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Coura, Florence Taciana Veriato; Fries, Daniela Deitos; Dias, Daniel Lucas Santos; Sousa, Renata Rodrigues Jardim; Santos, Adriane Pereira da Silva dos; Paiva, Leliane Santos

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Metabolism of carbohydrates in Estilosantes cv. Campo Grande under different systems of cultivation and nitrogen fertilization

Florence Taciana Veriato Coura *

Universidade Estadual do Sudoeste da Bahia, Brazil

Daniela Deitos Fries

Universidade Estadual do Sudoeste da Bahia, Brazil

ORCID: <http://orcid.org/0000-0002-3827-6905>

Daniel Lucas Santos Dias

Universidade Estadual do Sudoeste da Bahia, Brazil

Renata Rodrigues Jardim Sousa

Universidade Estadual do Sudoeste da Bahia, Brazil

Adriane Pereira da Silva dos Santos

Universidade Estadual do Sudoeste da Bahia, Brazil

Leliane Santos Paiva

Universidade Estadual do Sudoeste da Bahia, Brazil

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ABSTRACT. : The objective of this study was to verify the activity of invertases in Estilosantes under the influence of cultivation systems and nitrogen fertilization in two seasons. The experiment was conducted in the Xaraés area, where Estilosantes was planted according to a 2 x 2 factorial scheme with two cultivation systems (monoculture and mixed pasture) in the absence or presence of fertilization (75 kg of N ha⁻¹). A randomized block design with four replications was used. The neutral cytosol (Inv-N), vacuole acid (Inv-V) and cell wall acid (Inv-CW) invertase activity and reducing sugar contents in the leaves and stems were evaluated and analyzed by a 5% probability F-test. The interaction between cultivation systems and nitrogen fertilization was significant for Inv-N in the leaves in the summer, such that greater activity occurred in response to the mixed pasture system and nitrogen fertilization. Nitrogen increased the activity of the Inv-N in the stem in both periods, increased the activity of Inv-V in the leaf in the summer, and decreased the activity of Inv-CW in the fall. This cultivation system influenced the activity of Inv-N regardless of the period of the year and did not interfere with the activities of the acid invertases. The invertases did not show regular changes in relation to nitrogen fertilization.

Keywords: carbohydrates, legumes, mixed pasture, nitrogen, photoassimilates.

Introduction

Extractivism, the main form of exploitation of livestock in Brazil, has been causing damage to both the pastures and the soil due to the lack of replenishment of the nutrients withdrawn by the plants. Fertility depletion in soils causes several problems, from the physical and chemical characteristics to the quality of the fodder produced, which reduce the

supply of feed to the animal and impair its performance (Barcellos, Ramos, Viela, & Martha Jr., 2008).

Although the use of fertilization, especially nitrogen fertilization, has improved the productive performance of forages, it has been limited due to the high costs of chemical fertilizers, the pluviometric availability of the region and the frequent need for application (Barcellos et al., 2008).

The mixing of grasses with legumes can benefit the system since by fixing nitrogen through their symbiosis with bacteria of the genus *Rhizobium*, legumes can make this nutrient available to the grasses. This maintains the productivity and sustainability of the system at a reduced cost and for longer periods.

Productivity depends on the metabolism of plants, including mainly the processes of photosynthesis and translocation, which result in the production and distribution of carbohydrates. Plants have different types of carbohydrates, including soluble, insoluble, reserve and structural carbohydrates, and the predominance of these in the tissues as well as their chemical structure vary among species, organs, tissues and cells; throughout the day; and in different seasons (Souza, Moraes, & Ribeiro, 2005).

After the synthesis of glucose by photosynthesis, glucose must be sent to the drainage organs to be either used in growth or reserved. Thus, glucose is metabolized into sucrose so that it can be transported (Taiz, Zeiger, Møller, & Murphy, 2017). The metabolism of sucrose is vital to the life cycle of the plant, and its use as a source of energy and carbon requires that it be hydrolyzed. Invertase enzymes break sucrose into hexoses, providing the cells with carbohydrates for respiration, which allows energy production and the synthesis of compounds for growth and development. Invertases may also be involved in the transport of sucrose over long distances by creating a concentration gradient of this carbohydrate between the loading and unloading sites of the phloem (Eschrich, 1980; Roitsch & González, 2004).

