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Carbohydrate sources, alanine and calcium for *in vitro* multiplication of *Eucalyptus cloeziana* F. Muell.

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ABSTRACT - *Eucalyptus cloeziana* timber is used for the production of coal and at sawmills. The aims of this study were to evaluate the presence of different levels of sucrose, glucose, combinations of glucose and sucrose, alanine and calcium on the *in vitro* multiplication of *E. cloeziana*. The best level of sucrose was 15 g L^{-1}. The best level of glucose was also 15 g L^{-1} and it presents reduction in the oxidation of the tissues. Glucose and sucrose combinations did not present suitable results. The presence of L-alanine do not favor the multiplication. The best result for calcium supplementation was found at 1,112 mg L^{-1} $Ca(NO_3)_2.4H_2O$ and 192 mg L^{-1} $CaCl_2.2H_2O$ on solid medium, but the liquid medium avoid the oxidation of the explants.

Keywords: amino acid, eucalypt, glucose, micropropagation

RESUMO – Fonte de carboidratos, alanina e cálcio para multiplicação *in vitro* de *Eucalyptus cloeziana* F. Muell. A madeira do *Eucalyptus cloeziana* é usada para a produção de carvão em serrarias. Os objetivos desse estudo foram avaliar a presença de diferentes níveis de sacarose, glicose, combinações de glicose e sacarose, alanina e cálcio na multiplicação *in vitro* de *E. cloeziana*. A melhor concentração de sacarose obtida foi 15 g L^{-1}. A melhor concentração de glicose também foi 15 g L^{-1} e apresentou redução na oxidação dos tecidos. Combinações de glicose e sacarose não apresentaram resultados satisfatórios. A presença de alanina não favoreceu a multiplicação. O melhor resultado da suplementação com cálcio foi encontrado com 1,112 mg L^{-1} $Ca(NO_3)_2.4H_2O$ e 192 mg L^{-1} $CaCl_2.2H_2O$ no meio sólido, porém o meio líquido evitou a oxidação dos explantes.

Palavras-Chave: aminoácido, eucalipto, glicose, micropropagação

INTRODUCTION

Trees of the genus *Eucalyptus* are the woody species most cultivated in the world (Trueman *et al.* 2018), among them, the *Eucalyptus cloeziana* F. Muell. presents a durable timber, with density suitable for the production of coal and with excellent quality for sawmills (Alfenas *et al.* 2009), besides to be rustic and presenting a fast growth. However, this species demonstrates difficulties for vegetative propagation, especially with regard to their ability to rooting. An alternative to overcome this problem is the micropropagation, which is a technique of *in vitro* propagation, where the small explants (*i.e.* tissue pieces used to establish a culture) are cultivated in an axenic environment.

In vitro multiplication of plants is a phase of micropropagation. Several compounds can influence the *in vitro* multiplication phase, such as the presence of plant growth regulators (Oliveira *et al.* 2010, Resende *et al.* 2016, Lv *et al.* 2017, Alkowni *et al.* 2017), amino acids (Carvalho *et al.* 2013), carbohydrates (Hossain *et al.* 2005, Abdullah *et al.* 2013), vitamins (Silva *et al.* 2007, 2008, 2009), salts (George *et al.* 2008, Aranda-Peres *et al.* 2009, Machado *et al.* 2014), culture medium type (Ferreira *et al.* 2017), consistency of the culture medium (Oliveira *et al.* 2010, Silva *et al.* 2012), microbial fermented extract (Silva *et al.* 2013, Gollo *et al.* 2016), charcoal activate (North *et al.* 2012, Cardoso-Furlan *et al.* 2018), antioxidants (North *et al.* 2012) and gas exchange (Cassells & Walsh 1994).

In tissue culture, carbohydrate sources play an important role as a source of energy and for maintaining the osmotic potential (Cuenca & Vieitez 2000). Sucrose is a non-reducing sugar and these type of sugars are less reactive than the reducing sugars (Carvalho *et al.* 2013). Therefore, non-reducing sugars are more translocated (Taiz & Zeiger 2004). On the other hand, glucose, which is a

reducing sugar, can be readily taken up providing a better carbon source, by diffusion across the cut tissues surfaces and metabolized by *in vitro* tissue culture (Welander & Pawlicki 1994).

