Note

Organogenesis and transient genetic transformation of the hybrid *Eucalyptus grandis* × *Eucalyptus urophylla*

Giovana Bomfim de Alcantara[1]*; João Carlos Bespalhok Filho[1]; Marguerite Quoirin[2]

[1]*UFPR – Depto. de Fitotecnia e Fitossanitarismo – Setor de Ciências Agrárias, C.P. 5326 – 80035-050 – Curitiba, PR – Brasil.*
[2]*UFPR – Depto. de Botânica – Setor de Ciências Biológicas, C.P. 19031 – 81531-980 – Curitiba, PR – Brasil.*
**Corresponding author <giobomfim@ufpr.br>*

ABSTRACT: The hybrid *Eucalyptus grandis* × *Eucalyptus urophylla* presents high levels of productivity and potential for use in paper, cellulose and fiber industries. The bud organogenesis from leaf explants of two clones of *E. grandis* × *E. urophylla* was studied in order to verify the effect of several factors: subculture duration on multiplication medium, type of explants, entire and half leaves: basal and apical portions, and duration of the culture on a regeneration medium. Differences in organogenic capacity of the two clones tested were observed. The explant most recommended for organogenesis is the basal section of the leaf collected from shoot clusters subcultured every 17 days. Moreover, the leaf explants must be transferred to a fresh bud induction medium every five days. This study also aimed at evaluating factors affecting the genetic transformation of leaf explants with the *uidA* gene, via co-cultivation with *Agrobacterium tumefaciens*, such as the pre-culture of the explants on a specific medium, the duration of their co-culture with the bacteria and the addition of acetosyringone to the culture media. The best conditions for the expression of the *uidA* gene were two days of pre-culture of the leaf tissues, three days of co-culture with the bacteria and the addition of acetosyringone in pre- and co-culture media.
Key words**:** *Agrobacterium tumefaciens,* micropropagation, tissue culture, transgenic plant, woody species

Organogênese e transformação genética transiente do híbrido *Eucalyptus grandis* × *Eucalyptus urophylla*

RESUMO: O híbrido *Eucalyptus grandis* × *Eucalyptus urophylla* apresenta alta produtividade e potencial para indústrias de papel, celulose e fibras. A organogênese de explantes foliares de dois clones de *E. grandis* × *E. urophylla* foi estudada para verificar fatores como tempo de repicagem das plântulas matrizes em meio de multiplicação; tipo de explantes, folhas inteiras e de meias-folhas (porções basais e apicais) e dos dias que os explantes foliares permaneceram em meio de regeneração. Foram observadas diferenças na capacidade organogênica dos dois clones testados. A parte basal das folhas, coletadas de brotações repicadas a cada 17 dias, apresentou superioridade organogênica. Explantes foliares devem ser transferidos para novo meio de indução de brotações a cada cinco dias. Este estudo também teve como objetivo avaliar fatores que afetam a transformação genética de explantes foliares com o gene *uidA*, via co-cultivo com *Agrobacterium tumefaciens*, tais como a pré-cultura, a duração da co-cultura e a adição da acetoseringona no meio de cultura. As melhores condições para a expressão do gene *uidA* foram a cada dois dias de pré-cultura de explantes foliares, três dias de co-cultura com a bactéria e a adição de acetoseringona nos meios de pré e co-cultura.
Palavras-chave: *Agrobacterium tumefaciens,* cultura de tecidos, espécie lenhosa, micropropagação, planta transgênica

Introduction

Eucalyptus is the forest gender with the largest area under cultivation in the world: more than 17.8 million hectares, of which more than three millions are in Brazil (Bracelpa, 2007). This is due to its quick growth, short period before harvesting and its use in up to three successive rotations (Ho et al., 1998). The hybrid from *E. grandis* × *E. urophylla* has been largely used, especially in Brazil, due to its wood properties, rooting ability and resistance to tumor induced by *Cryphonectria cubensis*, which result in high levels of productivity and performance for paper, cellulose and fiber industries (Bracelpa, 2007).

Due to the economic importance of *Eucalyptus*, great investments were made in breeding programs and in the development of vegetative propagation techniques. However, there are other techniques, like genetic transformation, that can be incorporated in a genetic breeding program in order to increase yields, shorten production cycles and promote the development of high quality plantations (Diouf, 2003). Transformed tissues or cells with the ability to regenerate plants that express inserted genes are necessary for genetic transformation success.