Invertase enzymes are found in several isoforms with different biochemical properties and subcellular localizations: neutral cytosol invertase, vacuole acid invertase and cell wall acid invertase (Tymowska-Lalanne, & Kreis, 1998; Rohwer & Botha, 2001).

However, studies of carbon metabolism involving invertases as well as the characterization of the effects of the cultivation system and nitrogen fertilization on the activity of these enzymes in forage plants are scarce. The objective of this study was to verify the activity of invertases in *Estilosantes* cv. Campo Grande under the influence of two cultivation systems and nitrogen fertilization in two seasons.

Material and methods

The experiment was carried out from November 2013 to June 2015 in an area with *Brachiaria brizantha* cv. Xaraés, established 6 years ago, belonging to the Dairy Bovine Sector of the State University of Southwest of Bahia - "Juvino Oliveira" Campus, in the municipality of

Itapetinga, Bahia State, Brazil. The experimental area was located at the following coordinates: 15°38'46" S latitude, 40°15'24" W longitude, and the area has an average altitude of 280 m. The climate of the municipality, according to the Köppen classification, is of the type "Cw", defined as mesothermic wet and warm subhumid.

The meteorological data for the experimental period were obtained from the National Institute of Meteorology (INMET) and are presented from the 21st to the 20th of the following month (Figure 1).

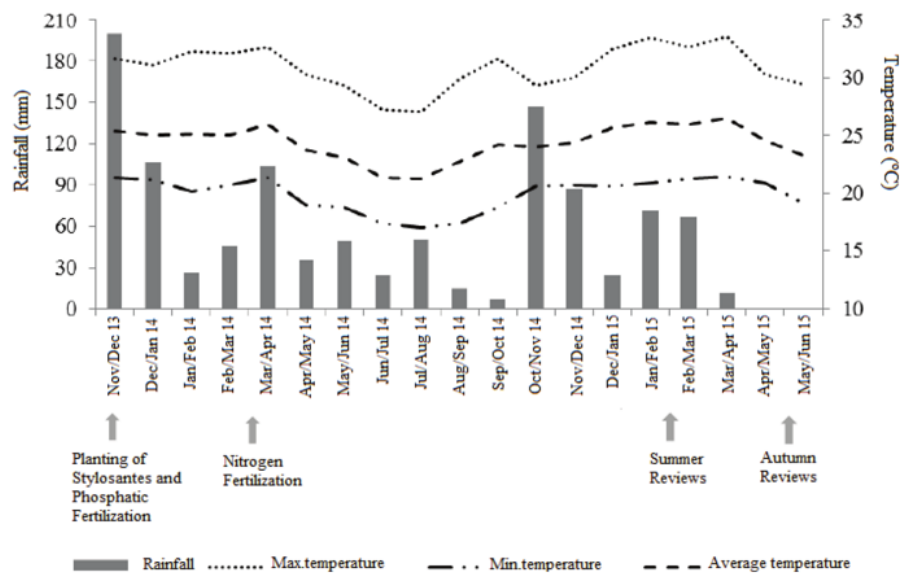


Figure 1

Precipitation (mm) and maximum, minimum and average temperatures (°C) during the experimental period (Nov. 2013 - June 2015). Source: INMET.

The cultivar Estilosantes cv. Campo Grande (Estilosantes) was evaluated using a 2 x 2 factorial scheme with two cultivation systems (1 - monoculture, 2 - Estilosantes mixed pasture with Xaraés grass) that were each evaluated in the absence of nitrogen fertilization and with a dose of 75 kg of nitrogen per hectare.

The design was a randomized complete-block design with four replications, which totaled 16 plots that were each 4 m wide by 3 m long (12 m²) and were arranged with a spacing of 50 cm between lines in the mixed pasture and 30 cm between rows in plots of the legume grown alone.

In November 2013, the area was prepared by cutting the Xaraés grass to a height of 5 cm from the ground. Then, the parcels were demarcated, the furrows were opened in the plots that received the intercropping system and were cleaned, and the grooves were opened in the plots where Estilosantes was sown.

The soil sample was collected following a thick, zig-zag pattern at a depth of 0-20 cm near the experimental area, where ten individual samples were collected and then mixed to obtain a composite sample, which was then sent to the Soil Laboratory of the Department of Agricultural Engineering and Soils of the UESB for chemical analysis. The

chemical analysis revealed the following characteristics: pH = 5.8; P = 16 mg dm⁻³; K = 0.70 cmol_c dm⁻³; Ca = 1.6 cmol_c dm⁻³; Mg = 1.0 cmol_c dm⁻³; Al = 0.2 cmol_c dm⁻³; base sum = 3.3 cmol_c dm⁻³; cation exchange capacity at pH 7.0 = 5.7 cmol_c dm⁻³; base saturation = 58%; and organic matter = 16 g dm⁻³.

