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In vitro morphogenesis of *Eucalyptus grandis*: effects of antibiotics on explants

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ABSTRACT - *Effects of increasing cefotaxime, carbenicillin and timentin concentrations on the* in vitro *morphogenesis of* Eucalyptus grandis *explants were evaluated. It was observed that carbenicillin and timentin increased the frequency of explant regenerating calluses and decreased necrosis up to 600 mg L^{-1}, and that cefotaxime behaved similarly up to 300 mg L^{-1}. At this level there was an increasing frequency of explants and calluses showing necrosis. Hypocotyl and cotyledon explants presented similar morphogenetic results. Our results suggest that carbenicillin or timentin should be used in* Eucalyptus *tissue culture protocols to control endophytic bacteria for genetic transformation protocols in view of their positive effect on callogenesis.*

Key words: *Eucalyptus grandis*, morphogenesis, antibiotics, plant tissue culture.

INTRODUCTION

Seeking to overcome the variability inherent to seed propagation in long generation periods, late expression characteristics and incorporation of resistance and quality traits to elite genotypes some biotechnological approaches are currently being applied. Plant tissue culture procedures support these approaches by offering means of clonal propagation of superior individuals, plant rejuvenation and regeneration protocols for genetic transformation (Moralejo et al. 1998, Zobayed et al. 2000, Sartoretto et al. 2002). In this context, attempts are being made to develop wood plant *Agrobacterium*-mediated transformation protocols (Ke et al. 2001), although transgenic trees are usually obtained via biolistics (Merkle 2003). Nevertheless, *Agrobacterium*-mediated transformation has been the most used technique for obtaining transgenic plants (Savka et al. 2002). It is worth to note that, different regeneration frequencies are observed, which may be associated with the use of different explants such as leaves, cotyledons, embryos and hypocolyls (Cid et al. 1999, Sartoretto et al. 2002).

Positive (d´Ultra Vaz et al. 1993, Picoli et al. 2000) and negative (Nauerby et al. 1997, Ling et al. 1998, Picoli et al. 2002) effects of antibiotics are observed in *in vitro* culture of several species. Regardless of studies on regeneration

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and transformation protocols this is, as far as we know, the first report on antibiotic effects on *Eucalyptus in vitro* morphogenesis. Accordingly, this work aimed to evaluate the effects of increasing antibiotic concentrations on *in vitro* morphogenesis of *Eucalyptus* explants.

MATERIAL AND METHODS

Plant material

Eucalyptus grandis seeds from controlled crosses were supplied by Companhia Suzano Papel e Celulose (Itapetininga, São Paulo). Surface-sterilization was performed by seed immersion into 70% (v:v) ethanol for 5 min, followed by 5 min in a 10% (v:v) hydrogen peroxide solution, and a final dip in a 5% (w:v) calcium hypochlorite solution containing 0.1% (v:v) Tween 20. Following, the seeds were rinsed four times in sterile distilled water, blotted on filter papers, and then were germinated *in vitro* in sterile and moistened filter paper. Seeds were kept in the dark for the first seven days and afterwards were maintained under 16 h light regime, 30 mmol $m^{-2} s^{-1}$ light radiation provided by two fluorescent tubes (daylight 20 W, Osram) for additional 3 to 5 days. The culture room temperature was kept at 26±2 °C.

In vitro-grown seedlings (10-12 days after germination) were used as the source of explants. Hypocotyls and cotyledons were aseptically removed and transferred to shoot induction medium (SIM). The latter consisted of MS (Murashige and Skoog 1962) basal salts supplemented with 2.5 mg L^{-1} nicotinic acid, 10 mg L^{-1} thiamine, 1.2 mg L^{-1} pyridoxine, 100 mg L^{-1} myo-nositol, 1.5% (w:v) sucrose, 1.5% (w:v) glucose, 800 mg L^{-1} PVP, 100 mg L^{-1} arginine, 0.1 mg L^{-1} NAA, 0.1 mg L^{-1} TDZ and solidified with 0.6% agar at pH 5.7±0.1.

Antibiotic treatment

The antibiotics cefotaxime (União Química Farmacêutica Nacional SA, Brazil), timentin (SmithKline Beecham Farmacêutica, Brazil), and carbenicillin (Sigma Chem. Co., USA) at 0, 150, 300, 450 and 600 mg L^{-1} were added to SIM just after autoclaving (1.2 kg cm^{-2} at 121 °C; 15 min) and cooling. The antibiotics were filter-sterilized with Millipore filters (2.5 cm diameter; 0.22 µm; Millex). Recultures to SIM were performed every four weeks. Data on the frequency of explants or callus presenting oxidation or presenting necrosis (CN), explants callus without oxidation sectors (CR) and buds (B) were evaluated after 3 months. Letters that follow these variables identify first the explant source, hypocotyl (H) and cotyledon (C) and lastly the antibiotics timentin (T), cefotaxime (CE) and carbenicillin (CA).

