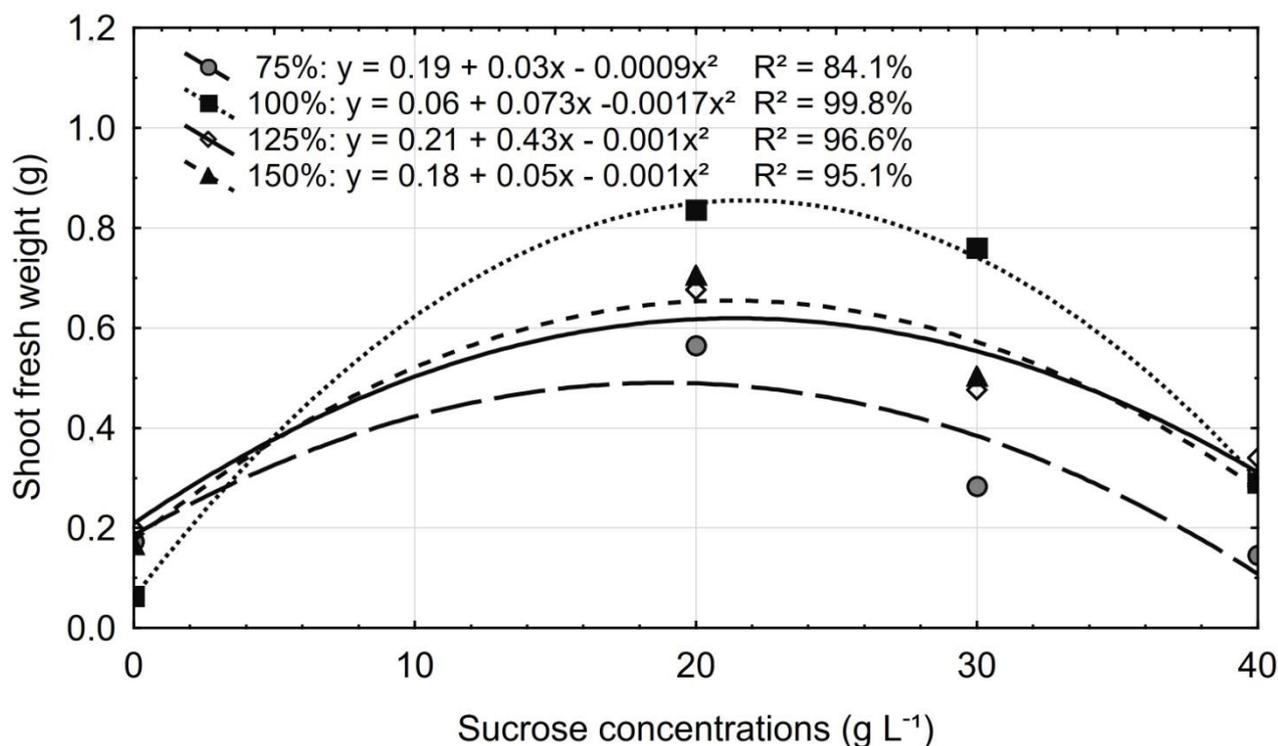
associated with nitrogen-fixing bacteria. Donato et al. (2004) evaluated the enzymatic activity of *in vitro* cultivated sugarcane varieties and found that the reduction of nitrogen levels did not affect the content of chlorophyll a and b, since nitrogen is one of the constituents of chlorophyll. The authors also verified that the constitutive activity of nitrate reductase and glutamine synthetase were also not affected. The presence of a constitutive nitrate reductase being expressed in the absence of nitrate, as well as the nonvariation in chlorophyll content, can be explained by both the fact that sugarcane is a nitrogen-fixing species and the fact that the sugarcane varieties evaluated are associated with nitrogen-fixing bacteria.



**Figure 2** - Number of leaves by explant of *Saccharum officinarum* 'RB 036091' as function of nitrogen and sucrose concentrations.



**Figure 3** - Number of shoots by explant of *Saccharum officinarum* 'RB 036091' as function of nitrogen and sucrose concentrations.



