IMPORTANCE OF MEDIA MINERAL COMPOSITION ON THE INDUCTION OF SOMATIC EMBRYOGENESIS IN *EUCALYPTUS GLOBULUS* LABILL

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Summary

Eucalyptus globulus is one of the main species for the pulp industry and it is the third most important forest species in Portugal. As other Eucalyptus species it is an obvious target for in vitro propagation (e.g. somatic embryogenesis) and genetic manipulation.

We have compared several published media (MS, 1/2MS, B5, WPM, DKW and JADS) both on the somatic embryogenesis induction and expression steps. MS, followed by B5, were the best induction media.

Since different media induced heterogeneous responses, with MS being the best medium to induce somatic embryos, we compared its mineral composition with the mineral content of zygotic embryos (explants). Simultaneously, and as the secondary somatic embryos are routinely maintained on this medium and used to regenerate plants, we also compared the mineral composition of zygotic and secondary somatic (grown in MS media) embryos. Fresh samples were digested with $HClO_4$ for Mg, Ca, Cu, Zn, Fe and Mn, and with H_2SO_4 for P, N and K analyses.. Preliminary results showed that zygotic embryos have different mineral proportions when compared to all basal media used, including the MS medium. Also, when compared to secondary somatic embryos, zygotic embryos showed higher levels of Mg (7.6:1), Cu (11.4:1), Zn (3.6:1), Fe (1.7:1), Mn (17.9:1), N (1.8:1), P (4.0:1) but less K (0.7:1), showing that SE induction conditions lead to changes in tissue mineral content relatively to zygotic embryos.

Keywords: *Eucalyptus*, medium composition, primary somatic embryos, woody species, mineral content

Resumen

Importancia de la composición mineral para la inducción de embriogénesis somática en *Eucalyptus globulus* Labill

El Eucalyptus globulus es una de las especies principales para la industria de la pasta de papel y la tercera en importancia entre las especies forestales en Portugal. Como sucede con otras especies de Eucalyptus, E. globulus es un blanco evidente para la propagación in vitro de, por ejemplo, la embriogénesis somática, así como para la manipulación genética.

En este trabajo comparamos el efecto de varios medios de uso común (MS, 1/2MS, B5, WPM, DKW y JADS) tanto en la inducción de la embriogénesis somática como en las etapas de expresión y encontramos que MS es el más eficaz inductor, seguido de B5. El hecho de que diferentes medios produjeran respuestas heterogéneas y MS fuese el medio más eficaz en la producción de embriones somáticos nos llevó a comparar su composición mineral con el contenido en minerales de embriones cigóticos (explantes). En paralelo, y dado que los embriones somáticos secundarios se suelen conservar en este tipo de medio para utilizarlos en la regeneración de plantas, también comparamos la composición mineral de embriones cigóticos y somáticos secundarios (desarrollados en MS). A tal fin, muestras recién obtenidas se sometieron a digestión con $HClO_4$ para el análisis de Mg, Ca, Cu, Zn, Fe y Mn, o con H_2SO_4 para el de P, N y K. En base a los resultados provisionales obtenidos, los embriones cigóticos presentan proporciones de minerales diferentes a las todos los medios basales estudiados incluido el MS. Asimismo, los embriones cigóticos muestran mayores niveles de Mg (7.6:1), Cu (11.4:1), Zn (3.6:1), Fe (1.7:1), Mn (17.9:1), N (1.8:1) y P (4.0:1) que los somáticos secundarios, pero menores niveles de K (0.7:1) que éstos, lo que demuestra que unas condiciones propicias para la inducción de embriogénesis somática alteran los contenidos en minerales de los tejidos con respecto a los embriones cigóticos.

Palabras clave: *Eucalyptus*, composición del medio, embriones somáticos primarios, especies madereras, contenido de minerales

Introduction

Somatic embryogenesis (SE) in *Eucalyptus globulus* is a recent advance in vegetative propagation that could have a great impact on tree breeding and commercial plantation forestry. Besides all the advantages of this technique the most promising application of somatic embryogenesis is in high-value clonal forestry. The commercial use of somatic embryo-derived plants is already a reality for conifers, but concerning Eucalyptus genus practical aplications of this technique are far from those published for conifers.