Statistical analysis

A statistical analysis was performed using a completely randomized design. Data were subjected to appropriate regression analysis based on the average of the experiments. Each treatment had five replicates, with 10 explants of each source, hypocotyls and cotyledons per Petri dish. The experiment was performed twice. Completely contaminated dishes or explants were not considered in the analysis.

RESULTS AND DISCUSSION

It was observed that increasing antibiotics concentrations promoted a reduction in the number of hypocotyl and cotyledon explants showing necrosis and oxidation. Cefotaxime was an exception; up to 300 mg L^{-1} it displayed the described behavior and tended to augment the necrosis frequencies at higher concentrations (Figures 1A and 1B). These Figures are consistent with Figures 1C and 1D, which clearly present positive effects on the frequency of explants with calluses which are still capable of following morphogenic routes, even at timentin and carbenicillin concentrations as high as 600 mg L^{-1}.

The increased necrosis frequency induced by cefotaxime suggests inhibitory or toxic effects on *Eucalyptus* explants, as previously observed (Sarma et al. 1995, Picoli et al. 2002). Antibiotics used in *Eucalyptus* genetic transformation should take this into account, as it can possibly increase transformation efficiencies. This is especially important when difficulties in eliminating *Agrobacterium* from plant tissues are observed (Landsmann et al. 1999), where higher antibiotic concentration might help without restricting the regeneration efficiency. Yet, the wide-spread use of cefotaxime in the transformation protocols (Moralejo et al. 1998, González et al. 2002) should also be reviewed based on these results.

Lower frequencies of eggplant explants regenerating shoots and roots were observed with increasing cefotaxime and timentin concentrations. Even though, in the

concentrations tested, the average number of shoots and roots decreased with cefotaxime, whilst with timentin no significant effect was observed (Picoli et al. 2002). Similarly, cefotaxime had deleterious effects on eggplant embryogenesis (Picoli et al. 2000), tomato (Ling et al. 1998) and tobacco organogenesis (Nauerby et al. 1997). Genotype interaction with responses to antibiotics is observed as improved growth of *N. plumbaginifolia* cell colonies by use of related cephalosporins and penicillins (Pollock et al. 1983). On the other hand, d'Ultra Vaz et al. (1993) reported that cefotaxime added to the medium was essential for passion fruit (*Passiflora edulis* f. *flavicarpa*) cell division.

In spite of well-documented positive effects of cefotaxime on other species (Yepes and Aldwinkle 1994, Barret and Cassells 1994), this seems not to be true for *Eucalyptus*, where timentin and carbenicillin presented better results. Possibly, these beneficial effects are related to penicillin metabolism, where one of the breakdown products, a natural weak auxin (phenyl acetic acid), may be contributing to morphogenic responses (Holford and Newbury 1992).

Despite of the presence of explants regenerating buds (Figures 2A, 2B and 2C), necrosis and oxidation are still present in a high frequency (Figures 1A, 1B and 2C).