Organic nitrogen is very important for the cellular growth and amino acids have been used as the source of nitrogen in the culture medium (Behrend & Mateles 1975). Alanine is an aliphatic amino acid used for biosynthesis of proteins. The nitrogen from amino acids is assimilated more quickly by the carbonic skeletons during the metabolism and synthesis of proteins, when compared to the nitrogen from the inorganic sources (Carvalho *et al.* 2004). Calcium is a macro element associated to the processes such as membrane structure and function, ions uptake, reactions with plant growth regulators and enzymatic activation (via calmodulin) (Malavolta *et al.* 1997).

Therefore, the aims of this study were to evaluate the presence of different levels of sucrose, glucose, glucose and sucrose combinations, alanine and calcium on the *in vitro* multiplication of *Eucalyptus cloeziana*.

MATERIAL AND METHODS

Plant material and source of explants

The seeds of *E. cloeziana* cv. LCFA026-IPEF, were originated from an experimental area located in Anhembi, SP, Brazil. The germination and *in vitro* multiplication were carried out according to the protocol established by Zorz (2016) and the explants used came from isolated shoots (0.7 mm length) originating from multiple shoots (*i.e.*, agglomerates or clumps).

In vitro multiplication on different carbohydrates, L-Alanine and calcium levels

The basal medium consisted of Woody Plant Medium (WPM) (Lloyd & McCown 1981) supplemented with 0.5 mg L^{-1} 6-Benzylaminopurine (BAP) and 0.05 mg L^{-1} 1-Naphthaleneacetic acid (NAA). The culture medium was solidified with 6 g L^{-1} agar before autoclaving. In order to evaluate the effects of sucrose on the *in vitro* multiplication, the following treatments were used 0, 15, 30, 45 and 60 g L^{-1}. For the effects of glucose on the multiplication were used the following levels (0, 15, 30, 45 and 60 g L^{-1} glucose). To evaluate the combination of glucose and sucrose were used the following levels (0, 7.5, 15.0, 22.5 and 30.0 g L^{-1} for each carbohydrate).

To analyze the influence of L-Alanine on multiplication, the concentrations of 0, 20, 40, 80 and 160 mg L^{-1} were tested and the basal medium was supplemented with 30 g L^{-1} sucrose. The experiment with different levels of calcium consisted in a two-way analysis of variance (ANOVA): Factor A was the medium consistency (*i.e.* solid and liquid) and the factor B were the calcium levels (120.54, 241.08 and 361.62 mg L^{-1} Ca). These calcium levels were obtained by the supplementation of the media with combinations of $Ca(NO_3)_2.4H_2O$ and $CaCl_2.2H_2O$. To obtain the calcium levels described above were used the following concentrations of 556, 1,112 and 1,668 mg L^{-1} $Ca(NO_3)_2.4H_2O$ and 96, 192 and 288 mg L^{-1} $CaCl_2.2H_2O$, respectively. The level of 120.54 mg L^{-1} Ca was the original level of the WPM medium (treatment control). The culture medium used in the calcium experiment were added with 30 g L^{-1} sucrose and solid medium was solidified with 6 g L^{-1} agar before autoclaving. The liquid medium was performed under stationary culture (*i.e. in vitro* culture without agitation) and the explants remained immersed with only one third in the culture medium.

In all the experiments, the survival percentage, oxidation percentage, callogenesis percentage, shoot number and explant length (cm) were evaluated after 30 days of *in vitro* culture.