Recently, we described a true-to-type plant regeneration protocol from secondary somatic embryogenesis and studied the influence of growth regulators, induction period, explant type and carbohydrate source on the SE primary induction (Pinto *et* al. 2002, Pinto *et* al. 2004b). We have also proved that the methodology used did not induce major genetic changes in the somatic embryos as evaluated by flow cytometry, and that our primary goal of "true-to-type" propagation was attained (Pinto *et* al. 2004a).

Our studies showed that induction of somatic embryos is a highly sensitive step, and its full control is of crucial importance to develop a robust protocol applicable to several genotypes. This is necessary to maintain a broad genetic base for clonal selection and management of genetic diversity (Park et al. 1998). Therefore, for the development of superior clonal varieties and development in high-value clonal forestry, it is important to have a high rate of SE induction.

The selection of a proper medium formulation plays a crucial role on the establishment of an efficient tissue culture system for plant regeneration (Kothari et al. 2004) but usually this selection is based on empirical approaches. Many researchers use the MS medium for several plant species, without previously screening other media, under a general belief that plants respond well enough to MS salts. Nevertheless, it is crucial to ensure the adequacy of the culture medium, when one intends to develop protocols for large scale plant production. Unfortunately, few works have addressed this area, and only one is reported to the Eucalyptus genus for the hybrid E. uropphylla x E. grandis. (Gribble et al. 2002). One of the strategies for defining the best medium is to analyze the mineral content of the plant tissue (e.g. explant) to model the mineral balance definition of the culture medium.

This work focuses on the conditions influencing the induction of embryogenic potential in mature zygotic embryos of Eucalyptus globulus Labill.. We also present preliminary studies of the mineral content of zygotic embryos and secondary somatic embryos.

Materials and Methods

Plant material and disinfection: Half-sib seeds of Eucalyptus globulus ssp. globulus Labill. (supplied by Celbi, Leirosa, Portugal) were surface-disinfected with a mixture of absolute ethanol and hydrogen peroxide for 15 min, washed twice in sterile distilled water (10 min each) and rinsed for 15 min with 0.1% (w/v) Benlate (Rhône-Poulenc). Then, seeds were imbibed over night in sterile distilled water.

Zygotic embryo explants were inoculated on different basal media: MS (Murashige an Skoog 1962), 1/2MS, B5 (Gamborg 1968), DKW (Driver and Kuniyuki 1984), WPM (Lloyd and McCown 1980), JADS (Correia 1993). All media were supplied with 3 mg/L NAA (3NAA) according to

Pinto et al (2002). Groups of 50 explants were distributed by five 90 mm diameter Petri dishes (each containing 10 embryos), for each medium tested..

Induction of somatic embryos took place in the dark, at 22- 24 ºC for three weeks.

Explants were then transferred to the same medium used during induction but without growth regulators (MSWH, 1/2MSWH, B5WH, DKWWH, WPMWH, JADSWH) for 12 weeks in the dark, for expression. Explants were monthly transferred to fresh medium.

All media were supplemented with 30g/L sucrose and 2.5g/L gelrite®, pH was adjusted to 5.8 and the media were autoclaved at 121ºC for 20 min. The JADS medium was made using stock solutions of chemicals purchased to Sigma (USA) and the MS vitamins from Duchefa (Haarlem, Netherlands). All other culture media, except JADS, sucrose, gelrite® and NAA (α-naphthalene acetic acid) were purchased to Duchefa (Haarlem, Netherlands).

Explants expression was analyzed in 50 replicates for each condition (n=50), 12 weeks after transfer to expression medium. The following parameters were analyzed with a magnifying binocular (Olympus SZ60): a) % of explants showing SE response and b) total number of somatic embryos.

Mineral composition determination

Fresh samples of mature zygotic embryos and secondary somatic embryos were digested with HClO4 – HNO3 for Mg, Ca, Cu, Zn, Fe and Mn (Mills and Jones, 1996). Those ions were determined by atomic absorption spectroscopy. A sulphuric acid digestion was carried out for N, P and K content and N and P were determined by molecular absorption spectroscopy and K by flame emission spectroscopy (Walinga et al., 1989).

Results

Medium effect on induction and expression

Germination of entire mature zygotic embryos was higher than 90% in all media tested. After two weeks on induction media, the germination process stopped and callus production started, mainly in cotyledons. After three weeks of induction, no visual differences were detected among the six media tested. A combination of whitish friable and compact calluses occurred simultaneously in the same explant while no phenolisation was observed.