Based on the results of the soil analysis and following the recommendations of the Soil Fertility Commission of the State of Minas Gerais (Alvarez & Ribeiro, 1999), the medium technological level was adopted; there was no need for liming since the soil presented a saturation by bases of 58%. Based on the recommendations of Cantarutti et al. (1999), it was not necessary to carry out fertilization with potassium because this nutrient presented good availability. On the other hand, phosphorus presented low availability, which required the application of 50 kg ha⁻¹ of P₂O₅ at the time of planting, which corresponded to 278 kg ha⁻¹ of Super Simple (333 g plot⁻¹).

In December 2013, soon after phosphatic fertilization, the sowing was performed manually. In the mixed pasture, 3 kg ha⁻¹ of pure and viable seeds (7.6 g of seeds plot⁻¹) was used, and 5 kg ha⁻¹ of pure and viable seeds was used for the monoculture (12.7 g seeds portion⁻¹). The seeds used had 95% purity and 60% germination.

The plots were kept clean by hand weeding so that there was no interference of invasive plants in the establishment of the legume. In March 2014, three months after planting, a uniform cut was made at a height of 15 cm from the soil using pruning shears, and nitrogen fertilization with urea was then performed manually, corresponding to 75 kg of nitrogen per hectare in the plots that received this treatment.

The evaluation of the enzymatic activities was performed in the middle-summer period (December 2014 to March 2015), which is considered the rainy season, and autumn (March to June 2015), which is considered the dry period according to the meteorological data provided by INMET.

The collections were carried out on March 2 and June 1, 2015. The third or fourth fully expanded leaf and intermediate stem parts of four plants were collected. At the time of collection, the plant material was placed in previously labeled aluminum foil bags and subsequently immersed in liquid nitrogen to stop the enzymatic activity. All material was stored in a freezer at -80°C for analysis of the enzymatic activity.

Extraction and incubation of the soluble invertases vacuole acid invertase (Inv-V) and cytosol neutral invertase (Inv-N) were performed as described by Zeng, Wu, Avigne, and Koch (1999), and extraction and incubation of the insoluble invertase cell wall acid invertase (Inv-CW) were performed according to Cazetta, Seebauer, and Below (1999).

Inv-V and Inv-N were extracted by the maceration and homogenization of 250 mg of fresh mass in 1.8 mL of ice-cold extraction buffer containing 100 mM potassium phosphate (pH 7.5), 5 mM MgCl₂, and 20 mM ascorbic acid, followed by centrifugation at 9,000 X g for 20 minutes at a temperature of 4°C. The supernatant was collected for incubation of Inv-V and Inv-N.

For extraction of Inv-CW, the pellet was resuspended and homogenized for 7 minutes with 1 mL of the following buffer: 200 mM sodium citrate (pH 4.8), 5 mM MgCl_2 , 20 mM ascorbic acid, and 1 M NaCl. Thereafter, a new centrifugation was carried out at $9,000 \times g$ for 20 minutes at a temperature of 4°C . The supernatant was collected for Inv-CW incubation.

Enzyme activity was performed in a reaction medium containing 200 mM sucrose and either 100 mM potassium phosphate (pH 7.5) and 0.5 mM MgCl_2 for Inv-N or 200 mM sodium citrate (pH 4.8), and 0.5 mM MgCl_2 for Inv-V and Inv-CW

Incubation was performed at time zero (T_0) and at sixty minutes (T_{60}). For T_0 , 200 μL of the sample was homogenized in an Eppendorf tube, and the reaction was immediately frozen on ice. Reactions at 0 minutes were determined to eliminate the pre-existing reducing sugars. For T_{60} , 200 μL of the sample was homogenized in an Eppendorf tube, and the incubation was carried out in a water bath at 30°C for 60 minutes. Then, the reaction was frozen on ice.

The activity of the enzymes was determined in the final product by reducing sugars by the dinitrosalicylic acid method (DNS) (Miller, 1959). The activity of the enzymes was obtained by determining the difference in the values after 60 minutes of incubation compared to those at T_0 . The results obtained were expressed in $\mu\text{mol gli g}^{-1} \text{FM h}^{-1}$.