Culture conditions and statistical analysis

All the media had their pH adjusted to 5.8 and were autoclaved at 1 kgf cm^{-2} and 120ºC for 20 min. The cultures were kept at 25 ± 2ºC under white fluorescent light (32 µmol m^{-2} s^{-1}) with a 16 h photoperiod. The treatments consisted of five replicates with five explants. The data was submitted to the Hartley´s test ($P<0.05$) and Lilliefors´s test ($P<0.05$), followed by analysis of variance (ANOVA) followed by Duncan´s test, both at the levels of $P<0.05$. Variables from counting were transformed to and variables from percentage were transformed to arcsinor $(n+0.5)^{0.5}$ according with the requirement. The statistical analyses were performed using the software SOC (Empresa Brasileira de Pesquisa Agropecuária 1990). The culture media used in all the experiments were placed in test tubes (2×10 cm) and were sealed with aluminum foil caps (the culture medium volume used was 6 mL per tube).

RESULTS AND DISCUSSION

Effects of sucrose on the *in vitro* multiplication

The presence of different levels of sucrose on the multiplication medium of *E. cloeziana* does not influence the explant length from 0 to 30 g L^{-1}, but concentrations of sucrose higher than 45 g L^{-1} decreased the length of the explants (Table 1). This result can be explained due to the low osmotic potential originated by high sucrose levels, which resulted in lowest water and nutrients uptake. Similar results were found in maize (*Zea mays* L.), which levels equals or above 50 g L^{-1} of sucrose decreased the shoot length (Gauchan *et al.* 2012) and in fox grape (*Vitis labrusca* L.), the level of 45 g L^{-1} sucrose also decreased shoot length (Carvalho *et al.* 2013).

The best concentration for shoot number was 15 g L^{-1} of sucrose, reaching 2.2 shoots per explant, but it does not differ of 0 and 30 g L^{-1}, resulting in 1.4-1.6 shoots per explant, respectively (Table 1). Normally, *Eucalyptus* species are *in vitro* cultivated at 30 g L^{-1} sucrose, as observed in *Eucalyptus benthamii* Maiden & Cambage (Brondani *et al.* 2013), *Eucalyptus globulus* Labill. (Cordeiro *et al.* 2014) and *Eucalyptus saligna* Sm. (Silva *et al.* 2015). Nevertheless, it is possible that *E. cloeziana* be more

sensitive to higher osmotic potential than other species or it requires a minor amount of energy.

A visual evaluation was made of the presence or absence of oxidation, where it was characterized by the release of phenolic compounds in the culture medium leaving it blackened due to the oxidizing substances (Assis *et al.* 2004). For the percentage of oxidation there were no statistical differences as well as for survival and callogenesis (Table 1).

Effects of glucose on the *in vitro* multiplication

The best result was found at 15 g L^{-1} glucose, which there was the highest shoot number per explant (5.5) and there was a suitable decrease in the oxidation (18%) (Table 2). Similar results were found in cork oak (*Quercus suber* L.), which the highest number of shoots occurred on glucose-containing medium (Romano *et al.* 1995) and in *Fagus sylvatica* L., whereas glucose presented the best result as carbon source for the *in vitro* multiplication (*i.e.* largest shoot number), when compared with different carbon sources (sucrose and fructose) (Cuenca & Vieitez 2000).

High levels of carbohydrates can stimulate the production of phenolic compounds (Zaprometov 1978), wherein these compounds induces oxidation in explants, what can result in low multiplication rate or death of explants. Moreover, reducing sugars such as glucose can be readily taken up and metabolized, potentially providing a better carbon source (Welander & Pawlicki 1994). Among the alternatives to avoid or reduce oxidation is recommended the addition of antioxidants, such as citric acid, ascorbic acid, activated charcoal or other antioxidants in the culture medium (Bassan *et al.* 2006, Werner *et al.* 2009).

The absence of glucose avoids the oxidation occurrence, but it decreased the shoot number per explant (3.7) (Table 2). The glucose levels does not interfered on the survival percentage of the explants and the absence of glucose on the culture medium has been decreased the callogenesis percentage (Table 2). The increase in glucose levels decreases the shoot number per explant and the lack of glucose and its excess (60 g L^{-1}) decreased the shoot length (Table 2).

