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RESEARCH ARTICLE

Effects of thermotherapy and meristem culture on forage production and nutrition value in elephant grass cultivars

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ABSTRACT

Elephant grass plays important role in dairy cattle production and has received special attention due to its potential in bioenergy and phytoremediation, as well as to its medicinal properties. The aim was investigating the effects of thermotherapy and meristem culture on elephant grass (*Cenchrus purpureus* (Schumach.) Morrone) forage production and nutritional value. Cultivars "Mineiro", "Taiwan A-147" and "Pioneiro" were subjected to the methods: thermotherapy plus meristem culture, meristem culture and mature stems (control). The experiment assessed the tiller number (TN), tiller height (TH), number of leaves/tiller (NLT), leaf/stem ratio (LSR), crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF), at three cuts performed every 60 days for 180 days. It was observed beneficial influence on the evaluated traits, which indicated how cleaning methods work. Cultivar "Mineiro", with more than 20 years of use, was more sensitive to the cleaning methods showing than cultivars "Taiwan A-147" (15 years of use) and "Pioneiro" (less than 10 years). It was observed that methods have affected the NT, TH, SDM, LSR, LDM, CP, and NDF. And, that the cultivars genotype effect in a different way the NT, TH, NLT, SDM, LSR, and CP. Overall, Cleaning methods produced an increase in the evaluated parameters over 100% for the cultivars Mineiro and Taiwan when compared with the traditional method and presented low or negative effect for Pioneiro cultivar This evidenced that the traditional propagation method affects forage production and quality over generations in vegetative propagated species. Basic biotechnology techniques such as meristem culture associated or not with thermotherapy can restore the productive potential being recommended for old asexually propagated cultivars with more than fifteen years of cultivation.

Keywords: Clonal cleaning, micropropagation, reinvigoration, growth dynamics, forage quality, bioenergy production, biofuel production.

INTRODUCTION

Elephant grass is one of the most important forage species used to feed livestock in the tropics; moreover, it is widely used for dairy farming purposes. Several studies focused on evaluating bioenergy production in recent years have described elephant grass as alternative source for biofuel production (Samson et al., 2005; Rocha et al., 2017) due to its high carbohydrate (Azeke, Eze, Ubong, Kuroshi, 2019) and cellulose contents, as well as to its high biomass production and fast growth (Campos et al., 2019). According to Marafon et al. (2020), total calorific value in elephant grass cultivars “Taiwan” and “Cameroon” ranged from 3.905 Kg^{-1} to 4.047 Kg^{-1} and from 4.033 Kg^{-1} to 4.042 Kg^{-1}, respectively; these values are like those recorded for sugarcane.

This forage species presents caespitose perennial-growth habit (it reaches 3-5-meter height), but it has low-quality seeds; thus, its propagation mainly happens in an asexual manner through 100-days-old vegetative stem fragments presenting two to three buds – this process is similar to that undergone by sugarcane plants (Ocumpaugh & Sollenberger, 1995). However, asexual reproduction can lead to issues such as accumulation of certain contaminants like viruses, bacteria, fungi, and mycoplasmas in plants over generations (Wang & Charles, 1991; Negawo et al., 2017). In addition, different symptoms triggered by viruses and mycoplasmas are not visible (Pierik, 1990).

Recent studies reported mosaic virus (Silva et al., 2013), bacterium species *Xanthomonas albilineans* (Meng et al., 2019) and diseases caused by phytoplasma (Kawube et al., 2015; Kogej et al., 2015; Asude et al., 2020; Kogej et al., 2020) in elephant grass. Decreased dry matter production and nutritional quality in plants are some of the effects resulting from pathogen accumulation. Reports issued by Embrapa researchers have corroborated these effects, since they pointed towards apparent forage production decrease in elephant grass (*Cenchrus purpureus*) over the years. Meristem culture is one of the fastest and most efficient techniques adopted to get pathogen-free plants. The hypothesis aimed at explaining that the lack of viral particles in meristematic region is associated with aspects such as the fast growth and development of these tissues, high protein synthesis level, vascular disconnection and the inactivation of viral particles (Kassanis & Varma, 1967; Mellor & Stace-Smith, 1977). This technique is widely used for the clonal cleaning of plants infected with viruses (Kartha &Gambog, 1975; Cronauer & Krikorian, 1985), fungi (Cronauer & Krikorian, 1985), bacteria (Meng et al., 2019) and phytoplasma (Kogej et al., 2020; Accosta et al., 2020; Tiwariet al., 2011).

The aforementioned pathogenic microorganisms often translocate through the phloem, a fact that hinders the use of antibiotics to control them, since they are not fully eliminated, and plants do not remain protected for long periods-of-time (Accosta et al., 2020). Thus, another strategy adopted to fight these microorganisms lies on associating thermotherapy with meristem culture. In the past, several studies have proved the effectiveness of thermotherapy in controlling viruses and mycoplasmas (Smith, 1950; Quak, 1977), since high temperatures hinder pathogen multiplication, and it enables meristematic tissues to undergo mitosis and to keep growing by producing pathogen-free tissues (Oehl & Hughes, 1980). However, new findings have suggested that high temperatures can activate hosts’ defense system through a virus-induced gene silencing (VIGS) process, which can prevent viral infections (Panattoni et al., 2013).

Few studies available in the literature have used elephant grass cultivation *in* *vitro* (Karasawa et al., 2021; Karasawa et al., 2002; Karasawa et al., 2006; Zanete et al., 1988; Herrera et al., 2012; Herrera et al., 2013; Crespo & Alvarez, 2014; Umami et al., 2016). However, to the best of our knowledge, none of these studies has evaluated zootechnical and agronomic traits of materials presenting different use times - which were obtained through meristem culture *in vitro* associated, or not, with thermotherapy, in comparison to the traditional cultivation method (mature stem) to indicate the feasibility of using this technique, as well as the benefits and losses that may result from its use. Thus, the aim of the current study was to assess the effects of thermotherapy and meristem culture techniques on forage production and nutritional value in elephant grass cultivars showing different use times, compared to the on mature stem propagation.

MATERIAL AND METHODS

Thermotherapy

The experiment was carried out in pots kept in greenhouse, at the Zootechnics Department of Federal University of Lavras, Lavras, MG, Brazil (latitude 21°14'30" S; longitude 45°00'10" W; altitude 918 m). The climate in the region is of the Cwb type, with rainy summer and dry winter.

Elephant grass (*Cenchrus purpureus*) cultivars "Mineiro", "Taiwan A-147" and "Pioneiro" deriving from Embrapa Elephant Grass Active Germplasm Bank (BAGCE - Banco Ativo de Germoplasma de Capim-elefante) were used in the experiment. Stems obtained in BAGCE were fractionated into cuttings comprising 3 - 4 buds, planted in 5-liter pots filled with soil and conducted until sprouts reached 20 cm (in height). Subsequently, thermotherapy treatment (T) was implemented at 30°C at night and 45°C during the day, for 20 days (Lassois et al., 2012, modified). After this period was over, the upper 20-cm section of stems presenting meristem was collected and prepared for disinfection.

Meristem culture

Apical stem fragments were disinfected in 1% sodium hypochlorite added with few drops of Tween® detergent, under stirring, for 20 minutes; they were rinsed 3 times with sterile distilled water. Disinfected materials were taken to laminar air flow cabinet and placed in petri dishes with sterile filter paper to remove their meristem under stereoscopic microscope. Isolated individual meristems (0.2 mm long) were removed from the samples and inoculated into 25 mm x 150 mm test tubes filled with 15 ml of MS culture medium (Murashige and Skoog 1962), WPM vitamins (Lloyd and McCown 1980), 3 % of sucrose, and supplemented with 0.7% of agar. The test tubes were sealed with parafilm and incubated under the following light and temperature conditions: 39 µmol m^{-2} s^{-1} and 25±2°C, respectively. The grown material was multiplied by using the best BAP phytoregulator concentration defined by Karasawa et al., (2021, 2002), as well as the best culture consistency medium defined by Karasawa et al., (2006). After grown the explants were rooted and acclimated.

