AGRONOMY SCIENCE AND BIOTECHNOLOGY

RESEARCH ARTICLE

Effects of thermotherapy and meristem culture techniques on macro and micronutrients content in elephant grass cultivars

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OPEN ACCESS

How to cite: Karasawa, M. M. G., Tavares, V. B., Pinto, J. C., Lédo, F. J. S., Pereira, A. V., & Pinto, J. E. B. P. (2023). Effects of thermotherapy and meristem culture techniques effect on macro and micronutrients conten in elephant grass cultivars. *Agronomy Science and Biotechnology*, 9, 1-19 https://doi.org/10.33158/ASB.r182.v9.2023

Received: January 12, 2023.
Accepted: March 22, 2023.
Published: May 31, 2023.

English by: Dayse Assis de Miranda

ABSTRACT

Elephant grass is a tropical forage crop highly used in dairy cattle production, in Brazil. It has been getting special attention, because of its bioenergy potential, medicinal properties, and bioremediation profile. The aim of the present study is to investigate the effects of thermotherapy based on clonal cleaning methods and meristem culture on the mineral content of elephant grass (*Cenchrus purpureus* (Schumach.) Morrone). Cultivars "Mineiro", "Taiwan A-147" and "Pioneiro" were subjected to the following methods: thermotherapy (T) combined to meristem culture (MC), meristem culture and mature stems (control). The experiment assessed the mineral contents of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) at three cuttings, which were performed every 60 days, for 180 days. There was lack of effects from these methods on the mineral content of approximately 66% of the carried out assessments, standing out unanimity for all cultivars, methods, and most of the cuts. T+MC was the only method showing positive effect on P and Ca content, in all cuts, in the cultivars Taiwan A-147 and Pioneiro, respectively. There was clear negative effect of cleaning methods on P and Ca content, in all cuts, for cultivar Mineiro, and on Mg, for cultivar Taiwan A-147. These results, along with the positive effects observed in vegetative and nutritional parameters shown in other articles published in this Journal, show that the clonal cleaning methods are strongly recommended for cultivars with more than 15 years of ripe stem propagation.

Keywords: Clonal cleaning, phosphorus, potassium, calcium, magnesium, sulfur, bioenergy production, micropropagation.

INTRODUCTION

It is necessary doubling food production (Ray, Mueller, West, et al., 2013), mainly of items such as cereals, meat, milk and eggs, in order to meet the sustainable development goals, set by the United Nations on hunger and to eradicate malnutrition by 2030 (Karasawa, 2021). The main animal–protein sources are meat and milk; approximately 95% of all beef and milk production in Brazil is mostly produced on pasture, which occupies 160 million hectares (Machado et al., 2021).
Elephant grass, *Cenchrus purpureum*, is one of the forages mostly used in cattle feed, especially in dairy farming, among all tropical forage species (Wanjala et al., 2013; Karasawa et al., 2023). The option made for using elephant grass was mainly linked to its great potential for dry matter production and good quality (Tcacenco, 1988). This species has the cespitous habit of perennial growth; normally it can reach 3 - 5 meters, in height. This forage's propagation is primarily asexual, and happens through vegetative stem fragments, at the age of 100 days, with 2 to 3 buds, as the produced seeds are of low quality (Ocumpaugh & Sollenberger, 1995).

This species has also been assessed in studies on bioenergy production, since it emerged as alternative source for biofuel production (Mohammed et al., 2015; Samson, et al., 2005; Rocha, et al., 2017) due to its high cellulose content level, fast growth, and biomass production (Campos et al., 2019), and carbohydrate content (Azeke, Eze, Ubong, & Kuroshi, 2019). Taiwan and Cameroon cultivars' total calorific value ranged from 3.905 - 4.047 Kg^{-1} and 4.033 - 4.042 Kg^{-1}, respectively, and these were the best results among all assessed cultivars (Marafon et al., 2020). It is essential observing that these values are similar to those recorded for sugarcane.

Mineral levels in plants have pointed out this species' potential to provide the aforementioned elements for animal nutrition, because they are essential inorganic nutrients for energy production and for macromolecule construction, such as nucleic acids, lipids, proteins and enzymes. Phosphorus, for example, acts in energy formation in ATP form, in DNA and RNA molecules' construction, in acid-base balance and in teeth formation. On the other hand, iron plays important role in binding and transporting oxygen in hemoglobin molecules and in the cellular respiration process, in cytochromes (Soetan, Olaiya, & Oyewole, 2010). In addition, many ions are important for the activity of enzymes in substrate processing, in metabolic activities (Nelson & Cox, 2014).