Effects of sucrose and glucose combinations on the *in vitro* multiplication

The increase of glucose and sucrose levels resulted in decreases the shoot length. Concentrations until 15 g L^{-1} sucrose and glucose (1:1 w/w) did not interfere in the shoot number per explant, but levels highest decreased (Table 3). There were no statistical differences for the percentage of survival, oxidation and callogenesis in different glucose and sucrose combinations.

The proportion of sucrose and glucose has an influence in the plant morphogenesis, mainly in cellular division rate (Borisjiuk *et al.* 1998). Some reports in the literature demonstrated suitable results for the combination of

Table 1. Survival percentage (S %), oxidation percentage (O %), callogenesis percentage (C %), shoot number (SN) and explant length (H cm) of *Eucalyptus cloeziana* after 30 days of *in vitro* multiplication, under different sucrose levels.

Sucrose (g L^{-1})	S %	O %[1]	C %[2]	SN	H cm
0	100	76	3	1.6 ab	1.1 a
15	100	63	2	2.2 a	1.1 a
30	100	57	2	1.4 ab	1.1 a
45	100	82	5	0.7 b	1.0 b
60	85	72	5	0.5 b	0.8 c
CV (%)	6.5	24.5	69.7	16.5	7.5

* Means within a column followed by the same letter for each parameter are not different at P<0.05 by Duncan's test. CV (%) = Coefficient of variation. [1] Data transformed by arcsin; [2] Data transformed by $(n+0.5)^{0.5}$.

Table 2. Survival percentage (S %), oxidation percentage (O %), callogenesis percentage (C %), shoot number (SN) and explant length (H cm) of *Eucalyptus cloeziana* after 30 days of *in vitro* multiplication, under different glucose levels.

Glucose (g L^{-1})	S %	O %[1]	C %[1]	SN	H cm
0	100	0 c	36 b	3.7 c	0.9 b
15	100	18 b	82 a	5.5 a	1.5 a
30	100	48 a	90 a	4.8 ab	1.4 a
45	100	30 b	90 a	4.1 bc	1.2 a
60	100	60 a	90 a	2.2 d	1.0 b
CV (%)	0	30.7	13.8	14.6	10.6

* Means within a column followed by the same letter for each parameter are not different at P<0.05 by Duncan's test. CV (%) = Coefficient of variation. [1] = Data transformed by arcsin.

Table 3. Survival percentage (S %), oxidation percentage (O %), callogenesis percentage (C %), shoot number (SN) and explant length (H cm) of *Eucalyptus cloeziana* after 30 days of *in vitro* multiplication, under different combination levels of glucose and sucrose.

Glucose (g L^{-1})	Sucrose (g L^{-1})	S %[1]	O %[1]	C %[1]	SN	H cm
0	0	100	39	39	0.7 a	0.9 a
7.5	7.5	100	36	36	0.7 a	0.8 ab
15.0	15.0	100	39	39	0.7 a	0.8 ab
22.5	22.5	90	45	45	0.2 ab	0.6 c
30.0	30.0	90	14	14	0.0 b	0.6 c
CV (%)		11.4	38.8	50.3	10.4	10.3

* Means within a column followed by the same letter for each parameter are not different at P<0.05 by Duncan's test. CV (%) = Coefficient of variation. [1] Data transformed by arcsin.

glucose and sucrose, as observed in banana (*Musa* spp. AAA), whereas the combination of sucrose and glucose at 30 g L^{-1} each, resulted in the best proliferation rate (Madhulatha *et al.* 2006). Similar results were also observed in *Dactylorhiza majalis* (Rchb.) P.F. Hunt & Summerh., which the combination of sucrose and glucose at 20 g L^{-1} each resulted in a superior shoot growth in comparison with the addition of isolated glucose (Wotavová-Novotná *et al.* 2007).