**Figure 4** - Shoot fresh weight of *Saccharum officinarum* 'RB 036091' as function of nitrogen and sucrose concentrations.

Results similar to those of the present study were observed in *Aechmea blanchetiana* for the variable leaf fresh weight, which decreased linearly with the increase of nitrogen concentration in the modified MS medium, where for each added mM of nitrogen there was a decrease of 0.0008 g in biomass (Kanashiro et al., 2007). For *Sinningia allagophylla* grown in culture medium with different concentrations of nitrate, the number of leaves did not present significant difference (Gomes & Shepherd, 2000). However, results contrasting to the present work were verified in the *in vitro* multiplication of shoots of citrus rootstocks, in which twice the concentration of nitrogen present in MS culture medium provided the best results for the number of new shoots and shoot length (Nagao et al., 1994). Notwithstanding, it has been reported a physiological and molecular correlation of carbon and nitrogen metabolism in plant growth; the presence of reduced carbon, mainly sucrose, stimulates nitrate assimilation (Jin et al., 2015). Nitrogen, together with sucrose, are the major components in the culture medium, contributing effectively both to cell metabolism and to the regulation of osmotic potential (Nagao et al., 1994).

The effect on shoot number and quality, as evidenced by the number of leaves per explant, number of shoots per explant, and shoot fresh weight (Figures 2, 3, and 4, respectively), showed different responses according to sucrose concentration. The best responses for these variables were verified at the concentration of 20 g L<sup>-1</sup>, that is, a lower concentration

than that commonly used in *in vitro* cultivation (30 g L<sup>-1</sup>) for many species. Langford & Wainwright (1987) cultivated rose shoots at different sucrose concentrations (0-40 g L<sup>-1</sup>) and did not find growth in culture medium without sucrose; however, a significant increase was observed when the sucrose concentration was reduced to 10 g L<sup>-1</sup>. Other authors have reported the positive effect of reducing sucrose concentrations, mainly associated with the success of acclimatization (Leite et al., 2000). This is because sucrose concentrations, normally used in tissue culture, may inhibit chlorophyll synthesis (Regueira et al., 2018). In the presence of sugars, seedlings do not develop photoautotrophic capacity, which can cause reduced growth and death of seedlings during the acclimatization phase (Shin et al., 2013; Shin et al., 2014). Some authors suggest to reduce the sucrose concentration in the last phase of *in vitro* cultivation aiming to promote increased photosynthesis and to accustom the plants to autotrophic nutrition conditions (Gribaudo & Fronda, 1993). Testing the influence of sucrose on photosynthesis and transpiration of *Actinidia deliciosa* explants grown *in vitro*, Argita et al. (2002) observed better results with 20 g L<sup>-1</sup> sucrose. The explants were exposed to 600 μmol CO<sub>2</sub> in the first 20 days, being then transferred to culture medium without sucrose until the end of the culture period. The same authors report that this condition provided development of the photosynthetic apparatus and better acclimatization.

Some authors defend the increase or maintenance of sucrose concentration aiming at an accumu-

lation of the carbohydrate reserves stored by the leaves, increasing the energy available to the seedlings during the processes of rooting (Rocha et al., 2013) and acclimatization (Hazarika, 2003). In this sense, when evaluating the *in vitro* root development of sugarcane cultivar RB867515, a 50% sucrose reduction compared to the usual concentration in culture medium (30 g L<sup>-1</sup>) negatively influenced root development after 60 days of cultivation, where the average values of root length and root fresh and dry weight were lower than in treatments with higher sucrose concentration (Ferreira et al., 2016). In *in vitro* development, photosynthesis is very low and cultures are dependent on the external support of carbohydrates (Couto et al., 2014). However, in the present study, sucrose was required for the development of *Saccharum officinarum* shoots, although in a concentration lower than that commonly used in *in vitro* cultivation.

### Conclusions

According to the results obtained in this study for sugarcane cultivar RB036091, we conclude that its multiplication is possible by reducing the sucrose concentration to 20 g L<sup>-1</sup>. Moreover, nitrogen concentration influences *in vitro* multiplication, being recommended the original concentration of the MS culture medium.

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