However, there is little information on *in vitro* organogenesis and on the factors involved in genetic transformation.

This study aims at evaluating factors for the organogenesis of buds from leaf explants and for genetic transformation of two clones of *E. grandis* × *E. urophylla*. The subculture duration on multiplication medium, type of explants, entire and half leaves: basal and apical portions, and duration of the culture on a regeneration medium were evaluated. We also evaluated factors affecting the genetic transformation of leaf explants with the *uidA* gene, via co-cultivation with *A. tumefaciens*, such as the pre-culture of the explants on a specific medium, the duration of their co-culture with the bacteria and the addition of acetosyringone.

Material and Methods

Prior to this work, *E. grandis* × *E. urophylla* seeds were germinated *in vitro* and two clones (11 and 827) were selected, based on the speed of onset of adventitious shoots. Both clones were used to study the factors that influence the organogenesis process and only clone 11 was used to study the genetic transformation.

Leaf explants were isolated from shoots of clones 11 and 827 and incubated for 30 d *in vitro*, at 25 ± 2°C under white fluorescent light (30 μmol m^{-2} s^{-1}) and a photoperiod of 16h. All culture media used in this study were those described by Tournier et al. (2003). These media were: multiplication medium (M), cellular reactivation medium (BIP), bud induction medium (BIT), shoot development medium (SDM), bacteria medium (C) and co-culture medium (A2). Shoots were cultivated in glass flasks (6 cm Ø × 9 cm high) with 35 mL of M medium and subcultured every 30 d. The explants were young leaves from these micropropagated shoots. They were collected and selected from the upper parts of the shoots and cut transversally into two parts. They were then cultivated in the dark for 2 d on BIP medium, followed by a solid A2 medium for 5 d. Afterwards, the explants were transferred to BIT medium and kept in the dark for 15 d, with subculture every 5 d. After 22 d of culture the explants were transferred to SDM medium for 68 d with subculture every 14 d. The leaf explants were kept in glass Petri dishes (10 cm Ø × 2 cm high) with 25 mL of culture medium.

Three tests were carried out with clones 11 and 827 in order to evaluate some factors that interfere with the organogenesis process. In the first test the effect of different dates of transfer to M medium (17, 26 and 35 d) was evaluated. In a second test entire and half leaves, basal and apical portions, were compared. The third test studied the effect of culture duration on a bud induction medium. The explants were transferred to this medium for 15 d, with subculture every 5, 10 or 14 d.

The number of explants forming callus, the number of buds per explant and of explants totally oxidized were recorded 90 d after the beginning of the initial culture. The experimental design was completely randomized, with five repetitions and ten explants per experimental unit and the experiments were repeated twice, the 2nd repetition being carried out two months after the first. The treatments were conducted in a 3 × 2 factorial arrangement (three periods of subculture or types of explants and two clones). First, the analysis of variance was applied; then the means were compared by Tukey´s test ($p < 0.05$). Statistical analysis was performed using SISVAR software (Ferreira, 1999).

Young leaves from micropropagated plants were inoculated with *A. tumefaciens* strain AGL1 containing the binary vector pKGWFS7 (Karimi et al., 2002). This vector contains the *npt*II selection gene, under the control of the nopaline synthase (*nos*) promoter and the *uidA* gene, under the control of the CaMV 35S promoter.

The bacteria were cultivated on YEB solid medium (Miller, 1972) supplemented with kanamycin (50 mg L^{-1}), ampicillin (50 mg L^{-1}) and rifampicin (50 mg L^{-1}), at 25 ± 2°C, for 48h. After this period, one colony was inoculated in 5 mL of YEB liquid medium and incubated at 28°C for 16h at 120 rpm. The bacterial culture was centrifuged and the pellet suspended in liquid C medium until the culture reached an OD_{600nm} of 0.5. Explants were removed from the BIP medium, immersed in the bacterial suspension and shaken for 30 min at 120 rpm. Explants were then dried on sterile filter paper, incubated on A2 medium, supplemented with acetosyringone (AS) (50 μM) and kept in the dark for 5 d.