After four weeks on expression medium, all explants showed browning and first embryogenic responses were observed mostly on cotyledon regions. After this period the number of adventitious roots newly formed, increased dramatically during this period, together with oxidation.

After 12 weeks on expression media, explant responses were heterogeneous and highly dependent on medium composition. Higher SE rates were obtained in MS_{WH} (30% of explants showed SE, in a total of 67 somatic embryos per 50 explants evaluated?) and $B5_{WH}$ (26%, a total of 44 somatic embryos), followed by $1/2MS_{WH}$ (10%, a total of 8 somatic embryos), $JADS_{WR}$ (8%, a total of 6 somatic embryos) and DKW_{WH} (6%, a total of 6 somatic embryos). SE was not observed on WPM_{WH}. MS revealed to be the best medium for both percentage of explants showing somatic embryogenesis response and the total somatic embryos formed per explant.

Somatic embryos formed were whitish, compact, and mostly at the globular stage, although other advanced stages could be found, showing some assynchronism of the process Germination and conversion were observed independently of the medium, although MS_{WH} continued to give the

highest number of cotyledonar embryos and of plantlets

Mineral Composition

The mineral composition of the six tested media differ largely (Table 2), with MS being the richest medium in nitrogen (both nitrate and ammonium), while JADS contains high levels of phosphate and DKW is the richest in sulphate, calcium and magnesium.

Except for Fe supply that differed in JADS, the absence of KI in DKW, WPM and JADS, and the lower concentration of Zn in B5, no major differences were found among micronutrient composition. The same was observed for vitamins, as the most significant changes were the absence of glycine in B5 and of pyridoxine in DKW (Table 2).

As to embryo mineral composition, results showed that zygotic embryos have different mineral proportions when compared to all basal media used, including the MS medium. When compared to secondary somatic embryos, zygotic embryos showed higher levels of Mg (7.6:1), Cu (11.4:1), Zn (3.6:1), Fe (1.7:1), Mn (17.9:1), N (1.8:1), P (4.0:1) but less K (0.7:1), showing that SE have different tissue mineral content, indicating that mineral composition during SE must be better clarified (Table 3).

Discussion and Conclusions

The combination of minerals necessary for plant development/morphogenesis is dependent on the species, and usually determined by empirical manipulation of one or a combination of existing published formulations (Ramage and Williams, 2002).

Concerning the combination of mineral nutrients in somatic embryogenesis media, we hypothesized, for *Eucalyptus globulus*, that the medium having a mineral proportion/composition similar or close to the explant mineral proportion/composition will give higher SE induction rates. To test this hypothesis we chose four media (MS, B5, DKW, WPM) largely used in woody species micropropagation and morphogenesis. Besides, we also used the JADS specifically designed for *E. grandis* micropropagation (Correia 1993) but with no use, up to moment, in *E globulus* SE studies.

A previous analysis of MS, B5, ½ MS, JADS, DKW and WPM mineral compositions led us also to the hypothesis that explant responses, during induction/expression steps, may be mostly due to macronutrient content differences, as all other experimental conditions such as pH, growth regulators or carbohydrate, were similar, and no large differences are found in the micronutrient and vitamin composition. As far as we know, all reports concerning somatic embryogenic response for the Eucalyptus genus just used MS and B5 media for induction (Muralidharan and Mascarenhas, 1995; Prakash and Gurumurthi, 2005).

Although no explanation is given and no reference is made to the use/effectiveness of other media in those works (Muralidharan and Mascarenhas, 1995; ; Prakash and Gurumurthi, 2005), results presented here confirm the authors' decisions. In fact, these preliminary results show that: 1) zygotic embryos have different mineral proportions when compared to all basal media used, including the MS medium; 2) this last medium has, however, the mineral proportion most close to the zygotic embryo one.