The reducing sugars were obtained from the extract of the soluble invertases, and quantification was performed by the dinitrosalicylic acid (DNS) method (Miller, 1959).

The results were submitted to analysis of variance, and the measurements were compared by means of the F-test, and the 5% probability level was adopted for both tests using the program Statistical Analysis System (SAS) version 9.2 (SAS, 2017).

Results and discussion

The interaction between the cultivation systems and nitrogen fertilization was significant ($p < 0.05$) for only cytosol neutral invertase (Inv-N) activity in the leaves and style of plants in the summer period.

The highest activity of Inv-N in the leaf was influenced by the intercropping system in the presence of nitrogen fertilization. In general, the intercropping system, both in the absence and in the presence of nitrogen fertilization, stood out compared to the single-crop system, with greater Inv-N activity in the leaf. However, in the stem, there was a significant difference for only the fertilization treatment, indicating that the presence of fertilization positively influenced the increase in cytoplasmic invertase activity (Table 1).

Table 1

Activity of cytosol-neutral invertase (Inv-N), acid vacuole invertase (Inv-V) and cell wall acid invertase (Inv-CW) in the leaves and stems of *Estilosantes* cv. Campo Grande in a monoculture system or mixed pasture system with 0 or 75 kg of nitrogen per hectare during the summer period.

System of cultivation		Fertilization (kg ha ⁻¹)		Average	CV ¹
		0	75		
		Inv-N (μmol gli·g ⁻¹ FM h ⁻¹)			
Leaf	Monoculture	48.85 aB	47.88 aB	48.36	15.10
	Mixed pasture	97.15 bA	135.66 aA	116.41	
	Average	73.00	91.77	82.38	
Stem	Monoculture	26.96	98.00	62.48 A	53.01
	Mixed pasture	62.80	145.34	104.07 A	
	Average	44.88 b	121.67 a	83.28	
		Inv-V (μmol gli·g ⁻¹ FM h ⁻¹)			
Leaf	Monoculture	35.25	44.15	39.70 A	24.68
	Mixed pasture	32.65	56.80	44.73 A	
	Average	33.95 b	50.48 a	42.21	
Stem	Monoculture	75.57	52.63	64.10 A	44.51
	Mixed pasture	77.55	119.02	98.29 A	
	Average	76.56 a	85.83 a	81.19	
		Inv-CW (μmol gli·g ⁻¹ FM h ⁻¹)			
Leaf	Monoculture	50.47	43.38	46.93 A	44.59
	Mixed pasture	15.05	41.59	28.32 A	
	Average	32.76 a	42.49 a	37.62	
Stem	Monoculture	33.58	45.63	39.61 A	45.02
	Mixed pasture	28.54	19.72	24.13 A	
	Average	31.06 a	32.68 a	31.87	

¹Coefficient of Variation. Means followed by distinct lowercase letters in a row differ from each other ($p < 0.05$). Means followed by uppercase letters in a column differ from each other ($p < 0.05$).

The activity of Inv-N in the plants, acting predominantly in the mature and fully expanded tissues, undergoes changes in relation to the development of the plant. According to the literature, there are significant changes in intensity in the enzymatic levels of neutral and acid invertases at different stages of maturation Leite, Crusciol, Lima, and Silva (2009).

According to Koch (2004), neutral or alkaline invertase located in the cytoplasm is considered a maintenance enzyme since it is involved in the degradation of sucrose when the activities of cell wall acid invertase and sucrose synthase are low. Thus, the values found in this study corroborate those found by the aforementioned author since the activity of acid invertases is inferior to the activity of neutral invertase.

As for acid invertase activity in the vacuole (Inv-V), there was a significant difference ($p < 0.05$) due to fertilization, but only in the leaf. On the other hand, the activity of cell wall acid invertase (Inv-CW) was not significantly different between the systems of cultivation or fertilization treatments in the leaf or in the stem of *Estilosantes* in the summer period.

Fertilization had a positive effect on Inv-V activity in the leaf, while the cultivation system did not alter the activity of this enzyme. In the stem, there was no difference caused by the fertilization treatments or the cultivation system.