Acclimatization

Seedlings generated *in vitro* from materials deriving from T+MC and MC treatments were removed from the test tubes; their roots were washed and planted in 300-ml plastic cups filled with Plantimax® substrate. Mature stems (ST) were longitudinally fractionated in 1 cm long remaining one bud and three root protuberances. Subsequently, they were planted in 300-ml plastic cups filled with Plantimax® substrate. Seedlings were left to acclimate in greenhouse, under 50% shading, for 30 days.

Plant growing in greenhouse

Acclimated seedlings were transplanted into pots (10 liters) filled with soil classified as red-yellow latosol with the following features: pH in water (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%.

The soil was previously dried, sieved and fertilized with 16.06 g of potassium chloride, 24.45 g of single superphosphate, 3.0 g of urea, 3.0 g of magnesium sulfate, 0.17 g of boric acid, 0.06 g of copper sulfate and 0.01 g of zinc sulfate. Seedlings were irrigated daily and fertilized with N, every 30 days, by alternating ammonium and sulfate sources. Mean temperatures recorded inside the greenhouse ranged from 20°C to 26°C.

Assessments

Three cuts were performed 5 cm above the ground, every 60 days, during the experiment (180 days), to determine the following parameters: tiller number (TN), tiller height (TH), number of leaves/tiller (NLT), leaf/stem ratio (LSR), leaf dry matter (LDM), stem dry matter (SDM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and crude protein content (CP).

The tiller number and the number of leaves per tiller were determined by counting, whereas tiller height was determined by measuring it from ground level to the ligule of the last expanded leaf.

Stem and leaf dry matter (g/Kg) were determined through gravimetric technique based on using heat at two different stages, namely: pre-drying in forced air ventilation oven at 60°C for 72 hours, which was followed by final drying in oven at 105°C for 12 hours (Association of Official Agricultural Chemists [AOAC], 1990).

ADF and NDF contents were determined based on the Van Soest method (A.O.A.C 1990), whereas CP or N content was determined based on the method indicated by the *Association* of *Official Analytical Chemists* (AOAC) (1990).

Experimental design and statistical analysis

Pots were arranged in randomized blocks with six repetitions, whereas treatments were arranged in split-plot desing in space and time (Petersen, 1994).

Plots comprised three elephant grass cultivars (Mineiro, Taiwan A-147 and Pioneiro), whereas subplots comprised the herein analyzed three seedling obtainment methods (T+MC, MC, and ST, with three repetitions). Three cuts performed at 60, 120 and 180 days after planting were allocated in the time subplot.

Statistical analysis was performed at significance levels $P<0.05$ and $P<0.01$ for the F test and for results recorded in the Scott-Knott test.

The herein adopted statistical model was:

yijkl = μ + ci + bj + eij + mk + cmik + eijk + ls + eijks + eijkst
Wherein:
Yijkl= value observed for cultivar *“i”* based on method *“k”* in block *“j”* and cut *“l”*;
μ= general mean;
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 experimental error associated with the plot;
mk= effect of method *“k”* - *k* = 1, 2, 3;
cmik= effect of the interaction between cultivar *“i”* and method *“k”*;
eijk= effect of experimental error associated with the subplot;
ls= effect of cuts *“s”* - *s* = 1, 2, 3;
eijks= experimental error associated with cuts; and
eijkst= experimental error associated with the interaction among cultivars, cuts, and methods.

RESULTS AND DISCUSSION

Tiller number (TN)

Results in the F test - joint analysis (Tables 1 and 3) - have shown statistically significant NT for factors such as cultivar (Cv) and method (M) at $P<0.05$, as well as for cuts (CUT) at $P<0.01$. This analysis has also shown highly significant interactions between factors Cv x M and Cv x CUT, as well as among Cv x M x CUT, at $P<0.01$; this finding has shown that these factors can influence one another. However, there was not interaction between M x CUT (Table 3).

Scott Knott test was applied to the herein tested factors (seedling obtainment methods, cultivars, and cuts); results have shown that these seedling obtainment methods did not statistically affect the NT (Table 1) of the three investigated cultivars at the first cut, which was carried out at 60 days after planting. However, T+MC and MC clonal cleaning methods have shown positive and stronger effects on the NT of Cv. “Mineiro” (more than 20-year use) at the second (at 90 days) and third cuts (at 180 days). Cv. “Taiwan A-147” (approximately 15-year use) has only shown statistically significant difference in NT at the third cut - the MC method recorded the best NT results. On the other hand, Cv. “Pioneiro” (less than 10-year use) recorded the best results for ST.

The MC and T+MC clonal cleaning methods enabled mean tiller density to increase by 126.34% and 137.47% in the oldest cultivar (“Mineiro”), as well as by 128.08% and 114.72% in the intermediate-use cultivar (“Taiwan A-147”). On the other hand, in the long term, these very same clonal cleaning methods reduced by 7.85% and -7.86% the NT of the recent-use cultivar (“Pioneiro”). The larger tiller number observed for cultivars “Mineiro” and “Taiwan A-147” subjected to the investigated clonal cleaning methods is justified, since the older the use of plant propagation materials, the higher the accumulation of pathogenic contaminants in them (Wang & Charles, 1991). On the other hand, the negative effect of the MC and T+MC techniques on the recent-use cultivar may have happened due to the elimination of beneficial microorganisms.

Literature reports have shown that sugarcane meristems ranging from 0.1 mm to 0.2 mm long were able to eliminate viruses (Cheong et al., 2012, Ramgareeb et al., 2010), whereas meristems ranging from 0.2 mm to 0.3 mm long were often capable of eliminating phytoplasmas (Tiwari et al., 2011); micropropagated plants recorded tiller density increase by 88% (Santana et al., 1992) in comparison to the value (30%) recorded for the traditional method (Perez & Rodriguez, 1987). On the other hand, sugarcane meristems ranging from 0.5 mm to 1.5 mm long were capable of effectively eliminating fungi and most bacteria (Cha-um et al., 2006). Moreover, meristems ranging from 0.1 mm to 0.5 mm could eliminate viruses showing increasing cassava yield, in comparison to the control (Deepthi & Makeshkumar, 2016). Since many pathogens do not always manifest themselves in a visible manner (Kumar et al., 2021), and based on results observed in our experiment, assumingly, the application of clonal cleaning techniques – such as 0.2 mm meristem culture - in association (or not) with thermotherapy must have enabed contaminants' removal from elephant grass cultivars.

Based on the comparison of the NT among elephant grass cultivars (Table 1) subjected to the same method and cut, Cv. "Pioneiro" recorded the best NT results at the first cut in all tested methods. At the method T+MC the Cv. "Pioneiro" recorded NT value 144.67% higher than that recorded for Cv. "Mineiro", as well as was 130.81% higher than that recorded for Cv. "Taiwan A-147". For the method MC the Cv. "Pioneiro" recorded NT value 151.13% higher than that observed for Cv. "Mineiro", as well as 116.25% higher than that observed for Cv. "Taiwan A-147". And, for the ST method the Cv. "Pioneiro" recorded tiller density of 200.08% in comparison to Cv. "Mineiro", as well as 160.44% in comparison to Cv. "Taiwan A-147".

At the second cut Cv. "Pioneiro" also recorded NT values higher than those recorded for the other cultivars, in all tested methods, although these values were less expressive than at the first cut. And at the third cut, Cv. "Pioneiro" also recorded higher NT production in all tested methods; however, its tiller density did not statistically differ from that observed for Cv. "Mineiro", when it was subjected to the T+MC method; and it recorded values like those recorded for Cv. "Taiwan A-147", when it was subjected to the MC method.