Asexual reproduction is a problem because plants accumulate pathogenic contaminants, such as viruses, bacteria, fungi, and mycoplasmas, over generations (Wang & Charles, 1991; Negawo, Teshome, Kumar, Hanson, & Jones, 2017). This accumulation of pathogens has negative effect on forage production and quality since it reduces the amount of dry matter and the nutritional quality over the years. Researchers from "Dairy Cattle Embrapa" observed these elephant grass effects. Although many symptoms are visible and easy to diagnose, overall, symptoms caused by viruses and mycoplasmas are not visible and often go unnoticed (Pierik, 1990; Stone, 1978; Kumar et al., 2021). The observed effects also met scientific publications showing the presence of mosaic virus (Silva et al., 2013), bacteria *Xanthomonas albilineans* (Meng et al., 2019) and diseases caused by phytoplasma (Kogej, Dermastia, & Mehle, 2020; Kawube et al., 2015; Asudi, Muyekho, Midega, & Khan, 2020).

Clonal cleaning is a technique widely used to propagate plants infected by viruses (Cronauer & Krikorian, 1985; Kartha & Gambog, 1975), bacteria (Meng et al., 2019), fungi (Cronauer & Krikorian, 1985) and phytoplasma (Accosta, Pantoja, & Perez-Lopez, 2020; Kogej et al., 2020; Tiwari, Tripathi, Lal, Sharma, & Chiemsombat, 2011). Meristem culture, chemotherapy and thermotherapy are among the techniques mostly used to remove pathogens (clonal cleaning). However, using antibiotics to control the aforementioned pathogens is difficult because they often translocate through the phloem, and it prevents the pathogens from being fully eliminated; moreover, this process does not protect plants for long periods-of-time (Accosta et al., 2020). Thus, the association between thermotherapy and meristem culture is widely used as strategy.

Micropropagation through meristem culture mainly focuses on clonal cleaning and herbaceous, shrub and tree species' multiplication (Grattapaglia & Machado, 1998). This technique is seen as the most efficient and fastest ones to produce contaminant-free plants, mainly of virus-free ones (Torres, Teixeira, & Pozzer, 1998). Assumingly, lack of viral particles in meristematic tissues is related to the accelerated growth and development of these tissues, as well as to vascular disconnection, high protein synthesis level and viral particles' inactivation (Mellor & Stace-Smith, 1977; Kassanis & Varma, 1967). This technique is widely used for clonal cleaning in plants infected by viruses (Kartha & Gambog, 1975; Krikorian and Cronauer, 1984), fungi (Pescador & Koller, 1992; Krikorian & Cronauer, 1984), bacteria (Meng et al., 2019) and phytoplasma (Kogej et al. 2020; Accosta et al., 2020; Tiwari et al., 2011).

According to Cha-um, Hein and Kirdmanee (2006), meristems (0.5 - 1.5 mm) are capable of efficiently removing fungi and most bacteria. Studies conducted with sugarcane have shown that meristems (0.2 - 0.3 mm) often eliminate phytoplasmas (Tiwari et al., 2011) and that meristems (0.1 mm - 0.2 mm) are able to eliminate viruses (Ramgareeb et al., 2010; Cheong et al., 2012). They increase the tiller density of micropropagated plants over 100% (Karasawa et al., 2023), by 88% (Santana et al., 1992), and by 30% in comparison to the traditional method (Perez & Rodriguez, 1987). Meristems (0.1 and 0.5 mm) were able to eliminate viruses in cassava plants and to increase their production in comparison to the control (Deepthi & Makeshkumar, 2016).

Thermotherapy consists of subjecting plants contaminated with active growth to environments with the highest temperature tolerated by their species for two weeks or more, without interruption (Quak, 1977; Boxus, Quorin, & Laine, 1977). Thermotherapy efficiency in controlling viruses and mycoplasmas has been proven for more than half a century, since the studies by Smith (1950) and Quak (1977). Higher temperatures would prevent, or reduce, pathogens' multiplication in comparison to meristematic tissues that keep normal cell cycles to produce pathogen-free tissues (Oehl & Hughes, 1980; Torres, Teixeira, &Pozzer, 1998). However, Panattoni et al. (2013) believe that high temperatures would activate the host's defense system due to induced viral silencing (VIGS), which would help avoiding viral infection.

So far, none of the studies focused on using elephant grass culture *in vitro* (Zanete, Pailo, & Moraes, 1988; Umami, Akashi, Gondo, Ishigaki, & Tanaka, 2016; Crespo & Alvarez, 2014; Karasawa, Pinto, Pinto, & Pereira, 2002; Karasawa, Pinto, Pereira, & Pinto, 2006; Herrera, García, Cruz, & Romero, 2012; Herrera, Garcia, Fortes, Cruz, & Romero, 2013). They have assessed the effect of clonal cleaning techniques on macro and micronutrient concentration in cultivars presenting different asexual propagation ages.

Because many pathogens do not always manifest themselves visibly (Pierik, 1990; Stone, 1978; Kumar et al., 2021) and based on reports in previous study, the option was made to apply clonal cleaning techniques based on using meristem culture (MC) - 0.2 mm – in combination to thermotherapy (T + MC), or not. The aim of the present research was to assess clonal cleaning methods' (T+MC and MC) effect on macro and micronutrients' concentration in elephant grass cultivars presenting different asexual propagation time in comparison to the control (CO).