One possible explanation for the results found in the present study is the fact of *E. cloeziana* explants are very inclinable to oxidize, moreover, the sucrose when is hydrolisated by autoclaving, form the compound named hydroxymethylfurfural, which is toxic, growth inhibitor and induce explant oxidation (Wang *et al.* 2012). Therefore, future studies can be carry out for sterilizing the culture medium is suggested to choose the filtering process as an alternative to avoid oxidation by autoclaving (Reis & Zydney 2007).

Effects of L-Alanine on the *in vitro* multiplication

The supplementation of the culture medium with alanine decreased the oxidation percentage at 80 and 160 mg L^{-1}, being 39 and 36%, respectively, but also decreased the shoot number (Table 4). Survival and callogenesis percentage and shoot length did not differ statistically. However, in the level of 80 and 160 mg L^{-1} there were a small reduction in the survival rate (90 and 95%, respectively).

Alanine presented good results for rooting in fox grape (*Vitis labrusca* L.), which increased the root number per explant (Carvalho *et al.* 2013). Nevertheless, the preference for certain amino acid as the source of nitrogen can be associated to the genetic differences among the plants. Moreover, amino acids has specific functions in plants in answer against stresses (Rai 2002). Thus, due to the lack of studies with the use of alanine as a source of organic carbon for *eucalyptus*, became relevant to investigate the effects caused by this amino acid on the *in vitro* multiplication of *E. cloeziana*.

Although of the importance of the amino acids to the process and development of the plants, some are considered growth inhibitors. Mainly, when occurs the combination of two or more amino acids added to the culture medium, due to the interaction among them (George *et al.* 2008). For example, in the culture of oat embryos was observed that the amino acids, phenylalanine and tyrosine had a negative interaction (Harris 1956). Similarly can have occurred in the present study of *in vitro* multiplication of *E. cloeziana*, because the original composition of WPM medium is supplemented with 1 mg L^{-1} glycine (*i.e.* an amino acid), therefore, glycine and alanine may have had a negative interaction presenting results below of the control (Table 4). Moreover, it is possible that endogenous amino acids of *E. cloeziana* can have influenced a negative interaction with the alanine.

Effects of calcium under solid and liquid medium

The variable shoot number presented interaction between the factors calcium levels and medium consistency (Table 5). The best result for shoot number of explants was found at 1,112 mg L^{-1} $Ca(NO_3)_2.4H_2O$ and 192 mg L^{-1} $CaCl_2.2H_2O$ on solid medium, reaching 0.9 shoots per explant (Table 5). Similar results were found in *Eucalyptus saligna* using $CaCl_2$ as calcium source in MS (Murashige & Skoog 1962) liquid medium (Silva *et al.* 2015) and in *Lavandula angustifolia* Mill. using $CaCl_2.H_2O$ as calcium source in MS solid medium (Machado *et al.* 2014). The great advantage in the present study was the combination of two sources of calcium to avoid elevated levels of chlorine, whereas high chlorine levels can induce oxidation in the tissues. The survival, oxidation, callogenesis percentage and explant height did not presented interaction between these factors (Table 5).

Calcium is an element relatively immobile in most plant species and the *in vitro* environment (*i.e.* it presents high relative humidity, approximately 95 to 100%) limits the calcium uptake due to low transpiration of the explants cultivated in flasks (Silva *et al.* 2015), what results normally in calcium deficiency in the *in vitro* environment. Therefore, highest calcium levels are desired in the culture medium, mainly for plants that present hyperhydricity or shoot-tip necrosis. Plants of the genus *Eucalyptus*, normally presented hyperhydricity under liquid medium, as observed in *E. phylacis* (Bunn *et al.* 2005), in hybrid *E. grandis* ×

Table 4. Survival percentage (S %), oxidation percentage (O %), callogenesis percentage (C %), shoot number (SN) and explant length (H cm) of *Eucalyptus cloeziana* after 30 days of *in vitro* multiplication, under different L-Alanine levels.