With respect to NT means observed in all three cuts, Cv. "Pioneiro" subjected to the T+MC method recorded NT value 111.25% higher than that recorded for Cv. "Mineiro", and 119.01% higher than that shown by Cv. "Taiwan A-147". Mean tiller density recorded for Cv. "Pioneiro" subjected to the MC method was 121.06% higher than that observed for Cv. "Mineiro", and 119.01% higher than that evidenced by Cv. "Taiwan A-147". And, for the ST method the mean tiller density recorded for Cv. "Pioneiro" was 165.99% higher than that observed for Cv. "Mineiro", and 148.18% higher than that recorded for Cv. "Taiwan A-147".

Differences observed for tiller density (NT) in cultivars subjected to the herein investigated seedling obtainment methods have evidenced differences in their genetic potential and ability to respond to clonal cleaning methods. Results have indicated that Cv. "Pioneiro" (Table 1), which is the recent-use one, has higher genetic potential for tillering than the other two older cultivars. In addition, the T+MC and MC clonal cleaning methods had positive influence on tiller density, and reduced the difference observed for this parameter in comparison to the ST method. This outcome has indicated that the herein analyzed clonal cleaning methods could restore the tillering potential of older cultivars such as "Mineiro" and "Taiwan A-147". Moreover, it was possible seeing that, in the long term, the investigated clonal cleaning methods had negative effects on tiller density in Cv. "Pioneiro".

Although clonal cleaning was capable of increasing NT in materials used for more than 15 years, it had harmful effects on the tillering of the recent-use cultivar (less than 10-year use in mature stem propagation processes). It is worth emphasizing that clonal cleaning is an efficient method used to eliminate all microorganisms found in plant tissues, including the non-pathogenic ones, such as microorganisms that help nitrogen fixation and soil nutrient absorption processes. This is an important finding since, according to reports, elephant grass can show endophytic nitrogen-fixing bacteria associated with root (Videira et al., 2013; Videira et al., 2012) and apical tissues (Dos Santos et al., 2021); these bacteria act as growth and salt tolerance promoters (Li et al., 2016). The negative effect of the analyzed clonal cleaning methods on TN in the Pioneiro cultivar may have happened due to the elimination of beneficial microorganisms. This factor was also observed for the older materials; however, the effect of pathogenic microorganisms on them may have outweighed the effect of beneficial microorganisms on them, to the extent that the investigated clonal cleaning methods have only shown positive outcomes.

Clonal cleaning studies carried out with sugarcane (*Saccharum officinarum*) recorded tiller density increase by 88% in micropropagated plants (Santana et al., 1992) and by 30% in comparison to plants subjected to the traditional method (mature stem) (Perez & Rodriguez, 1987) and by 12-14% in comparison to seed-borne plants (Anderlini & Kostka, 1986). Micropropagated ginger plants (*Zingiber officinalis*) recorded shoot production increase by 130-140% in comparison to seed-borne plants (Smith & Hamill, 1996). Micropropagated banana plants (*Musa* spp.) belonging to cultivars "New Guinea Cavendish" (Drew & Smith, 1990) and "Chinese Cavendish" recorded increase in shoot production by 10% and 40%, respectively, in comparison to buds obtained from the mother-plant through conventional propagation (Johns, 1994). On the other hand, micropropagated yam plants (*Colocasia esculenta*) recorded budburst 60% higher than that of plants propagated through mature stem (Johnston et al., 1997), whereas micropropagated *Curcuma longa* plants produced 41.5 % more tillers (Salvi et al., 2002).

Studies available in the literature about micropropagated elephant grass plants have only compared clones produced *in vitro* to each other (Crespo & Alvarez, 2014; Herrera et al., 2012; Herrera et al., 2013), without comparing them to plants produced through the traditional method. Reports comparing materials showing different use times in traditional propagation (mature stem) compared with clonal cleaning methods were not found in the literature.

The tiller number in all cultivars has significantly increased from the first to the third cut (Table 1), in all tested methods ($P<0.05$). Cv. "Mineiro" recorded increase in this parameter by 267% and 84.6% (T+MC), by 248.8% and 86.0% (MC), and by 222.2% and 84.3% (ST) at the second and third cuts, respectively. However, Cv. "Taiwan A-147" recorded increase in this parameter by 219.2% and 71.4% (T+MC), by 197.5% and 88.8% (MC), and by 206.3% and 66 % (ST) in these very same same cuts, respectively. On the other hand, Cv. "Pioneiro" recorded increase in this parameter by 177.2% and 81.2% (T+MC), by 180.1% and 78.2% (MC), and by 136.4% and 114% (ST) at the two aforementioned cuts, respectively. Cv. "Mineiro" genotype was more responsive to cutting application than Cv. "Taiwan A-147" and "Pioneiro", respectively; Cv. "Taiwan A-147" was more responsive to cutting than Cv. "Pioneiro".

Cuts were performed 5 cm (in height) above the grond every 60 days, based on reports found in the literature, which have shown that the 60-day interval was the most recommended to enable high yields (Ferraris, 1978). However, Lounglawan et al., (2014) have shown that cuts applied every 45 and 60 days did not show difference in DM and nutrient yield; consequently, cutting height did not influence these parameters. Cuts performed every 60 days in the current study, regardless of the method adopted to obtain seedlings, was an excellent way to increase cultivars' tillering.

Tiller height (TH)

Joint statistical analysis based on the F test has shown that tiller height (Table 1) was significantly affected by cultivars (Cv) and cuts (CUT) at P<0.01 level, as well as by seedling-obtainment methods (M) at P<0.05 level. In addition, there were interactions between Cv x M, Cv x Cu, M x Cu, and among Cv x M x Cu; this outcome has shown that the evaluated factors have influenced TH in a highly significant manner (P<0.01), so both results depended on the involved factors.

The MC seedling-obtainment method has only positively affected TH (Table 1) in Cv. "Pioneiro", at the first cut, which was performed at 60 days, whereas cultivars "Mineiro" and "Taiwan A-147" did not have their TH affected by clonal cleaning methods, such as T+MC and MC. The T+MC and MC methods inhibited Cv. "Taiwan A-147" growth (in height) at the second cut, in comparison to the traditional method, whereas cultivars "Mineiro" and "Taiwan A-147" did not show any different in this parameter, in this same cut. Cv. "Pioneiro" subjected to the MC method presented the tallest plants at the third cut, whereas Cv. "Mineiro" did not show any effect resulting from this treatment; Cv. "Taiwan A-147" subjected to the T+MC method has shown strong HT (height of tiller) inhibition. The MC method had intermediate inhibition effect on HT, whereas the ST method had no inhibitory effect, at all, on this parameter (Table 2).

Studies conducted with ginger (Smith & Hamill, 1996) and papaya (Drew & Vogler, 1993) plants also have shown lower growth in plants subjected to micropropagation. On the other hand, studies conducted with sugarcane (Santana et al., 1992), banana (Drew & Smith, 1990) and *Curcuma longa* (Salvi et al., 2002) plants recorded higher height values for micropropagated plants. Based on the current results, plants' response to the herein applied treatments was mostly associated with their genotype, phenological age and reproductive stage. Such a response can range from lack of effect, as observed for Cv. "Mineiro", to positive influence from treatments, as observed for Cv. "Pioneiro", and to increasing negative influence from treatments as plants age, as observed for Cv. "Taiwan A-147". However, this observation is not conclusive because the investigated cultivars were also affected by cuts applied every 60 days.