MATERIAL AND METHODS

Thermotherapy

The experiment was carried out in greenhouse, at the Animal Science Department of Federal University of Lavras. Lavras County - MG – the aforementioned county is located at latitude 21°14'30" S and longitude 45°00'10" W, at mean altitude of 918 m. It presents Cwb climate, with rainy summers and dry winters. Mineiro, Taiwan A-147 and Pioneiro elephant grass cultivars provided by Embrapa's Active Elephant Grass Germplasm Bank (BAGCE) were treated with thermotherapy (Figure 1). BAGCE culms were divided into cuttings with 3 - 4 buds to carry out the treatment; they were planted in late July 1999, in vessels filled with 5 liters of soil, and observed until shoots reached 20 cm in height. Thermotherapy started at temperatures of 30°C at night and 45°C during the day, for 20 days (Lassois, Lepoivre, Swennen, Houwe, & Panis, 2012). Subsequently, the upper 20-cm of the stems, with apical meristem, were collected and prepared for disinfection.

Figure 1. Greenhouse details showing plants of elephant grass in the pots and the thermotherapy control panel with a red square.

Meristem culture

Stem fragments with apical meristem were disinfested at the Laboratory of Tissue Culture and Medicinal Plants of DAG (Department of Agriculture), at UFLA, in Lavras County, MG. It was done by using 1% sodium hypochlorite and 3 drops of Tween®, under agitation for 20 minutes. Then, 3 rinses were performed in sterile distilled water. The disinfested materials were taken to the laminar air flow and placed in petri dishes filled with sterile filter paper. Meristems were removed with the aid of stereoscopic microscope (Figure 2). Each meristem (0.2 mm, in diameter) was isolated and inoculated in test tubes (25 mm x 150 mm) filled with 15 ml of MS culture medium (Murashige & Skoog, 1962), WPM vitamins (Lloyd & McCown, 1980), 3 % sucrose - supplemented (or not) with 0.7% agar (Karasawa et al., 2006). The test tubes were incubated at temperature of 25±2°C, and 2000 lux lighting, after they were sealed. Developed meristems were multiplied, according to Karasawa et al., 2002, rooted and acclimatized.

Acclimation

Elephant grass plantlets produced *in vitro* through the T+MC and MC treatments were removed from the test tubes. Their roots were washed and, subsequently, planted in 300 ml plastic cups filled with Plantimax® substrate (Figure 2b). In addition, untreated culms (1 cm of mature stem) were longitudinally fragmented; one bud and three root protuberances were left on them. These fragments were planted in 300-ml plastic cups filled with Plantimax® substrate, in separate; they composed the experimental control. Seedlings remained in environment under high relative humidity, forced ventilation and 50% shading, for 30 days, in greenhouse, for acclimation purposes. Then, they were sent to the greenhouse at the Department of Animal Science (DZO – UFLA), where they were grown and assessed for 180 days.

Figure 2. Steps involved in meristem culture (drawn by Karasawa, M.M.G.), and plant acclimatization details (thermotherapy + tissue culture, tissue culture and ripe stem, respectively, from the left to the right).

Growing in greenhouse

Acclimated seedlings were planted in pots, in separate (Figure 3) and taken to the greenhouse of the Department of Animal Science (DZO). Each pot was filled with 10 L of red-yellow latosol, presenting the following features: water pH (1: 2.5) = 5.7; P and K (Mehlich I) = 18.0 and 69.0 mg/dm^3; Ca, Mg and Al = 2.6; 1.0 and 0.0 cmolc/dm^3, respectively; V = 59.2% – soil was collected in Coronel Pacheco City, MG.

The dry soil was previously sieved and fertilized with 16.06 g KCl; 24.45g SSP; 3.0 g urea; 3.0 g $MgSO_4$; 0.17 g H_3BO_3; 0.06 g $CuSO_4$ and 0.01 g $ZnSO_4$. Seedlings were irrigated on a daily basis and fertilized with nitrogen on a monthly basis. Ammonium and sulfate sources' application were alternated. Mean temperatures inside the greenhouse ranged from 20 °C to 26°C. Cuts were made every 60 days, over 180 days, 5 cm above ground over the experimental time; evaluations of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) contents were made during three cuts.