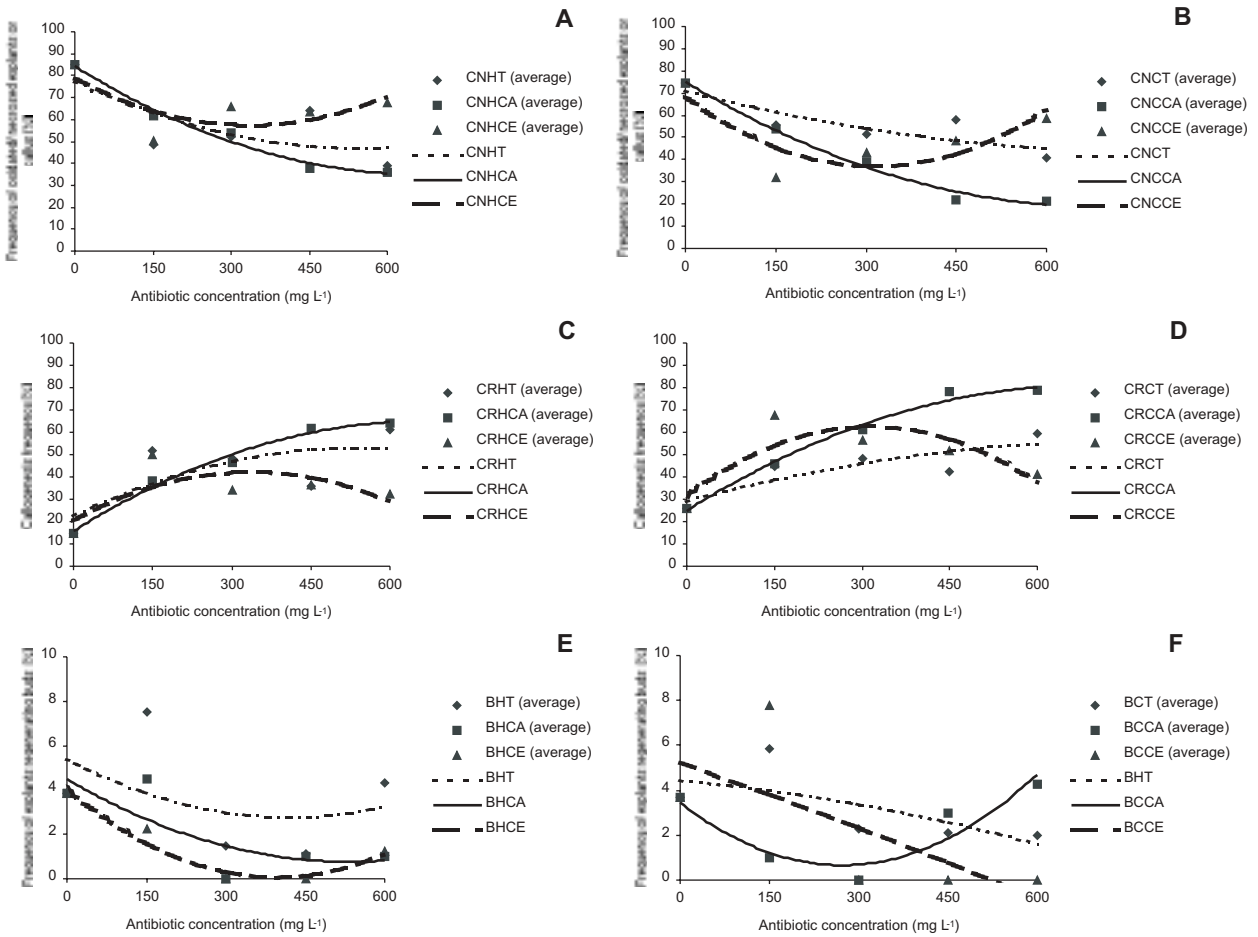

Figure 1. Antibiotic effects on the *Eucalyptus* morphogenic responses. Observed and estimated frequency of: **A** – hypocotyl explants or callus presenting oxidated/necrosed sectors; **B** – cotyledon explants or callus presenting oxidated/necrosed sectors; **C** – of hypocotyl explants presenting callus with non-oxidated sectors; **D** – of cotyledon explants presenting callus with non-oxidated sectors; **E** – of hypocotyl explants presenting buds; **F** – of cotyledon explants presenting buds. Frequency of explants or callus oxidation or presenting necrosis (CN), explants presenting callus with non-oxidated sectors (CR) and buds (B). Letters that follow these variables identify first the explant source, hypocotyl (H) and cotyledon (C), and lastly the antibiotics timentin (T), cefotaxime (CE) and carbenicillin (CA), respectively

Besides that, there is also a low frequency of explants regenerating buds (Figures 1E and 1F). On the other hand, little difference was observed in a comparison of the explant source (Figures 1A, 1C and 1E compared to Figures 1B, 1D and 1F).

The occurrence of necrosis, phenol exudation and explant oxidation (Figure 2C) are still problems to be solved regarding *Eucalyptus* regeneration protocols. The detrimental effect of phenolic compounds on morphogenic processes was described in the studies by Gill and Gill (1994). As far as the explant sources are concerned, Nugent et al. (2001) and Tibok et al. (1995) found that *E. globulus* shoot development occurred at a higher frequency from hypocotyl explants. On the other hand, Cid et al. (1999) observed different results depending on the growth regulators associated to *E. grandis* vs. *E. urophylla* cotyledon, hypocotyl and cotyledonary node explants.

Even though *Eucalyptus* is a recalcitrant species, genotype variation may have contributed to low regeneration frequencies since the explants were taken from seeds. Another variable is that *Eucalyptus* species respond differently to light stimuli, while light favored *E. globulus* (Nugent et al. 2001), longer periods in the dark provided better morphogenic responses in *E. camaldulensis* and *E. grandis* (Muralidharan and Mascarenhas 1987, Lainé and David 1994). Contrarily, Fett-Neto et al. (2001) observed that rhizogenesis of *E. globulus* was inhibited in the presence of light, while it did not affect *E. saligna* rooting capacity, suggesting the need for optimizing regeneration protocols.