The activity of this enzyme is related to the storage of sugars, osmotic regulation and responses to abiotic stresses. In addition, vacuolar invertase can control the primary pathway of sucrose cleavage in expanding or mature tissues, contributing to the flux of hexose through

tonoplasts and mediating the entry of these into cytoplasmic metabolism (Roitsch & González, 2004; Yao et al., 2009).

Nitrogen fertilization benefits the development of the plant, with nitrogen being the main nutrient for maintaining the productivity of forage grasses; nitrogen promotes increases in leaf elongation, the number of green leaves, axillary bud formation and the tillering rate Silva, Nascimento Júnior, and Euclides (2008) and thereby ensures higher yields of dry mass and greater renewal of the tiller population.

Thus, the increase in the activity of Inv-V in the leaf, together with sucrose degradation, suggests a higher carbon (hexoses) requirement to supply energy needs both for growth and for the formation of new leaves and tillers.

The metabolism in the leaves responds to the energy needs and growth of the plant such that there is synchrony between the source organs and drainage organs. High carbohydrate content in leaves promotes plant growth and the storage of carbohydrates in the reserve organs (Taiz et al., 2017).

The interaction between the cultivation systems and nitrogen fertilization was significant ($p < 0.05$) for only the activity of cytosol neutral invertase (Inv-N) in the leaf of Estilosantes plants in the fall period. However, in the stem, although the interaction was not significant, there was a difference between the cultivation systems and between the fertilization treatments (Table 2).

Table 2

Activity of cytosol neutral invertase (Inv-N), acid vacuole invertase (Inv-V) and cell wall acid invertase (Inv-CW) in the leaves and stems of Estilosantes cv. Campo Grande in a monoculture system or mixed pasture system with 0 or 75 kg of nitrogen per hectare in the autumn period.

System of cultivation		Fertilization (kg ha ⁻¹)		Average	CV ¹
		0	75		
Inv-N (μmol gli·g ⁻¹ FM h ⁻¹)					
Leaf	Monoculture	34.02 bA	73.71 aA	53.86	31.87
	Mixed pasture	37.16 aA	36.90 aB	37.03	
	Average	35.59	55.31	45.45	
Stem	Monoculture	25.90	81.75	53.82 B	21.57
	Mixed pasture	113.77	194.14	153.95 A	
	Average	69.83 b	137.95 a	103.89	
Inv-V (μmol gli·g ⁻¹ FM h ⁻¹)					
Leaf	Monoculture	90.18	120.46	105.32 A	20.76
	Mixed pasture	83.21	97.26	90.24 A	
	Average	86.70 a	108.86 a	97.78	
Stem	Monoculture	126.08	105.94	116.01 A	38.05
	Mixed pasture	84.38	106.00	95.19 A	
	Average	105.23 a	105.97 a	105.60	
Inv-CW μmol gli·g ⁻¹ FM h ⁻¹)					
Leaf	Monoculture	55.35	27.96	41.66 A	42.74
	Mixed pasture	47.88	24.61	36.25 A	
	Average	51.62 a	26.28 b	38.95	
Stem	Monoculture	40.53	25.03	32.78 A	33.85
	Mixed pasture	67.63	24.64	46.14 A	
	Average	54.08 a	24.84 b	39.46	

¹Coefficient of variation. Means followed by distinct lowercase letters in a line differ from each other ($p < 0.05$). Means followed by uppercase letters in a column differ from each other ($p < 0.05$).

Contrary to what occurred in the autumn season, there was a greater influence of the Inv-N activity on the leaf when the plants were cultivated singly in association with the presence of nitrogen fertilization. In the stem, the highest activity of this enzyme was in the intercropping system, and the nitrogen fertilization influenced the greater activity of the Inv-N.

The cytoplasmic invertase, which exhibits greater activity in tissues whose expansion has already been completed, is important in the differentiation of plant development stages. The modification of the drains occurs during the flowering process, when there is a greater energetic requirement for the formation of seeds and / or fruits. According to Gobbi, Garcia, Ventrella, Garcez Neto, and Rocha (2011), the style begins its reproductive period in April and ends in May with the maturation of seeds, when it is greatly influenced by the photoperiod, which explains the values found in this study (Table 2).

The activity of vacuole acid invertase (Inv-V) in the leaf and in the stem did not significantly differ between the cultivation systems or the fertilization treatments. However, the activity of cell wall acid invertase (Inv-CW) in both the leaf and the stem was significantly different between the fertilization treatments, such that nitrogen reduced its activities (Table 3).