With respect to the TH recorded for the cultivars at the first cut, Cv. "Pioneiro" presented the tallest plants in all tested methods (Table 1). Cv. "Pioneiro" subjected to the T+MC method recorded TH 316.45% higher than that observed for Cv. "Mineiro" and 319.03% higher than that recorded for Cv. "Taiwan A-147". Cv. "Pioneiro" subjected to the MC method recorded HT 304.87% higher than that recorded for Cv. "Mineiro" and 275.69% higher than that observed for Cv. "Taiwan A-147". Cv. "Pioneiro" subjected to the ST method recorded TH 372.5% higher than that shown by Cv. "Mineiro" and 293.84% higher than that recorded for Cv. "Taiwan A-147".

With respect to the TH at the second cut, cultivars subjected to the T+MC and MC clonal cleaning methods have shown TH similar to each other, whereas Cv. "Taiwan A-147" subjected to the ST method (mature stem) produced the tallest plants; they were 136.58% higher than those recorded for Cv. "Mineiro" and 128% higher than those observed for Cv. "Pioneiro".

Table 1. Tiller number (TN), tiller height (TH), number of leaves/tiller (NLT), leaf/stem ratio (LSR), Mineiro, Taiwan A-147 and pioneiro, in three cuts (CUT), in relation to the methods (M) of obtaining seedlings (S): thermotherapy + meristem culture (T+MC), meristem culture (MC), and control (ST).

		MINEIRO			TAIWAN A-147			PIONEIRO		
	CUT	**T + MC**	**MC**	**ST**	**T + MC**	**MC**	**ST**	**T + MC**	**MC**	**ST**
TN (n°)	**1º**	15.67 _Cb_**A**	15.00 _Cb_**A**	12.83 _Cc_**A**	17.33 _Cb_**A**	19.50 _Ca_**A**	16.00 _Cb_**A**	22.67 _Ca_**A**	22.67 _Ca_**A**	25.67 _Ca_**A**
	2º	57.50 _Ba_**A**	52.33 _Bb_**A**	41.33 _Bb_**B**	55.33 _Ba_**A**	58.00 _Ba_**A**	49.00 _Bb_**A**	62.83 _Ba_**A**	63.50 _Ba_**A**	60.67 _Ba_**A**
	3º	106.00 _Aa_**A**	97.33 _Ab_**A**	76.17 _Ab_**B**	94.83 _Ab_**B**	109.50 _Aa_**A**	81.33 _Ab_**B**	113.83 _Aa_**B**	113.17 _Aa_**B**	130.00 _Aa_**A**
Factors		**Cv = ***		**M = ***		**CUT = ****				
Interactions		**Cv x M = ****		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ****	
TH (cm)	**1º**	16.17 _Cb_**A**	20.50 _Cb_**A**	15.00_Cb_**A**	17.50 _Bb_**A**	22.67 _Bb_**A**	19.00 _Bb_**A**	51.17 _Aa_**B**	62.50 _Aa_**A**	55.83 _Aa_**B**
	2º	40.33 _Aa_**A**	34.17 _Aa_**A**	39.17 _Ab_**A**	35.83 _Aa_**B**	39.67 _Aa_**B**	53.50 _Aa_**A**	40,33 _Ba_**A**	38.67 _Ba_**A**	41.50 _Bb_**A**
	3º	23.00 _Ba_**A**	25,00 _Bb_**A**	19.50 _Bb_**A**	18.83 _Bb_**C**	25.17 _Bb_**B**	47.00 _Aa_**A**	26,17 _Ca_**B**	33.50 _Ba_**A**	21.83 _Cb_**B**
Factors		**Cv = ****		**M = ***		**CUT = ****				
Interactions		**Cv x M = ****		**Cv x CUT = ****		**M x CUT = ****			**Cv x M x CUT = ****	
NLT (n°)	**1º**	7.50 _Ab_**A**	8.33 _Ab_**A**	7.17 _Ac_**A**	8.00 _Ab_**A**	9.00 _Ab_**A**	9.00 _Ab_**A**	11.83 _Aa_**A**	13.00 _Aa_**A**	12.00 _Aa_**A**
	2º	7.33 _Aa_**A**	7.67 _Ab_**A**	7.50 _Aa_**A**	7.33 _Aa_**B**	11.50 _Aa_**A**	8.17 _Aa_**B**	8.00 _Ba_**A**	7.83 _Bb_**A**	7.33 _Ba_**A**
	3º	8.33 _Aa_**A**	8.00 _Aa_**A**	8.17 _Aa_**A**	8.50 _Aa_**A**	8.00 _Ba_**A**	9.67 _Aa_**A**	8.00 _Ba_**A**	9.33 _Ba_**A**	7.67 _Ba_**A**
Factors		**Cv = ***		**M = ns**		**CUT = ns**				
Interactions		**Cv x M = ns**		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ns**	
LSR	**1º**	1.79 _Aa_**A**	1.65 _Aa_**A**	1.64 _Aa_**A**	1.85 _Aa_**A**	1.42 _Aa_**B**	1.65 _Aa_**A**	0.97 _Ab_**A**	0.98 _Ab_**A**	0.91 _Ab_**A**
	2º	1.15 _Bb_**A**	1.22 _Ba_**A**	1.24 _Ba_**A**	1.33 _Ba_**A**	0.81 _Bb_**B**	0.55 _Bc_**C**	1.01 _Ab_**A**	1.05 _Aa_**A**	0.98 _Ab_**A**
	3º	1.29 _Ba_**A**	1.38 _Ba_**A**	1.25 _Ba_**A**	1.23 _Ba_**A**	1.02 _Bb_**B**	0.75 _Bc_**C**	1.07 _Aa_**A**	1.08 _Ab_**A**	0.91 _Ab_**B**
Fatores		**Cv = ****		**M = ****		**CUT = ****				
Interactions		**Cv x M = ****		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ***	

Capital letters in italics (column) compare the cuts within the method for the same cultivar (Cv), lowercase letters (in the row) compare the cultivars for the same method (M) in each cut, and capital letters in bold (in the row) compare the different methods for an even cultivate and cut. ns = not significant, * = significant at 5%, ** = significant at 1% by the Scott Knott test.

Table 2. Stem dry matter (SDM), Leaf dry matter (LDM), Neutral detergent fiber (NDF), Acid detergent fiber (ADF) and percentage (%) of crude protein (CP) for the cultivars (Cv) Mineiro, Taiwan A-147 and pioneer, in three cuts (CUT), in relation to the methods (M) of obtaining seedlings (S): thermotherapy + meristem culture (T + MC); meristem culture (MC) and control (ST).