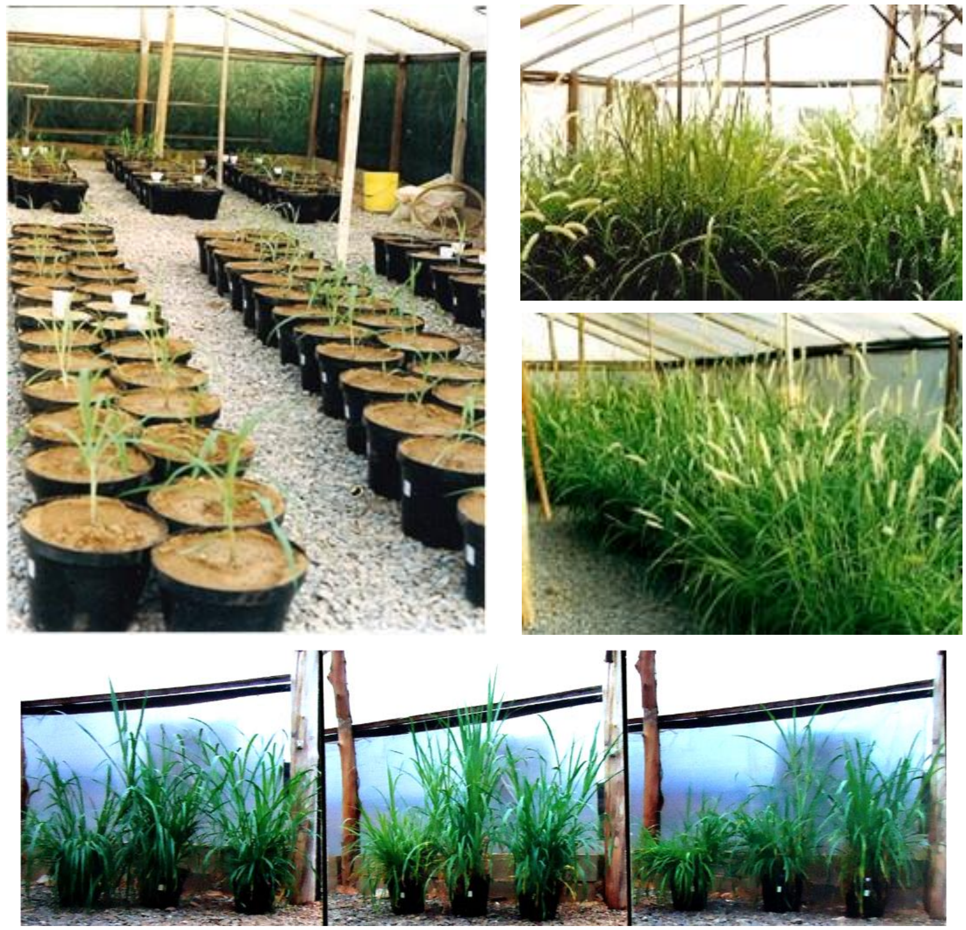

Figure 3. Starting experiment (upper left), first cut (upper middle), second cut (upper right), third cut (down): left Mineiro, middle Taiwan A-147, and right Pioneiro.

Analyses

Three cuts were made 5 cm above the ground, at 60-day intervals, for 180 days to determine phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) levels. The contents of minerals P, K, Ca, Mg and S were first observed through nitro-perchloric digestion; Ca and Mg content was assessed through atomic absorption spectrophotometry; the P content was assessed through colorimetry, K content through flame photometry (Malavolta, Vitti, & Oliveira, 1989) and S content through turbidimetry (Blanchar, Rehm, & Caldewel, 1965).

Experimental design and statistical analysis

Pots planted with the seedlings were distributed in randomized blocks (Figure 3), with six replications. Treatments were distributed into subdivided plots, in space and time (Petersen, 1994). The three elephant grass cultivars (Mineiro, Taiwan A-147 and Pioneiro) were distributed in the plots. Methods applied to get the seedlings (T+MC, MC and CO, with 3 repetitions, each) were randomized in each sub-plot. Three cuts were made in the time sub-plot every 60 days, starting from planting date.

Statistical analysis was performed through F and Scott tests, at significance levels P<0.05 and P<0.01, respectively. The used statistical model was:

yijkl = μ + ci + bj + eij + mk + cmik + eijk + ls + eijks + eijkst

Wherein:

yijkl = value observed for cultivar "*i*", for method "***k***", in block "***j***" and in cut "***l***"

μ = overall average;

ci = effect of cultivar *"i"* on the plot - *i = 1, 2, 3;*

bj = effect of block "*j*" - j = 1, 2, 3, 5, 6;

eij= effect of plot-associated experimental error;

mk = effect of method "k" - *k* = 1, 2, 3;

cmik = effect of interaction between cultivar "i" and method "k"

eijk = effect of experimental error associated with the subplot;

ls = effect of "s" cuts - s = 1, 2, 3;

eijks = experimental error associated with cuts; and

eijkst = experimental error associated with interaction among cultivars, cuts and methods.

RESULTS AND DISCUSSION

Phosphorus (P) content

Methods adopted to get the seedlings only affected phosphorus content in Cv Taiwan A-147, in the first cut, wherein T+MC produced the highest values, the MC method showed intermediate values and CO recorded the lowest values for this nutrient (Table 1). Taiwan A-147 and Pioneiro cultivars showed the highest P concentrations through the T+MC and MC methods; they did not differ from each other, whereas Cv Mineiro showed the lowest value for this element. On the other hand, there was no difference in P concentration in the control between cultivars. Difference in P content based on the method T+MC adopted to get seedlings has shown that different genotypes have different ability to absorb this element.