Table 3

Reducing sugar contents in the leaves and stems of *Estilosantes* cv. Campo Grande in the monoculture system or mixed pasture system with 0 or 75 kg of nitrogen per hectare during the autumn and summer.

System of cultivation		Fertilization (kg ha ⁻¹)		Average	CV ¹
		0	75		
Summer					
Leaf	Monoculture	187.21	157.79	172.50 A	16.37
	Mixed pasture	159.56	150.90	155.23 A	
	Average	173.39 a	154.35 a	163.87	
Stem	Monoculture	258.99	312.28	285.64 A	35.30
	Mixed pasture	309.52	304.96	307.24 A	
	Average	284.26 a	308.62 a	296.44	
Autumn					
Leaf	Monoculture	303.63	300.77	302.20 A	22.83
	Mixed pasture	224.35	234.86	229.61 A	
	Average	263.99 a	267.82 a	265.90	
Stem	Monoculture	273.50	357.57	315.53 A	21.26
	Mixed pasture	224.76	303.87	264.32 A	
	Average	249.13 a	330.72 a	289.92	

¹Coefficient of Variation. Means followed by distinct lowercase letters in a line differ from each other (p < 0.05). Means followed by uppercase letters in a column differ from each other (p < 0.05).

Carbohydrates are derived from the reduction of carbon that occurs in the biochemical stage of photosynthesis and have several roles in plant metabolism, such as carbon storage and translocation as well as protection against adverse conditions, such as restricted or excessive water, high salinity and extreme temperatures (Keller & Pharr, 1996).

Sucrose is nonreductive in nature, can be translocated and stored in the cell vacuoles and is not metabolized until it is necessary Souza, Moraes, and Ribeiro (2005). In the autumn season, which occurs from mid-April to mid-June according to the INMET meteorological data, climatic conditions underwent strong oscillations with low temperatures

and no precipitation. These conditions caused variations mainly in the intensity of vacuole acid invertase activity, which is related to the process of osmoregulation under conditions of water deficiency. Even so, this enzyme was not influenced by the cultivation systems or by nitrogen.

As for the reducing sugar contents, the interaction was not significant ($p > 0.05$) between the cultivation systems and fertilization treatments in either the plant components or the seasons. For the cultivation systems and fertilization treatments, there were no significant differences between the components of the Estilosantes plant in the evaluated seasons of autumn and summer (Table 3).

The activity of vacuole and cell wall acid invertases can be high or low under conditions that are favorable for growth or under unfavorable conditions, such as water stress, a short photoperiod and low temperatures (Gayler & Glasziou, 1972; Legendre, 1975). The fluctuations in sugar contents during plant growth are a consequence of the level of the acid invertase enzyme (Gayler & Glasziou, 1972; Lingle, 1999) since it has a close and inverse relationship with the content of sucrose and total sugars; this is in agreement with the findings of other authors (Hawker, 1985; Su, Cruz, Moore, & Maretzki, 1992; Zhu, Komor, & Moore, 1997; Terauchi et al., 2000).

Other studies have emphasized the reduction in the activity of this enzyme under conditions of low temperatures and with the occurrence of the maturation process, as in sugarcane, in which the reduced activity of this enzyme is correlated with an increase in sucrose concentration in the stem (Zhu, Komor, & Moore, 1997; Echeverria, 1998; Terauchi et al., 2000). However, Rose and Botha (2000) demonstrated a significant correlation between the sucrose content and the level of neutral invertase.

As there was no variation in the reducing sugar contents, the changes that occurred in the activity of the invertases as a function of the mixed pasture or the nitrogen in the summer and in the autumn may have been to maintain the reductive sugar concentrations required for cellular activities.

According to Vilhar, Kladnik, Blejec, Prem, and Chourey (2002), there is evidence that sugars function not only as energetic compounds or precursors of other cellular components but also as signals within tissues defined to maintain a distinct stage of differentiation or to proceed within the program, indicating that invertases play a crucial role in the generation of metabolic signals.

Conclusion

The cultivation system influences the activity of neutral cytosol invertase in Estilosantes cv. Campo Grande independent of the period of the year. However, it does not interfere with the activities of acid invertases.

The vacuole and cell wall acid invertases do not present regular changes in relation to nitrogen fertilization; thus, they can increase or reduce their activity when the plants are cultivated under these conditions.

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Author notes

Author for * correspondence. E-mail: florenceveriato@yahoo.com.br