		MINEIRO			**TAIWAN A-147**			**PIONEIRO**		
	CUT	**T + MC**	**MC**	**ST**	**T + MC**	**MC**	**ST**	**T + MC**	**MC**	**ST**
LDM	**1º**	13.50 *C*a**A**	13.33 *C*a**A**	9.00 *C*a**B**	12.50 *C*a**A**	12.83 *C*a**A**	12.33 *C*a**A**	12.33 *B*a**A**	11.67 *C*a**A**	10.50 *B*a**A**
	2º	42.50 *B*a**A**	43.67 *B*a**A**	36.17 *B*a**B**	43.50 *B*a**A**	41.33 *B*a**A**	32.33 *B*a**B**	42.17 *A*a**A**	40.17 *B*a**A**	37.50 *A*a**A**
	3º	56.00 *A*a**A**	60.50 *A*a**A**	46.17 *A*a**B**	50.83 *A*a**A**	52.33 *A*a**A**	41.33 *A*a**B**	42.50 *A*b**A**	47.83 *A*b**A**	42.00 *A*a**A**
Factors		**CV = ns**		**M = ****		**CUT = ****				
Interactions		**CV x M = ns**		**CV x CUT = ****		**M x CUT = ***			**CV x M x CUT = ns**	
SDM	**1º**	7.66 *B*b**A**	8.33 *B*b**A**	5.50 *C*b**B**	9.00 *B*b**A**	7.67 *B*b**B**	6.83 *B*b**B**	12.83 *B*a**A**	12.17 *B*a**A**	11.50 *B*a**A**
	2º	37.83 *A*a**A**	36.33 *A*b**A**	29.83 *B*b**B**	58.00 *A*a**A**	55.33 *A*a**A**	34.33 *A*a**B**	42.50 *A*a**A**	38.83 *A*b**A**	39.33 *A*a**A**
	3º	43.50 *A*a**A**	43.83 *A*a**A**	39.00 *A*a**A**	57.33 *A*a**A**	53.67 *A*b**A**	41.33 *A*a**B**	46.33 *A*b**A**	44.50 *A*b**A**	39.83 *A*a**A**
Factors		**Cv = ****		**M = ****		**C UT= ****				
Interactions		**Cv x M = ****		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ***	
NDF	**1º**	82.69 *A*a**A**	81.51 *A*a**A**	84.88 *A*a**A**	81.89 *A*a**A**	79.63 *A*a**A**	76.15 *A*c**B**	81.02 *A*a**A**	81.19 *A*a**A**	82.74 *A*b**A**
	2º	78.73 *A*a**A**	76.49 *B*a**A**	78.05 *B*a**A**	82.25 *A*a**A**	78.61 *A*a**A**	80.40 *A*a**A**	79.66 *A*a**A**	78.94 *A*a**A**	75.11 *B*b**B**
	3º	77.73 *B*b**A**	76.81 *B*a**A**	76.44 *B*a**A**	79,.93 *A*a**A**	77.62 *A*a**B**	77.32 *A*a**B**	77.55 *A*b**A**	78.43 *A*a**A**	76.84 *B*a**A**
Factors		**Cv = ns**		**M = ****		**CUT = ****				
Interactions		**Cv x M = ****		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ***	
ADF	**1º**	34.00 *C*a**A**	34.28 *C*b**A**	35.13 *B*b**A**	34.95 *C*a**A**	33.23 *C*b**A**	34.12 *C*b**A**	35.76 *B*a**A**	37.41 *B*a**A**	38.34 *B*a**A**
	2º	48.77 *A*a**A**	46.68 *A*a**A**	45.45 *A*a**A**	46.85 *A*a**A**	46.18 *A*a**A**	48.87 *A*a**A**	43.80 *A*b**A**	44.13 *A*a**A**	41.59 *A*b**A**
	3º	38.90 *B*a**A**	38.96 *B*a**A**	38.06 *B*a**A**	40.43 *B*a**A**	39.76 *B*a**A**	38.39 *B*a**A**	40.54 *A*a**A**	40.70 *A*a**A**	40.12 *A*a**A**
Factors		**Cv = ns**		**M = ns**		**CUT = ****				
Interations		**Cv x M = ns**		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ns**	
CP	**1º**	20.66 *A*a**B**	21.03 *A*a**B**	23.48 *A*a**A**	21.21 *A*a**A**	21.37 *A*a**A**	22.46 *A*a**A**	18.36 *A*b**B**	19.58 *A*a**A**	19.78 *A*b**A**
	2º	12.86 *B*a**B**	14.44 *B*a**A**	14.60 *B*a**A**	12.83 *B*a**A**	10.67 *B*c**B**	13.05 *B*a**A**	12.36 *B*a**B**	12.23 *B*b**B**	13.51 *B*a**A**
	3º	9.12 *C*a**C**	10.52 *C*a**B**	12.76 *C*a**A**	10.03 *C*a**A**	9.01 *C*b**C**	9.05 *C*b**B**	9.80 *C*a**B**	10.06 *C*a**B**	11.71 *C*a**A**
Factors		**Cv = ****		**M = ****		**CUT = ****				
Interations		**Cv x M = ****		**Cv x CUT = ****		**M x CUT = ns**			**Cv x M x CUT = ****	

Capital letters in italics (column) compare the cuts within the method for the same cultivar, lowercase letters (in the row) compare the cultivars for the same method in each cut, and capital letters in bold (in the row) compare the different methods for an even cultivate and cut. ns = not significant, * = significant at 5%, ** = significant at 1% by the Scott Knott test.

Table 3. Analysis of variance of the tiller number (NT); tiller height (TH); number of leaves/tiller (NLT); stem dry matter (SDM) and leaf (LDM); leaf/stem ratio (LSR); crude protein (CP); neutral detergente fiber (NDF) and acid (ADF); obtained in the evaluation of three cultivars (Cv), submitted to three methods (M) of obtaining seedlings and three cuts (CUT).

SOURCES OF VARIATION	MEAN SQUARES									
	DF	NT	TH	NLT	SDM	LDM	LSR	CP	NDF	ADF
Blocks	5	15.98	58.56	4.999	138.90	100.50	0.02171	1.193	10.240	27.370
Cultivar (Cv)	2	3724.82*	3322.30**	38.117*	856.02**	198.90ns	2.23954**	27.096**	0.608ns	3.575ns
Error a	**10**	**572.77**	**76.47**	**7.777**	**76.83**	**59.79**	**0.04551**	**2.183**	**3.587**	**13.584**
Method (M)	2	90.45*	335.06*	11.191ns	231.04**	620.78**	0.55540**	33.074**	31.531**	0.708ns
Interaction (Cv x M)	4	797.27**	515.99**	3.052ns	442.55**	47.27ns	0.35030**	9.492**	26.602**	6.996ns
Error b	**30**	**196.25**	**63.19**	**5.002**	**28.68**	**23.86**	**0.02807**	**1.447**	**5.169**	**8.990**
Cut (CUT)	2	95440.93**	2628.69**	31.747ns	21491.97**	19943.04**	2.36372**	1647.056**	180.021**	1597.415**
Error c	**10**	**185.37**	**47.63**	**8.184**	**44.09**	**41.54**	**0.04276**	**0.935**	**20.216**	**11.511**
Interaction (Cv x CUT)	4	520.16**	2890.01**	40.525**	317.95**	142.78**	0.90442**	16.852**	63.584**	61.316**
Interaction (M x CUT)	4	150.43ns	197.13**	2.738ns	64.16ns	96.17*	0.07148ns	1.008ns	4.728ns	9.071ns
Interaction (Cv x M x CUT)	8	294.11**	171.55**	5.765ns	112.91*	19.14ns	0.08344*	3.687**	20.929*	12.765ns
Error d	**80**	**83.37**	**55.10**	**6.044**	**41.31**	**31.03**	**0.03232**	**0.928**	**10.058**	**8.878**
CV a (%)		40.64	26.72	32.15	27.42	23.02	17.90	10.06	2.39	9.20
CV b (%)		23.79	24.29	25.79	16.75	14.54	14.06	8.19	2.87	7.49
CV c (%)		23.12	21.09	32.99	20.77	19.19	17.35	6.58	5.68	8.47
CV d (%)		15.50	22.68	28.35	20.10	16.59	15.09	6.56	4.01	7.44
Overall average		**58.90**	**32.72**	**8.67**	**31.97**	**33.59**	**1.192**	**14.69**	**79.17**	**40.05**

DF= degree of freedom, nsNot significant. * and **Significant at 5 and 1% 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.
Sub subplot=Cut.

As for the third cut, the TH of cultivars "Mineiro" and "Pioneiro" subjected to the T+MC method recorded the highest TH; Cv. "Pioneiro" subjected to the MC method recorded the highest TH; and Cv. "Taiwan A-147" subjected to the traditional method produced the tallest plants – this cultivar recorded TH 245.02% higher than that observed for Cv. "Mineiro" and 215.30% higher than that evidenced by Cv. "Pioneiro". On the other hand, Cv. "Taiwan A-147" subjected to the T+MC method produced plants 18.13% shorter than those recorded for Cv. "Mineiro" and 28.04% shorter than those of Cv. "Pioneiro"; whereas Cv. "Pioneiro" subjected to the MC method recorded the highest TH – it was 134% higher than that recorded for Cv. "Mineiro" and 133.09% higher than that observed for Cv. "Taiwan A-147".