The effects of pre-culture, co-culture and the use of AS (50 μM) were analyzed in the following treatments: 0, 1 and 2 d of pre-culture; 0, 3 and 5 d of co-culture; pre- and co-culture with AS, pre-culture with AS and co-culture without AS, pre-cultivation without AS and co-cultivation with AS and pre- and co-culture without AS.

Histochemical analyses (Jefferson et al., 1987) of *uidA* gene expression were conducted 5 and 12 d after inoculation with *A. tumefaciens* and the percentage of the area of leaf tissue with a blue color was estimated visually. The two evaluations were carried out on ten explants/treatments and the experiments were repeated twice, the second repetition being applied one month after the first.

Results and Discussion

The callus formation varied according to the clone. For clone 11 it occurred 10 d after the isolation of leaf explants and for clone 827 after 14 d. The calli remained green for approximately 25 d while they were kept in the dark. They gradually turned brown and, in most of the explants in which buds regenerated, this browning occurred in only part of the calli. In general, the formation of buds occurred 20 to 30 d after the transfer of explants to a bud induction medium (Figure 1a and 1b).

Differences in regenerative capacity between clones 11 and 827 were observed (Table 1). Clone 11 yielded a higher percentage of explants regenerating buds and a larger number of buds per explant than clone 827, ex-

Table 1 – Callus induction and organogenesis of buds in leaf explants of clones 11 and 827 of *E. grandis* × *E. urophylla* according to subculture duration on multiplication medium, with evaluation after 90 d in culture.

Days	Clone 11	Clone 827
Explants forming callus (%)		
17	30.00 a*B	56.00 aA
26	30.00 aA	36.00 bA
35	26.00 aA	24.00 cA
Explants regenerating buds (%)		
17	32.00 aA	10.00 aB
26	12.00 bA	6.00 bB
35	2.00 cA	2.00 cA
Number of buds/explant		
17	5.00 aA	2.72 bB
26	4.43 bA	3.63 aB
35	2.40 cB	2.90 bA
Oxidation (%)		
17	38.00 cA	34.00 cA
26	58.00 bA	58.00 bA
35	72.00 aA	74.00 aA

*Means followed by the same small letter in the column, for duration of the subculture, and means followed by the same capital letter in the line, for clones, do not differ (Tukey, $p < 0.05$).

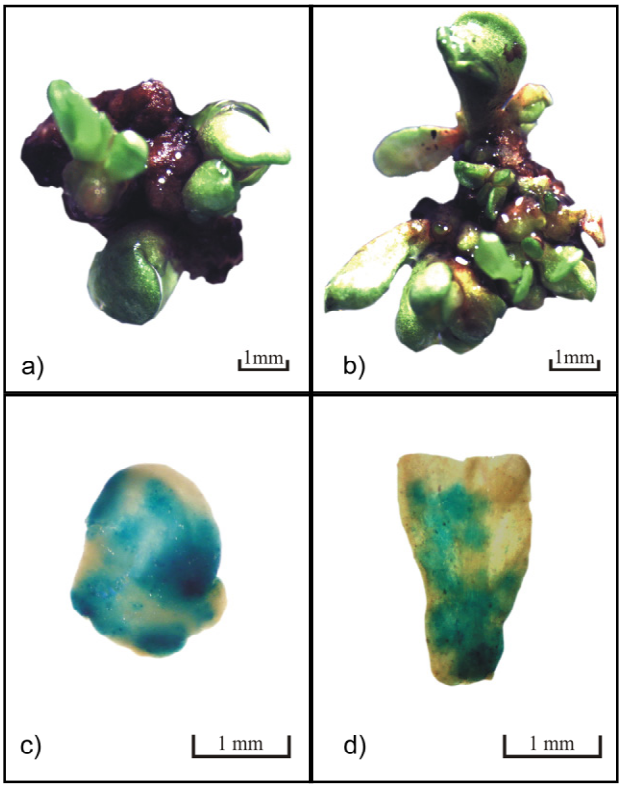

Figure 1 – Bud organogenesis from in leaf explants of clone 11 of *E. grandis* × *E. urophylla*. a) after 30 days; b) after 68 days on the bud induction medium. Expression of *uidA* gene in leaf explants of *E. grandis* × *E. urophylla*. c) 5 d after inoculation with *A. tumefaciens*, d) 12 d after inoculation with *A. tumefaciens*.

cept for treatment with subculture every 35 d. In general, for both clones, the increase of the frequency of transfer of shoots from which the leaves were collected increased the regenerative responses, as can be evidenced by the percentage of formation of calli with buds.