The highest P content, in the second cut, was observed for the control method (CO) when it was applied to Cv Mineiro, whereas Cv Taiwan A-147 showed the highest values when it was subjected to the T+MC method; Cv Pioneiro results did not differ among methods applied to the tested seedlings (Table 1). Cultivars were also differently affected by the tested methods: T+MC showed better response in Cv Taiwan A-147, intermediate response in Cv Pioneiro and worse response in Cv Mineiro. All cultivars responded similarly to the MC method and Mineiro and Pioneiro cultivars responded similarly and quite well to the CO method.

T+MC and MC methods produced the lowest P contents in Cv Mineiro, at the third cut, whereas methods MC and CO produced lower P values in cultivars Taiwan A-147 and Pioneiro (Table 1). The methods differently affected the response of the cultivars when it comes to P content. Cv Pioneiro showed the best P absorption in comparison to the other cultivars through the T+MC method, whereas methods MC and CO led to similar values in Mineiro and Pioneiro cultivars and to higher ones in Tawian A-147.

The comparison among the three cuts showed the highest P levels in the first cut, and values kept on decreasing untill the third cut, under all methods applied to get to the tested seedlings, in all cultivars (Table 1). This finding evidenced that cuts had negative effect on the content of this nutrient in the herein assessed forage.

Table 1. Contents (%) of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) in the dry weight of cultivars (CV) Mineiro, Taiwan A-147 and Pioneiro, in three cuts (CUT), in relation to the three methods (M): thermotherapy + meristem culture (T + MC), meristem culture (MC) and control (CO).

		MINEIRO			TAIWAN A-147			PIONEIRO		
Nutrients	**CUT**	**T + MC**	**MC**	**CO**	**T + MC**	**MC**	**CO**	**T + MC**	**MC**	**CO**
P	**1º**	0.39 AbA[1]	0.40 AbA	0.42 AaA	0.51 AaA	0.46 AaB	0.39 AaC	0.42 AbA	0.43 AaA	0.41 AaA
	2º	0.30 BcB	0.31 BaB	0.36 BaA	0.41 BaA	0.30 BaB	0.29 BbB	0.35 BbA	0.33 BaA	0.36 BaA
	3º	0.25 CbB	0.26 CaB	0.28CaA	0.27 CbA	0.23 CbB	0.21 CbB	0.30 CaA	0.28 CaB	0.27 CaB
Factors			**CV** = 0.006382*		**M** = 0.008415**		**CUT** = 0.364619**			
Interactions			**CV x M** = 0.023940**		**CV x CUT** = 0.009097**		**M x CUT** = 0.002369*		**CV x M x CUT** = 0.001634*	
K	**1º**	1.36 AaA	1.45 AaA	1.45 AaA	1.43 AaA	1.34 AbA	1.40AaA	1.34 AaA	1.33 AbA	1.38 AaA
	2º	1.24 BaA	1.24BaA	1.31 BaA	1.21 BaA	1.26 AaA	1.26 BaA	1.31 AaA	1.31 AaA	1.24 BaA
	3º	1.25 BaA	1.29 BaA	1.31 BaA	1.28 BaA	1.29 AaA	1.24 BaA	1.31 AaA	1.26 AaA	1.29 BaA
Factors			**CV** = 0.8753^{ns}		**M** = 1.3374^{ns}		**CUT** = 15.9456**			
Interactions			**CV x M** = 1.9550^{ns}		**CV x CUT** = 2.3871*		**M x CUT** = 1.5917^{ns}		**CV x M x CUT** = 1.3913^{ns}	
Ca	**1º**	0.13 AaB	0.14 AaA	0.15 AaA	0.13 AaA	0.14 AaA	0.14 AaA	0.14 AaA	0.12 AbB	0.11 AbC
	2º	0,11 BbB	0.12 AbB	0.15 AaA	0.15 AaA	0.15 AaA	0.13 AaB	0.12 AbA	0.12 AbA	0.11 AbB
	3º	0,10 BbB	0.13 AaA	0.12 BaA	0.12 BaA	0.11 BbB	0.11 BbA	0.12 BaA	0.10 BcB	0.10 AcB
Factors			**CV** = 0.003413**		**M** = 0.001017**		**CUT** = 0.007246**			
Interactions			**CV x M** = 0.001007**		**CV x CUT** = 0.000631**		**M x CUT** = 0.000557**		**CV x M x CUT** = 0.000563**	
Mg	**1º**	0.28 AbA	0.30 AbA	0.32 AbA	0.31 AaB	0.34 AaA	0.36 AaA	0.31 AaA	0.30 AbA	0.32 AbA
	2º	0.25 BaB	0.26 BaB	0.31 AaA	0.25 BaB	0.25 BaB	0.27 BbA	0.26 BaB	0.27 BaB	0.30 AaA
	3º	0.24 BbA	0.25 BaA	0.25 BbA	0.25 BbB	0.26 BaB	0.29 BaA	0.28 BaA	0.26 BaA	0.28 BaA
Factors			**CV** = 0.004341*		**M** = 0.017686**		**CUT** = 0.048467**			
Interactions			**CV x M** = 0.000717^{ns}		**CV x CUT** = 0.004854**		**M x CUT** = 0.001177^{ns}		**CV x M x CUT** = 0.000952^{ns}	
S	**1º**	0.19 AaA	0.19 AaA	0.19 AaA	0.19 AaA	0.19 AaA	0.19 AaA	0.16 AbA	0.17 AbA	0.17 AbA
	2º	0.16 BaA	0.16 BaA	0.17 BaA	0.15 BaA	0.15 BaA	0.16 BaA	0.16 AaA	0.15 BaA	0.16 AaA
	3º	0.14 CbA	0.14 CaA	0.14 CbA	0.14 BbA	0.14 BaA	0.14 CbA	0.16 AaB	0.15 BaB	0.17 AaA
Factors			**CV** = 0.000160^{ns}[2]		**M** = 0.001184*		**CUT** = 0.015439**			
Interactions			**CV x M** = 0.000359^{ns}		**CV x CUT** = 0.003639**		**M x CUT** = 0.000048^{ns}		**CV x M x CUT** = 0.000371*	