Based on the comparison of different cuts, by taking into consideration the same method and cultivar, cultivars "Mineiro" and "Taiwan A-147" overall grew more in height from the first to the second cut; such a growth has decreased afterwards. On the other hand, Cv. "Pioneiro" has shown decrease in TH from the first cut onwards. Cv. "Mineiro" subjected to the T+MC method recorded increase by 249.41% in TH, Cv. "Taiwan A-147" recorded TH increase by 204.74%, whereas Cv. "Pioneiro" recorded TH decrease by 21.18%. According to Lounglawan et al. (2014), the 45- or 60-day cutting interval should not affect plant height. On the other hand, Passos, (1999) has stated that the tallest Napier grass plants would be observed at 90 days because plants at this age would have higher reserve levels. However, we believe that the higher height of the assessed plants was influenced by their reproductive stage, since the flowering period of the investigated cultivars - which happened at different times - coincided with the highest plants belonging to all cultivars and subjected to all tested methods.

Number of leaves per tiller (NLT)

Joint analysis based on the F test ($P<0.05$) has shown that NLT was only significantly affected by factor "cultivar" (Cv), which interacted with CUT, at $P<0.01$ level. This outcome has shown that different cuts affected the investigated cultivars in different ways (Tables 1 and 3).

The analysis of factors has confirmed that NLT was not affected by the investigated seedling-obtainment methods (Table 1) at the first and third cuts. However, Cv. "Taiwan A-147" subjtected to the MC method recorded the largest NLT at the second cut. Similar study conducted with yam plants (Johnston et al., 1997) has shown that micropropagation did not affect NLT in comparison to the conventional propagation method, whereas studies conducted with micropropagated ginger plants has shown smaller NLT than that recorded for seed-borne plants (Smith & Hamill, 1996). Studies conducted with *Curcuma longa* (Salvi et al., 2002) reported increased number of leaves per tiller.

Based on the comparison of cultivars, by taking into consideration the seedling-obtainment methods, Cv. "Pioneiro" subjected to the T+MC method produced the largest NLT at the first cut; NLT results recorded for this cultivar were significantly higher - 157.73% and 147.88% - than those recorded for cultivars "Mineiro" and "Taiwan A-147", respectively (Table 1). In addition, Cv. "Pioneiro" subjected to the MC method recorded NLT values 156.06% and 144.44% higher than those recorded for Cv. "Mineiro" and "Taiwan A-147", respectively. Moreover, the number of leaves per tiller recorded for Cv. "Pioneiro" subjected to the ST method outperformed the number of leaves per tiller recorded for cultivars "Mineiro" and "Taiwan A-147" by 167.36% and 133.33%, respectively. CM was the only method showing significant difference in NLT at the second cut; Cv. "Taiwan A-147" subjected to this method recorded NLT production 140.93% higher than that recorded for Cv. “Mineiro” and 146.87% higher than that observed for Cv. “Pioneiro”. Difference in NLT was not observed at the third cut.

Based on the comparison among different cuts, it appears that “Pioneiro” was the only cultivar showing consistent effect on NLT between the first and the second cuts. NLT has decreased by 32.38% in plants subjected to the T+MC method, by 39.77% in plants subjected to the MC method and by 38.91% in plants subjected to the ST method (mature stem). It is important to mention that the trait “number of leaves per tiller” is often under genetic control. However, the reproductive stage of cultivar “Pioneiro”, which happened at the first cut, may also have influenced this character. Or, yet, it may have happened due to some random factor that did not have plausible explanation at this time.

Animal nutrition requires the highest NLT possible since leaf is the organ where nutrients are concentrated in, as well as the most digestible and nutritious fraction of plants. Since this trait is, overall, of the genetic control type, one should focus on selecting the genotypes showing the largest NLT. However, since cuts also enable significant increase in the tiller number, this factor should be evaluated before making the decision about the genotype to be used to achieve the highest yield per planted area. On the other hand, older-use cultivars recorded increased tiller number in micropropagated plants. Thus, using clonal cleaning methods appears to be the most appropriate way to increase leaf production in old cultivars propagated through mature stems.

Leaf / Stem ratio (LSR)

Joint analysis applied to LSR (Tables 1 and 3), based on the F test, recorded statistically significant results for factors Cv., M and CUT, at $P<0.01$ level. Cv x M and Cv x Cu interactions were also observed, at $P<0.01$ level; this outcome has evidenced that these factors have strong influence on one another. Cv x M x Cu interaction was significant at $P<0.05$; this finding has evidenced the mutual influence of the evaluated factors on effects observed in the investigated cultivars.

Based on the analysis applied to factors, the investigated seedling-obtainment methods have significantly affected LSR in Cv. “Taiwan A-147”, at the first cut; T+MC and ST recorded the highest LSR values (Table 1). Seedling-obtainment methods have positively influenced LSR in Cv. “Taiwan A-147” (Table 1) - the T+MC method enabled LSR increase by 241.82% and 164%, whereas the MC method enabled LSR increase by 147.27% and 136%, at the second and third cuts, respectively. The LSR of Cv. “Pioneiro” was only influenced by clonal cleaning methods at the third cut – it recorded LSR increase by 117.58%, on average, whereas the LSR in Cv. “Mineiro” remained unchanged, regardless of the applied method.

Based on responses from all three cultivars to the same method, cultivars “Mineiro” and “Taiwan A-147” subjected to the T+MC method recorded LSR value 187.63% higher than that recorded for Cv. “Pioneiro” (Table 1), at the first cut. Whereas the very same cultivars subjected to the MC method recorded LSR value 156.63% higher than that observed for Cv. “Pioneiro”, on average, and those subjected to the ST method recorded LSR value 180.76% higher than that shown by Cv. “Pioneiro”, on average.

With respect to the second cut, Cv. “Taiwan A-147” subjected to the T+MC method recorded LSR 115.65% higher thant that recorded for Cv. “Mineiro” and 131.68% higher thant that observed for Cv. “Pioneiro”; cultivars “Mineiro” and “Pioneiro” subjected to the MC method recorded LSR 140.12% higher than that shown by Cv. “Taiwan A-147”, on average. Whereas, cultivar Mineiro subjected to the ST method recorded the highest LSR; it was 225.45% higher than that recorded for Cv. “Taiwan A-147” and 126.53% higher than that evidenced by Cv. “Pioniero”.

MC and ST methods had effect on LSR at the third cut. Cv. “Mineiro” subjected to the MC method recorded LSR 135.29% higher than that observed for Cv. “Taiwan A-147” and 127.78% higher than that shown by Cv. “Pioneiro”; whereas Cv. “Mineiro” subjected to the ST method recorded LSR 166.67% higher than that recorded for Cv. “Taiwan A-147” and 137.36% higher thant that observed for Cv. “Pioneiro”.

Based on the comparison of all three cuts, by taking into consideration each method and the same cultivar (Table 1), all evaluated methods had negative effect on LSR values recorded for cultivars “Mineiro” and “Taiwan A-147”. Plants subjected to the T+MC and MC methods have shown consistent decrease in LSR by 35.74% and 24.39% (Cv. “Mineiro”), and by 28.10% and 66.67% (Cv. “Taiwan A-147”) from the first to the second cut, respectively, although LSR values recorded for the aforementioned cultivars remained unchanged from the second to the third cut. Cv. “Pioneiro” was indifferent to the applied cuts, since its LSR remained constant in all tested methods.