The use of young leaves accounted for the best regenerative capacity of explants collected from shoots, subcultured every 17 d, compared to shoots transferred every 26 or 35 d (Table 1). When the shoots were transferred, all the oldest leaves and oxidized calli were removed. Consequently, leaves collected from shoots transferred every 17 d were younger than shoots transferred every 26 d, which in turn were younger than those cultured for 35 d on the same medium. The best organogenetic potential of young explants is related to the fact that young tissues are less differentiated and their cells are metabolically more active when cultured under suitable hormonal and nutritional conditions (Famiani et al., 1994).

The use of young tissues is recommended for *Eucalyptus* genetic transformation, normally cotyledons and hypocotyls, 2 and 15 d after germination, zygotic embryos and young leaves (González et al., 2002). However, for most species of *Eucalyptus*, the ideal age or the best phase of seedling development for a high explant regeneration rate has not yet been defined. Barrueto Cid et al. (1999) compared primary leaves from 20-, 30-, 40- and 50-d-old seedlings of *E. grandis* × *E. urophylla*. A poor bud formation capacity (13 ± 11%) was detected on 50 day-old leaves. However, differently from the observations made in the present study, a poor bud formation capacity was also detected on 20 day-old leaves.

Differences in regenerative potential between clones 11 and 827 were found, showing the regenerative superiority of clone 11 (Table 2). This clone yielded a higher number of calli with buds, except when entire leaves were used, and larger number of buds per explant. Conversely, clone 827 did not form buds. For clone 11, there was a higher number of callus and callus with buds when the leaf was divided into basal and apical portions, the basal portion yielding the best result, followed by the apical portion. These results are consistent with those observed on *E. gunnii* leaves that did not present cellular competence for bud regeneration when the region near the petiole was removed (Hervé et al., 2001). According to the authors, the regeneration was originated from meristems located in the leaf base and connected to the vascular bundles (Hervé et al., 2001).

There was a higher percentage of explants forming callus for the clone 827 but the bud formation was higher for clone 11 (Table 3). Subculture on a regeneration medium every 5 d was more favorable to the bud formation and number of buds than subculture every 10 or 14 d.

Table 2 – Callus induction and organogenesis of buds in leaf explants of clones 11 and 827 of *E. grandis* × *E. urophylla* according to different types of leaf explants, evaluated after 90 d in culture.

Type of leaf explants	Clone 11	Clone 827
Explants forming callus (%)		
Basal	30.00 a*B	48.00 aA
Apical	32.00 aB	44.00 aA
Entire leaves	16.00 bB	28.00 bA
Explants regenerating buds (%)		
Basal	42.00 aA	12.00 aB
Apical	34.00 bA	10.00 aB
Entire leaves	10.00 cA	6.00 aA
Number of buds/explant		
Basal	5.10 aA	3.04 aB
Apical	5.40 aA	2.26 aB
Entire leaves	3.00 aA	2.00 aB
Oxidation (%)		
Basal	28.00 bB	40.00 bA
Apical	34.00 bB	46.00 bA
Entire leaves	74.00 aA	66.00 aA

*Means followed by the same small letter in the column, and by the same capital letter in the lines do not differ (Tukey, $p < 0.05$).

Table 3 – Callus induction and organogenesis of buds in leaf explants of clones 11 and 827 of *E. grandis* × *E. urophylla* according to subculture duration on a regeneration medium (5, 10 and 14 d), evaluated after 90 d in culture.

Type of leaf explants	Clone 11	Clone 827
Explants forming callus (%)		
5	28.00 a*B	60.00 AA
10	32.00 aB	62.00 AA
14	38.00 aB	64.00 aA
Explants regenerating buds (%)		
5	30.00 aA	6.00 aB
10	20.00 bA	4.00 aB
14	12.00 cA	4.00 aB
Number of buds/explant		
5	4.86 aA	2.12 aB
10	4.00 aA	1.50 aB
14	2.82 bA	1.74 aB
Oxidation (%)		
5	42.00 aA	34.00 aB
10	48.00 aA	34.00 aB
14	50.00 aA	32.00 aB

*Means followed by the same small letter in the column, and by the same capital letter in the lines do not differ (Tukey, $p < 0.05$).