L-Alanine (mg L^{-1})	S %[1]	O %[1]	C %[2]	SN	H cm
0	100 a	66 a	0.5	1.4 a	0.9 a
20	100 a	63 a	1.0	0.9 ab	0.9 a
40	100 a	82 a	1.0	1.4 a	0.9 a
80	90 a	39 b	0.5	0.5 bc	0.8 a
160	95 a	36 b	1.0	0.4 c	0.8 a
CV (%)	10.1	27.7	42.2	11.3	12.5

* Means within a column followed by the same letter for each parameter are not different at P<0.05 by Duncan's test. CV (%) = Coefficient of variation. [1] Data transformed by arcsin; [2] Data transformed by $(n+0.5)^{0.5}$.

Table 5. Survival percentage (S %), oxidation percentage (O %), callogenesis percentage (C %), shoot number (SN) and explant length (H cm) of *Eucalyptus cloeziana* after 30 days of *in vitro* multiplication, under different types of medium consistency and calcium levels.

Liquid medium						
$Ca(NO_3)_2.4H_2O$ (mg L^{-1})	$CaCl_2.2H_2O$ (mg L^{-1})	S %[1]	O %[1]	C %[1]	SN	H cm
556 (control)	96 (control)	100	0	0	0.25 aA	0.7
1,112	192	95	0	0	0.15 aB	0.6
1,668	288	90	0	0	0.05 aB	0.6
Solid medium						
$Ca(NO_3)_2.4H_2O$ (mg L^{-1})	$CaCl_2.2H_2O$ (mg L^{-1})	S %[1]	O %[1]	C %[1]	SN[2]	H cm
556	96	100	10	0	0.2 cA	0.8
1,112	192	100	25	0	0.9 aA	0.8
1,668	288	100	30	0	0.5 bA	0.7
CV (%)		16.5	51.9	0.0	15.4	11.7

* Means followed by the same lower case letter for the same medium consistency (*i.e.* liquid medium or solid medium only) and means by the same capital letter for different medium consistency (*i.e.* solid and liquid) are not different at P<0.05 by Duncan's test. CV (%) = Coefficient of variation. [1] Data transformed by arcsin, [2] Data transformed by $(n+0.5)^{0.5}$. Control = Original level of WPM medium (Lloyd & McCown 1981).

E. urophylla (McAlister *et al.* 2005, Hyodo *et al.* 2013), in *E. grandis* × *E. nitens* (McAlister *et al.* 2005) and in *E. saligna* (Silva *et al.* 2015). However, the liquid culture of *E. cloeziana* did not induce hyperhydricity (*i.e.* typical glassy, vitrescent and stunted form of the explant appearance) (Data not show).

Survival and callogenesis percentage did not differ statistically. There were statistical differences in the oxidation percentage and shoot length for medium consistency. Liquid medium avoided the occurrence of oxidation in the explants and decreased the shoot length (Table 5). Similar results were found in *E. saligna* cultivated in liquid medium under different levels of $CaCl_2$, which the levels of 0 to 880 mg L^{-1} resulted in 4-10% oxidation (Silva *et al.* 2015). Another alternative to decrease the chlorine concentration in the culture medium and increase the absorption of calcium is the use of a chelating agent, however, there are no such researches yet in *Eucalyptus*.

In summary, the best results found in these study were: (1) The best level of sucrose was 15 g L^{-1}, but this level do not decrease oxidation in tissues and until 30 g L^{-1} sucrose can be used with no negative effects on *in vitro* multiplication of *E. cloeziana*; (2) The best level of glucose was 15 g L^{-1} and it presents reduction in the oxidation of the tissues of *E. cloeziana*; (3) The combinations of glucose and sucrose levels did not present suitable results for *in vitro* multiplication; (4) The presence of L-alanine on the culture medium do not favor the multiplication and (5) The best result for calcium supplementation in the multiplication of *E. cloeziana* was found at 1,112 mg L^{-1} $Ca(NO_3)_2.4H_2O$ and 192 mg L^{-1} $CaCl_2.2H_2O$ on solid medium, but the liquid medium avoid the oxidation of the explants. These results can be useful to optimize the micropropagation protocol of *E. cloeziana*.

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