[1]Capital letters in italic (column) compare cuts within method for the same cultivar, tiny letters (in the line) compare cultivars in the same method in each cut, and bold letters (in the line) compare different methods in the same cultivar and cut.

[2]ns = not significant, * = significant 5%, ** = significant at 1% by F and Scott Knott test.

Lounglawan, Lounglawan and Suksombat (2014) discusses that cuttings applied every 45 or 60 days would provide the highest concentrations of nutrients and that cutting height that did not influenced these parameters. Próspero and Peixoto (1972) assessed elephantgrass at the age of 45 days and recorded 0.21% P. On the other hand, Guerrero, Fassbender and Blyndestain (1970) found P values ranging from 0.32% to 0.38% when increasing N doses were applied. Cut-off intervals of 60 days were used in the current study, and values higher than those reported by the aforementioned authors were observed in our the first cut, under all tested methods, including the traditional one. Values in the present research were similar to those recorded by Guerreiro, Fassbender and Blyndestain (1970) and higher than those recorded by Próspero and Peixoto (1972), in the second cut.

Values herein recorded in the third cut were higher than those reported by Próspero and Peixoto (1972) and lower than those obtained by Guerreiro, Fassbender and Blyndestain (1970). Briefly, the methods adopted to get the seedlings influenced significantly ($P<0.05$) the cultivars (Cv) and highly significantly ($P<0.01$) the methods of obtaining seedlings (M) and the cuts (CUT). Interaction was also observed between Cv x M and Cv x CUT, at $P<0.01$, and between M x CUT and Cv x M x CUT, at $P<0.05$ (Tables 1 and 2).

Potassium content (K)

Potassium (K) content was not affected by the herein tested methods in the assessed cultivars, at any of the adopted cuts (Table 1). However, cultivars showed different responses to the method used to get the CT seedlings, at the first cut. Cv Pioneiro and Taiwan A-147 showed lower K–content response.

The comparison among cuts showed their negative influence on K content in Cv Mineiro, under the three methods applied to the tested seedlings, and lower and similar values at the second and third cuts (Table 1). Cuts only influenced Cv Taiwan A-147 under the T+MC and CO methods. The second and third cuts produced similar and lower values under these methods. Cv Pioneiro presented the same effects under the CO method; lower results were observed at the second and third cuts. Potassium (K)_ content was only influenced by the cuts (CUT) at $P<0.01$; there was interaction between Cv x CUT at $P<0.05$ (Tables 1 and 2). Próspero and Peixoto (1972) assessed elephantgrass at the age of 45 days and recorded 5.07 K. This value is much higher than that recorded in the current study, under all methods applied methods to get the seedlings (Table 1).

Calcium content (Ca)

Calcium (Ca) content was differently affected by the methods applied to get the seedlings of cultivars assessed at the first cut. The highest Ca content value recorded for Cv Mineiro was observed under methods MC and CO, whereas the highest concentration of this element in Cv Pioneiro was observed under method T+MC, intermediate values were observed under method MC and lower ones under method CO. The comparison of responses from the three cultivars against the same methods for seedlings' obtainment showed similar responses under T+MC, whereas Mineiro and Taiwan A-147 cultivar results under the MC and CO methods were better than those recorded for Cv Pioneiro under the same methods.