The regeneration medium was defined for the hybrid *E. grandis* × *E. urophylla* as a bud-inducing medium (Tournier et al., 2003). These authors use this medium with subculture every 5 d. However, as the regenerative response of explants varies not only with the species but also with the clone, different clones were used in this study. A variation in the organogenic response between the clones was observed. Unlike clone 827, clone 11 provided the response observed by Tournier et al. (2003) and presented better regenerative capacity with transfer every 5 d. Thus, the increase of the frequency of transfer of the leaf explants on a regeneration medium had a positive influence on organogenesis in clone 11.

For clone 827, no difference between the regenerative responses was verified (Table 3). This may be due to the absence or the insufficiency of external stimuli such as the culture medium composition and luminosity which induce the formation of buds. In this case frequency of transfer did not affect the organogenesis process. In general, the two genotypes responded differently to the same stimulus because of morphological, biochemical and physiological variations resulting from the differentiated expression of the genes (Srivastava, 2001). Studies suggested considerable variation in the regenerative capacity of explants in response to genetic differences. In a study with eight clones of *E. grandis*, differences between the different clones were observed in the demand for growth regulators with variations in regenerative potential (Lainé and David, 1994).

The pre-culture influenced *uidA* gene expression in the explants. The explants cultured for 2 d before bacteria inoculation had a higher rate of expression than explants cultured for 1 d or without pre-culture (Figure 2a). Reports in the literature indicate several durations of pre-culture according to the species. For *E. camaldulensis* hypocotyl segments, the best transient expression of the *uidA* gene was obtained with a 3 d pre-culture, followed by a 2 d co-culture (Ho et al., 1998). Moralejo et al. (1998) reported a longer pre-culture duration, between 4 and 6 d, for the increase in the level of *uidA* expression in cotyledons and hypocotyls of *E. globulus*. For *Vigna mungo*, the pre-culture of explants on a regeneration medium did not increase the level of *uidA* expression (Saini and Jaiwal, 2007).

The mechanisms that act in promoting *A. tumefaciens*-mediated genetic transformation during pre-culture are still not well understood. However, some reserachers indicate that the pre-culture favors the binding of the bacteria with plant tissue. This occurs due to the active metabolism of these cells, the synthesis of cells on the surface of the wounded tissues and the production of compounds inducing *vir* region in the bacterial plasmid (Tzfira and Citovsky, 2006). In addition to increasing the efficiency of genetic transformation of some species, pre-culture on a medium containing 2.4-D and