Furthermore, when compared to secondary somatic embryos, zygotic embryos showed higher levels of Mg, Cu, Zn, Fe, Mn, N, P but less K, showing that, with the protocol used here led to significant differences in tissue ineral composition of the zygotic

embryo (explant) and the resulting secondary somatic embryos. This generalized decrease of mineral level in secondary somatic embryos (when compared to zygotic embryos) could be the explanation for problems related with maturation resulting in low germination and convertion in plants (decreasing therefore the effectiveness of the SE process), once we presently use secondary somatic embryos to regenerate plants. Pullman et al. (2003) refer that the nutrional, osmotic and hormonal environments surronding an embryo are well know to control embryo growth. Optimization of these envirinments is critical for growth and development of high- quality vigours somatic embryos. These authors propose to optimize the nutrional environment to somatic embryos based on the analysis of female gamethophyte and zygotic embryos metal contents of *Pinus taeda* L.

In conclusion, minerals appear to play an important role in the regulation of plant morphogenesis, and in particularly somatic embryogenesis. Our preliminary results suggested that MS gave the best SE induction results followed by B5. Also, our data showed that, although mineral proportion of all media differs from the mineral proportion found in zygotic embryos, MS has the most close profile. This data suggests that SE induction may be improved if the medium salt composition (e.g. MS) is adjusted to similar mineral proportions to those found in the explant (zygotic embryos), but further studies on the formulation of a newly and optimized medium for SE process in *E globulus* are needed. Also, there are many challenges in the area of embryo quality and vigor in order to secondary somatic embryogenesis became commercial avaible for this specie.

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Table 1: Embryogenic response after 12 weeks on the expression medium for the six media tested

Induction medium	Expression medium	Induction (%) (n=50)	Total No. of Somatic embryos	Somatic embryo type		
				Globular	**Cotyledonar**	**Plant**
MS	MS_{WH}	30	67	39	28	8
½ MS	½ MS_{WH}	10	8	3	2	3
B5	$B5_{WH}$	26	44	22	20	2
DKW	DKW_{WH}	6	6	0	5	1
WPM	WPM_{WH}	0	0	0	0	0
JADS	$JADS_{WH}$	8	6	3	2	1

Table 2: Mineral composition of plant tissue culture media used.

mM	MS	B5	1/2 MS	DKW	WPM	JADS
Macro Elements						
$Ca(NO_{3)2}.4H_2O$						5.000
$Ca(NO_3)_2.2H_2O$				8.300	2.350	
$CaCl_2$	2.990	1.020	1.500	1.010	0.650	
KH_2PO_4	1.250		0.630	1.950	1.250	3.000
K_2SO_4				8.950	5.680	
KNO_3	18.700	24.730	9.400			8.000
$MgSO_4$	1.500	1.010	0.730	3.000	1.500	
$MgSO4.7H_2O$						3.000
$(NH_4)_2SO_4$		1.010				
NaH_2PO_4		1. 0900				
NH_4NO_3	20.610		10.300	17.600	5.000	4.000
Micro Elements						
$CoCl_2.6H_2O$	0.000	0.000	0.000			0.000
$CuSO_4.5H_2O$	0.000	0.000	0.000	0.000	0.000	0.005
$Na_2EDTA.2H_2O$						0.200
$FeSO_4.7H_2O$						0.200
FeNaEDTA	0.100	0.100	0.100	0.120	0.100	
H_3BO_3	0.100	0.048	0.100	0.078	0.100	0.050
KI	0.005	0.005	0.005			
$MnSO_4.H_2O$	0.100	0.059	0.100	0.200	0.130	0.075
$Na_2MoO_4.2H_2O$	0.001	0.001	0.001	0.002	0.001	0.001
$ZnSO_4.7H_2O$	0.030	0.007	0.030	0.072	0.030	0.015
Vitamins						
Glycine	0.0266		0.0266	0.0266	0.0266	0.0266
Myo-Inositol	0.5600	0.5600	0.5600	0.5600	0.5600	0.5600
Nicotinic acid	0.0041	0.0081	0.0041	0.0081	0.0041	0.0041
Pyridoxine HCL	0.0024	0.0049	0.0024		0.0024	0.0024
Thiamine HCL	0.0003	0.0030	0.0003	0.0059	0.0030	0.0003

Table 3: Total levels of macro and micronutrients in zygotic embryos and secondary somatic embryos.

mg/Kg fw	Mg	Ca	Zn	Cu	Fe	Mn	N	K	K	P
Zygotic embryos	1739.4	826.2	40.9	16.0	66.5	315.5	21656.2	2913.3	2913.3	4139.7
Secondary somatic embryos	228.4	542.5	11.2	1.4	39.5	17.6	11807.7	4379.0	4379.0	1039.5

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