Calcium (Ca) content in Cv Mineiro, at the second cut, was affected by the tested methods similarly to what has happened at the first cut (Table 1): the lowest result was recorded under T+MC, whereas cultivars Taiwan A-147 and Pioneiro showed the highest values under T+MC and MC. The comparison of the effects from each method on Ca absorption by the assessed cultivars, at the second cut, showed that T+MC and MC methods led to higher Ca content in Taiwan A-147 cultivar than in Mineiro and Pioneiro, which produced similar values. With respect to the CO method, Mineiro and Taiwan A-147 cultivars showed similar values, but they were higher than that recorded for Cv Pioneiro.

Calcium (Ca) content recorded for Cv Mineiro, at the third cut, was similarly affected by the tested methods, just as at the first and second cuts. It led to the lowest values recorded under method T+MC. Cv Taiwan A-147 accounted for the lowest Ca value under the MC method, whereas Cv Pioneiro recorded the highest Ca content under the T+MC method (Table 1). Cultivars also behaved differently under the assessed methods; there was a higher Ca absorption under the T+MC method by the Taiwan A-147 and Pioneiro cultivars, whereas the MC and CO methods led to highest Ca content in Cv Mineiro and to intermediate values in Taiwan A-147.

The comparison among the three cuts evidenced that the methods applied to get the seedlings were differently affected by the adopted cuts (Table 1). Cv Mineiro registered higher Ca values, under the T+MC method, at the first cut; values recorded at the second and third cuts were similar and lower. Cultivars Taiwan A-147 and Pioneiro did not suffer the effect of cuts under the T+MC method. Cuts did not influence Cv Mineiro under the MC method. Cv Taiwan A-147 suffered the negative effect from the third cut; it recorded the lowest calcium content. Cv Pioneiro recorded the highest Ca content under the MC method, at the second cut; it recorded similar and inferior results at the first and third cuts. Cuts affected Ca content in the Taiwan A-147 and Mineiro cultivars under the CO method; Mineiro recorded the lowest values at the third cut.

Tested factors Cv, M and CO affected Ca content at $P<0.01$; interaction was recorded for Cv x M, Cv x CUT, M x CUT and Cv x M x CUT at $P<0.01$ (Tables 1 and 2). The herein recorded Ca values under the different tested methods, at the three applied cuts, were similar to those recorded by Próspero and Peixoto (1972), who found 0.14% Ca in elephant grass seedlings at the age of 45 days.

Magnesium content (Mg)

Magnesium (Mg) content was affected by the tested methods in Cv Taiwan A-147, at the first cut; it recorded higher values under the MC and CUT methods and lower values under the T+MC method (Table 1). The comparison of the methods' effect on the cultivars showed that the Taiwan A-147 and Pioneiro cultivars presented the highest Mg values under the T+MC method, whereas the MC and CO methods accounted for similar and lower values in Mineiro and Pioneer cultivars, respectively.

All cultivars were similarly affected by the methods at the second cut. The CO method produced the best Mg absorption outcomes, whereas the other methods led to similar and inferior results (Table 1). The effect of the methods on cultivars' behavior showed that T+MC and MC similarly affected the three of them, whereas the CO method led to the highest Mg content in Mineiro and Pioneiro cultivars.

Table 2: Analysis of variance of the content in dry matter of phosphorus (P), Calcium (Ca), Magnesium (Mg), potassium (K) and sulfur (S), obtained in the evaluation of three cultivars, submitted to three methods of obtaining seedlings and three cuts.

Variation sources	DF	P	Ca	Mg	K	S
Block	5	0.000362	0.000273	0.000906	0.8748	0.000295
Cultivar (Cv)	2	0.006382*[1]	0.003413**	0.004341*	0.8753^ns	0.000160^ns
Error a	**10**	**0.001287**	**0.000114**	**0.000667**	**2.0004**	**0.000329**
Method (M)	2	0.008415**	0.001017**	0.017686**	1.3374^ns	0.001184*
Interaction (Cv x M)	4	0.023940**	0.001007**	0.000717^ns	1.9550^ns	0.000359^ns
Error b	**30**	**0.000971**	**0.000108**	**0.000494**	**0.8169**	**0.000307**
Cutting (CUT)	2	0.364619**	0.007246**	0.048467**	15.9456**	0.015439**
Error c	**10**	**0.001737**	**0.000430**	**0.000740**	**0.7343**	**0.000145**
Interaction (Cv x CUT)	4	0.009097**	0.000631**	0.004854**	2.3871*	0.003639**
Interaction (M x CUT)	4	0.002369*	0.000557**	0.001177^ns	1.5917^ns	0.000048^ns
Interaction (Cv x M x CUT)	8	0.001634*	0.000563**	0.000952^ns	1.3913^ns	0.000371*
Error d	**80**	**0.000687**	**0.000083**	**0.000581**	**0.8833**	**0.000140**
CV a (%)		10.56	8.50	9.22	10.81	11.19
CV b (%)		9.17	8.26	7.93	6.91	10.81
CV c (%)		12.27	16.47	9.70	6.55	7.43
CV d (%)		7.72	7.22	8.60	7.18	7.29
Overall average		**0.340**	**0.126**	**0.280**	**13.09**	**0.16**

[1] ns Not significant. * and ** Significant at 5 and 1% of probability, respectively, by the F test.