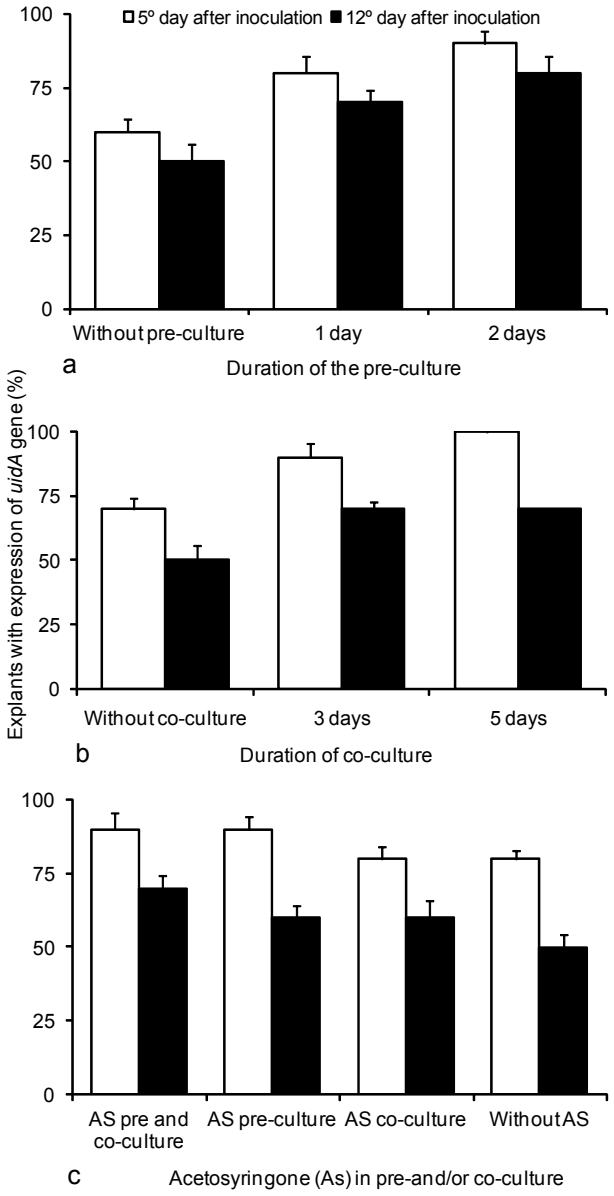

Figure 2 – Expression of *uidA* gene in leaf explants of *E. grandis* × *E. urophylla*, 5 and 12 d after inoculation with *A. tumefaciens*. a) Effect of pre-culture duration; b) effect of co-culture duration; c) effect of acetosyringone (50 μM) addition in pre- and co-culture media.

BAP is a favorable phase for regeneration. Pre-culture can promote physiological and morphological changes in explants and increase their morphogenetic competence, thus facilitating the regeneration process (Ainsley et al., 2001).

Five days after bacterial inoculation, the highest rate (100%) of explants expressing *uidA* gene was observed with the 5-d co-culture, followed by 3-d co-culture (91%). However, when the evaluation was carried out after 12 d, there was no difference in the rate of *uidA* expression between 3 and 5 d of co-culture (Figures 1c, 1d and 2b). In a study with *E. globulus*, apical segments were transformed and co-cultivated for periods that varied from 2 to 7 d, with the best results of callogenesis and total number of transformed events being obtained after a 6 d co-culture (Spokevicius et al., 2005). For hypocotyl segments of *E. camaldulensis*, 2 d were sufficient (Ho et al., 1998).

Five days after tissue inoculation with *A. tumefaciens*, there was an increase in the expression of the *uidA* gene when AS (50 μM) was added to the pre-culture medium or in both media. When evaluation was carried out 12 d after inoculation, only the use of AS in both pre-and co-culture media positively affected *uidA* expression (Figure 2c). Some authors have reported the use of AS in the bacterial solution in the pre-and/or co-culture, aiming the infection of plant tissue. A higher concentration (100 μM) was used in a bacterial solution for genetic transformation of apical segments of *E. globulus* (Spokevicius et al., 2005). However, in other studies with *E. camaldulensis* using leaf explants (Mullins et al., 1997) and hypocotyls (Ho et al., 1998) the use of AS in the protocol of transformation was not reported.

Phenolic compounds are very important for the process of induction and infection of plant tissues by *Agrobacterium*. The bacteria are attracted by signal molecules, such as phenolic compounds. After the initial connection of the bacteria with the plant tissue, these molecules induce virulence genes in the *vir* region of Ti plasmid that activate T-DNA transfer from *A. tumefaciens* into the plant tissues (Tzfira and Citovsky, 2006). The results of this study suggest that acetosyringone in the pre- and/or co-culture medium may prove useful for improving transformation rates. The low rate of transformation of *E. grandis* × *E. urophylla* tissues not treated with synthetic acetosyringone suggests that while natural exudates of leaf explants wounded may not be sufficient to elicit v*ir* region activity, the addition of exogenous acetosyringone may stimulate the bacterium to transfer T-DNA to host cells.

Conclusions

Clone 11 presented a higher capacity than clone 827. Leaf explants collected from material transferred every 17 d had a better regenerative capacity then those transferred every 26 or 35 d. The use of a specific part of the leaves affected the organogenesis of clone 11, the most efficient being the basal part. The increase in the frequency of transfer of leaf explants to a bud induction medium has a positive influence on the organogenesis of clone 11 explants. The efficiency of genetic transformation of hybrid *E. grandis* × *E. urophylla* is affected by the duration of pre- and co-culture and by the addition of acetosyringone to the culture media. The most efficient conditions during genetic transformation were a 2-d pre-culture followed by a 3-d co-culture and the use of acetosyringone in pre- and co-culture media, when evaluation was carried out 12 d after inoculation.

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