Results observed for cultivars “Mineiro” and “Taiwan A-147” corroborated the study by Mora & Silva, (1988), who reported reduced number of leaves after the reproductive stage and as plants aged; however, it is worth mentioning that Cv. “Pioneiro” did not show the same behavior in the current study. Santos et al. (1994) recorded LSR 0.76 for Cv. “Mineiro” and 0.82 for Cv. “Taiwan A-147”, whereas Santana et al. (1989) recorded LSR values 0.53 and 0.58 for the same cultivars, respectively. Zailan et al. (2018) conducted a study with elephant grass varieties in Malaysia and recorded LSR values ranging from 0.76 to 3.18. According to LSR values higher than 1.0 are the desirable ones because they indicate the high-quality forage. Thus, cultivars such as “Taiwan A-147” must be subjected to the T+MC method in order to reach the highest LSR. All three assessments conducted over 180 days in the current study have show mean LSR values higher than, or very close to, 1.0 in all tested methods. We believe that it happened because the herein used elephant grass was grown in pots, in greenhouse environment, under controlled conditions that have kept both temperature and humidity relatively constant. In addition, soil analyses were carried out and fertilization was performed as recommended.

Leaf dry matter (LDM)

Joint analysis applied to LDM, based on the F test, has indicated statistically significant values for factors M and CUT, at $P<0.01$ level (Table 2 and 3), as well as Cv x CUT ($P<0.01$) and M x CUT ($P <0.05$) interactions. This outcome has shown that both methods and cultivars were affected by cut applications.

Clonal cleaning methods had positive effect on LDM (Table 2) in Cv. “Mineiro” (more than 20-year use) at the first cut; T+MC and MC produced the highest LDM values - these cleaning methods recorded LDM 149.06% higher than that of ST. Cv. “Pioneiro” (most recent use) subjected to these very same methods recorded LDM increase by 114.28%, on average. Whereas, Cv. “Taiwan A-147” was not affected by them. Cultivars “Mineiro” and “Taiwan A-147” subjected to the T+MC and MC methods recorded LDM similar to, and higher than, that of the traditional method at the second cut, respectively, whereas the LDM recorded for cultivar “Pioneiro” (recent use) was not affected by these methods. Cultivar “Mineiro” subjected to the herein investigated clonal cleaning methods recorded LDM increase by 119.11%, on average, whereas Cv. “Taiwan A-147” recorded LDM increase by 131.19%. T+MC and MC methods promoted LDM increase by 126.16% in Cv. “Mineiro” and by 124.80% in Cv. “Pioneiro” at the third cut, in comparison to the traditional method (ST).

Cultivars subjected to the same seedling-obtainment methods did not show difference in LDM, neither at the first nor at the second cut (Table 2). However, cultivars “Mineiro” and “Taiwan A-147” subjected to the T+MC and MC methods recorded mean LDM yield 125.68% and 117.95% higher than that observed for Cv. “Pioneiro” at the third cut, respectively, whereas all cultivars subjected to the ST method have shown yield potential similar to each other. This outcome has evidenced that the LDM yield potential of genotypes “Mineiro” and “Taiwan A-147” was higher than that recorded for genotype “Pioneiro”. In addition, it emphasized the importance of using clonal cleaning methods in old-propagated plants (more than 15 years) by traditional propagation methods.

By comparing the effect of cuts on LDM production (Table 2), it was possible seeing that they led to significant LDM increase, from the first to the third cut, in cultivars “Mineiro” and “Taiwan A-147” subjected to all methods. The second and third cuts enabled LDM increase by 314.81% and 131.76% in Cv. “Mineiro” subjected to the T+MC method, respectively; cultivars subjected to the MC method recorded LDM increase by 327.61% and 138.54% at the second and third cuts, whereas cultivars subjected to the ST method recorded LDM increase by 401.89% and 127.65%, respectively. The T+MC method produced LDM increase by 348% and 116.85% in Cv. “Taiwan A-147”, at the second and third cuts, respectively, whereas the MC method produced LDM increase by 322.14% and 126.62%. The ST method produced LDM increase by 262.21% and 127.84%, in these very same cuts, respectively. This same behavior was only observed for Cv. “Pioneiro” subjected to the MC method – it recorded LDM increase by 344.22% and 119.07%, at the second and third cuts, respectively, whereas Cv. “Pioneiro” subjected to the other two methods (T+MC and ST) recorded LDM increase by 342.01% and 357.14%, only from the first to the second cut, respectively.

According to Lounglawan et al. (2014), the highest DM yield would be achieved in cuts performed every 45 or 60 days, although the cutting height did not will influence these parameters. On the other hand, Uvidia et al. (2015) reported highly significant increase in DM yield as plants aged, whereas Mora & Silva, (1981) reported decreased leaf production from flowering onwards, and as plants aged. The current study has shown that cuts carried out at 5 cm from the ground, every 60 days, provided significant increase in LDM yield, as mentioned above. The highest LDM increase observed for all investigated cultivars and methods took place between the first and second cuts. Results reported in the current research were similar to those recorded by Passos et al. (2001) for cuts performed in cultivars “Mineiro” and “Pioneiro”, every 60 days. Leaf is the plant fraction mostly appreciated in animal nutrition, since it is the plant part holding nutrients, as well as the most digestible plant fraction (Wilson & Minson, 1980). Thus, increasing the production of this fraction means improving forage quality.

Stem dry matter (SDM)

Cv, M and CUT had statistically significant effect on SDM yield (Tables 2 and 3); there was Cv x M and Cv x CUT interaction, at $P<0.01$ level, as well as Cv x M x CUT interaction, at $P <0.05$ level.

Seedling-obtainment methods have only influenced SDM in cultivars “Mineiro” and “Taiwan A-147”. T+MC and MC methods applied to Cv. “Mineiro” produced SDM higher than that produced by the ST method (Table 2) – they recorded SDM increase by 139.27% and 141.45%, on average, at the first cut, respectively; these very same methods applied to Cv. “Taiwan A-147” produced SDM increase by 131.77% and 112.29%, on average, at the first cut, respectively. Cultivar “Mineiro” recorded proportionate SDM increase by 126.82% (T+MC method) and 121.79% (MC method) at the second cut, whereas Cv. “Taiwan A-147” recorded SDM increase by 168.95% (T+MC method) and 161.17 % (MC method). At the third cut, both the cultivars “Mineiro” and “Taiwan A-147” subjected to the cleaning methods recorded SDM increase by 111.54% and 112.38%, respectively for T+MC and MC.

Cultivar “Pioneiro” has shown significantly higher SDM yield at the first cut, in response to all tested methods (Table 2). Cultivars’ response to the seedling-obtainment methods at the second cut has shown statistically significant difference in plants subjected to the MC method, since Cv. “Taiwan A-147” recorded SDM yield higher than that observed for the other cultivars, whereas cultivars “Taiwan A-147” and “Pioneiro” subjected to the ST method recorded SDM yield similar to, and higher than, that shown by Cv. “Mineiro”, respectively. Cultivars have also shown differential responses to the investigated seedling-obtainement methods at the third cut; cultivars “Mineiro” and “Taiwan A-147” subjected to the T+MC method recorded similar and higher yields, whereas Cv. “Mineiro” subjected to the MC method was the cultivar recording the highest SDM yield. The traditional method (ST) did not produce differences in SDM yield among the investigated cultivars.

The herein applied cuts had impact on the SDM of all investigated cultivars, which overall recorded significant increase in this parameter from the first to the second cut, except for Cv. “Mineiro’ subjected to the ST method, which recorded progressive SDM increase at all cuts (Table 2).

According to Lounglawan et al. (2014), cuts performed every 45 or 60 days enabled the highest total DM yield, whereas cut height did not influence this parameter. Uvidia et al., (2015) reported highly significant increase in DM yield as cultivars aged. On the other hand, Smith and Hamil (1996) have compared micropropagated ginger plants to seed-borne plants and found decreased SDM yield in the micropropagated ones.