Note: As the cuts are made in the same experimental subplot, there is no randomization of the cuts in the different blocks, since the measurements are taken at the same time in the same subplots. As a result, there is no independence of the measures taken over time. In this case, it is not possible to use only one residue to evaluate the effects of the cut and the cut x treatments interaction, being necessary the use of 2 experimental errors, one for each effect. Therefore, the analysis presents 4 errors.

Plot=Cultivar.

Subplot=Method.

Subsubplot=Cut.

The methods adopted to get the seedlings only affected Mg values in Cv Taiwan A-147 at the third cut: the lowest results were recorded under methods T+MC and MC (Table 1). There was behavioral difference between cultivars, under the same method, at this cut. The highest Mg content values recorded for Cv Pioneiro were observed under T+MC method. The MC method did not cause any difference between values recorded for the assessed cultivars, but Cv Taiwan A-147 and Pioneiro showed the highest Mg values under the CO method.

The assessed cultivars showed significant effect on Mg content, at $P<0.05$, under assessed methods (M) and cuts (CUT), at $P<0.01$ level there was interaction between Cv x CUT at $P<0.01$ (Tables 1 and 2). Magnesium (Mg) concentration recorded under the different herein tested methods, cultivars and cuts was more than 100% higher than values recorded by Próspero and Peixoto (1972), who found 0.10% Mg in elephant grass, at the age of 45 days. On the other hand, results in the present study are similar to those recorded by Vélez-Santiago, Arroyo-Aguilú and Rivera (1982), who found 0.30% Mg in elephant grass Cv. Mercker.

Sulfur content (S)

Sulfur content in the assessed cultivars was not affected by the tested methods (Table 1) in association with the adopted cuts, except for Cv Pioneiro, which recorded negative effect on S content in plants subjected to clonal cleanup methods (T+CM and MC), at the third cut, in the 180th experimental day.

The behavior of the assessed cultivars under the same tested methods showed that Mineiro and Taiwan A-147 cultivars presented S levels similar to, and higher than, those recorded for Cv Pioneiro, at the first cut. All cultivars showed similar behavior under the tested methods at the second cut. On the other hand, Pioneiro cultivar recorded higher S content than the other cultivars, at the third cut, under methods T+MC and CO – results recorded for the other cultivars were similar to each other. There was difference in S absorption under the MC method. The comparison of the effect from the three cuts on the cultivars, under each tested method presented progressive decrease in S content from the first to the third cut, under all methods, in Cv Mineiro (Table 1). This finding points out that the cuts had strongly affected this nutrient’s concentration, in this cultivar. Cultivar Taiwan A-147 showed decrease from the first to the second cut under methods T+MC and MC, but values remained similar at the second and third cuts, except for the CO method, which led to progressive decrease in it, from the first to the third cut. The third cut affected S content in Pioneiro cultivar under the MC method, and reduced such a value from the second to the third cut.

Overall, significant effects were detected in S content at $P<0.05$ under the tested methods (M) and at $P<0.01$ under the adopted cuts (CUT); there was interaction between Cv x CUT at $P<0.01$ and between Cv x M x CUT at $P<0.05$ (Tables 1 and 2). Sulfur contents recorded for the three herein assessed cultivars, methods and cuts were higher than those recorded by Próspero and Peixoto (1972), who found 0.15% S in elephant grass, at the age of 45 days.

CONCLUSIONS

Methods to get clonal cleanning did not affected mineral element contents in the assessed elephant grass cultivars in most referenced studies. However, some positive and negative results were observed for them.

Positive effects were observed in cultivar Taiwan A-147 for P content, and in cultivar Pioneiro for Ca content. The present results pointed out that pathogenic agents that have affected the absorption of this element probably were eliminated; however, further studies are needed to validate this observation at molecular level.

The negative effects observed on P and Ca content in Mineiro cultivar; and for cultivar Taiwan A-147 on Mg content may indicate that clonal cleaning methods also eliminated beneficial microorganisms that help these elements' absorption. Yet, further molecular studies are needed to validate this observation.

ACKNOWLEDGMENT

The authors thank CAPES (Coordination for the Improvement of Higher Education Personnel) for the first author scholarship and FAPEMIG (Minas Gerais Research Support Foundation) for the second author scholarship. We also thank BAGCE from Embrapa Gado de Leite for providing the study material. Good Deal revised the text. Technical revisions were by JEBPP and MMGK.

AUTHOR CONTRIBUTIONS

MMGK conceived and designed this research, performed the experiments, draw the tables, and wrote the article. VBT performed the experiments. AVP provided the plant material and performed the thermotherapy process. JEBPP and JCP conceived the research and supervised the experiment. FJSL performed the statistical analysis. All authors read and approved the manuscript.

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