Reduced SDM is desirable in animal nutrition, since it is where the largest portion of indegestible fibers – such as cellulose, hemicellulose (Mohammed et al., 2015) and lignin – are often found. However, the fraction of these components plays important role in biofuel production since they are directly linked to the total calorific value produced in these processes (Marafon et al., 2020), a fact that makes the increased SDM yield provided by clonal cleaning methods beneficial and quite interesting.

Neutral detergent fiber (NDF)

The analysis applied to NDF content, based on the F test, has shown statistically significant results for M and CUT at $P<0.01$ level. There was Cv x M and Cv x CUT interaction, at $P<0.01$ level, as well as Cv x M x CUT interaction at $P<0.05$ level (Tables 2 and 3).

The seedling-obtainment methods have only affected NDF contents in Cv. “Taiwan A-147” (Table 2). T+MC and MC provided NDF contents similar to, and higher than, those recorded for plants subjected to the ST method. Based on the comparison of NDF contents among cultivars, by taking into consideration the adopted seedling-obtainment methods, ST was the only method showing difference in NDF contents. The best result was recorded for Cv. “Taiwan A-147”, which recorded the lowest NDF contents; it was followed by cultivars "Pioneiro' and "Mineiro".

The T+MC and MC methods provided NDF content similar to, and higher than, that of Cv. "Pioneiro" at the second cut, whereas the two other cultivars did not show any difference in this parameter. Cultivars subjected to the T+MC and MC methods recorded NDF production similar to each other, whereas the ST method only Cvs Taiwan A-147 and Pioneiro showed similar results.

The tested seedling-obtainment methods at the third cut affected NDF contents in Cv. "Taiwan A-147"; NDF values recorded for plants subjected to the T+MC method were higher than those recorded for plants subjected to the other two methods. Cultivars subjected to the T+MC method have also behaved differently; cultivars "Pioneiro" and "Mineiro' recorded the lowest NDF contents.

Pereira et al. (2000) have shown increased NDF contents as plants aged; whereas Lounglawan et al., (2014) have shown that cuts performed every 45 or 60 days produced the highest NDF contents. However, cut height did not affect neutral detergent fiber contents. Zailan et al. (2018) recorded NDF values ranging from 66.11% to 71.59% in plants cutted at 20 cm from the ground, whose age ranged from 45 to 60 days. On the other hand, Habte et al., (2020) recorded NDF values ranging from 61% to 67% in the dry season, as well as from 63% to 69% in the rainy season. The aforementioned values were lower than those recorded in the current study. According to Alves et al. (2020) the lowest NDF content was recorded for 60-day-old plants and the highest one, for 90-day-old plants; thus, it is recommended using 60-day-old plants for animal feeding purposes, whereas 90-day-old plants should be used for biofuel production purposes.

Acid detergent fiber (ADF)

The ADF analysis has only shown statistically significant difference for CUT at $P<0.01$ level, as well as Cv x CUT interaction at $P<0.01$ level. Likewise, the herein adopted seedling-obtainment methods did not affect ADF levels in the investigated cultivars (Table 2). However, there was difference in cultivars' response to the applied methods; the lowest ADF level was recorded for Cv. "Pioneiro" subjected to T+MC.

The comparison among all three cuts has shown different behaviors among cultivars towards the tested methods (Table 2). Cultivar "Mineiro" subjected to T+MC and MC has shown increased ADF levels from the first to the third cut, whereas plants subjected to the ST method have shown increased ADF content from the first to the second cut, although it has subsequently decreased. Cv. "Taiwan A-147" recorded the lowest ADF contents at the first cut, and Cv. "Pioneiro" subjected to all tested methods recorded significant increase in ADF content from the first to the second cut.

Pereira et al. (2000) have shown increase in ADF content as plants aged, whereas Lounglawan et al. (2014) have shown that cuts performed every 45 or 60 days provided the highest ADF contents, as well as that cutting height did not influence this parameter. However, Alves et al., (2020) recorded the highest ADF values at 90 days. ADF values recorded in the current study were close to those reported by Zailan et al. (2018), who observed variation in ADF content ranging from 36% to 41.22% in 45 to 60-day-old plants that have been cut 20 cm from the ground. On the other hand, Habte et al. (2020) recorded ADF content ranging from 29% to 32% in the dry season, as well as from 34% to 40% in the rainy season.

According to Macedo Júnior et al. (2007), there is decrease in the number of leaves and increase in the proportion of stems as plants get older; consequently, the proportion of structural compounds in plants' cell wall, such as lignin, cellulose, and hemicellulose, which hinders their digestibility, also increases. Thus, Alves et al., (2020) recommended grazing and cutting to be performed at 60 days if plants are going to be used for animal feeding purposes, as well as cutting at 90 days if plants are going to be used for biofuel production purposes; this species has been described among the ones with the potential to due to its high cellulose level (Campos et al., 2019; Rocha et al., 2017; Azeke et al., 2019).

Crude protein (CP)

CP production was significantly affected by cultivars (Cv), methods (M) and cuts (CUT), at P<0.01 level. Interaction was also identified between the following factors: Cv x M, Cv x CUT, as well as among Cv x M x CUT, at P<0.01 level.

The T+MC and MC methods have negatively influenced CP production in Cv. "Mineiro" at the first cut, whereas only T+CM had negative influence on CP production in Cv. "Pioneiro"; Cv. "Taiwan A-147" did not show difference in CP production, regardless of the applied method (Table 2). CP production recorded for cultivars "Mineiro" and "Taiwan A-147" was similar to, and higher than, that recorded for Cv. "Pioneiro" in plants subjected to the T+MC and MC methods, at the first cut, respectively.

At the second cut T+MC was the only method producing negative effects on CP production in Cv. "Mineiro", whereas MC was the only method negatively affecting CP content in Cv. "Taiwan A-147". For Cv. "Pioneiro" the T+MC and MC methods, in their turn, reduced CP contents (Table 2). Cultivars have also shown different responses to the applied methods; the MC method produced the highest CP content in Cv. "Mineiro", intermediate CP content in Cv. "Pioneiro" and the lowest CP content in Cv. "Taiwan A-147".

At the third cut, the ST method produced the highest CP content in Cv. "Mineiro", whereas the MC method produced intermediate CP content and T+CM produced the lowest CP content in this cultivar (Table 2). Cv. "Taiwan A-147" presented inverse behavior, since the T+MC method produced the highest CP content in this cultivar, ST produced intermediate CP content and MC produced the lowest content of it. And Cv. "Pioneiro" subjected to the ST method has also recorded the highest CP content, whereas plants subjected to the T+MC and MC methods recorded lower CP contents, although they did not significantly differ from each other.

Based on the evaluation of all three cuts, it appears that all methods led to CP content decrease by approximately 50% in all investigated cultivars, from the first to the third cut. Pereira et al. (2000), Lounglawan et al. (2014) and Alves et al. (2020) have shown that the CP content has decreased as the cutting interval got longer, as well as that cutting height did not influence this parameter. On the other hand, Uvidia et al. (2015) reported CP content increase by 7% in 75-day-old plants; however, they have emphasized that although plants' age had significant effect on CP production increase, it has also reduced quality.

CONCLUSIONS

Applying meristem culture and thermotherapy showed to produce greater effect on the old cultivars Mineiro, intermediary effect over Taiwan A-147, and negative effect for the cultivar Pioneiro. This evidenced that the traditional propagation method affects forage production and quality over generations.

Basic biotechnology techniques such as meristem culture associated or not with thermotherapy can restore the productive potential being recommended for old asexually propagated cultivars with more than fifteen years of cultivation.

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