Nevertheless, marked effects of antibiotics and their concentration are observed for callogenesis, irrespective of the explant used (Figures 1A to 1F). It is interesting to note that regression equations display correlation coefficients as high as 98% (Table 1), corroborating the influence of antibiotics on *Eucalyptus* morphogenesis. Despite the indirect regeneration of shoots and embryos, which has an intermediary step with proliferation of less differentiated and organized cells, from which groups or isolated cells reacquire competence and follow morphogenic pathways, these results are of great importance. The reason is assumed to be that, besides adopting cefotaxime, most of the *Eucalyptus* regeneration and transformation protocols are based on indirect regeneration (Tibok et al. 1995, Moralejo et al. 1998, Cid et al. 1999, González et al. 2002).

Data on explants regenerating buds, in spite of the good coefficients of correlation, still demand further studies regarding the optimization of regeneration frequencies. Our results suggest that the use of timentin and carbenicillin in concentrations up to 600 mg L^{-1} would support callus induction while controlling endophytes and eliminating

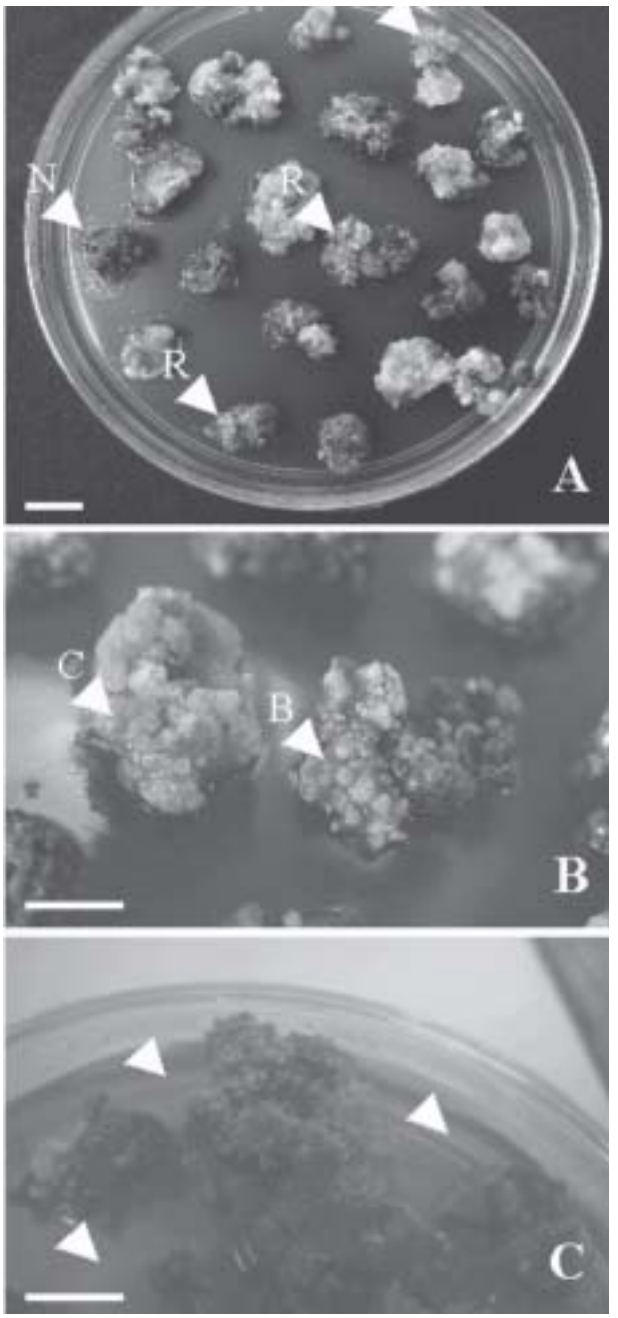

Figure 2. Regenerating hypocotyls and cotyledon *Eucalyptus* explants. **A** – *In vitro* culture showing necrosed (N) and regenerating explants (R), 300 mg L^{-1} carbenicillin; **B** – Detail of a callus (C) and a bud regenerating explant; **C** – Detail of a bud regenerating explant and phenol exudation. Bars = 10 mm

Table 1. Regression equations and correlation coefficients for the variables analyzed. Frequency of explants or callus oxidation or presenting necrosis (CN), explants presenting callus without oxidation sectors (CR) and buds (B)

Variable*	Regression	Coefficient of determination (R^2)
CNHCE	$Y = 0.0002X^2 - 0.1282X + 79.27$	0.4876
CNHCA	$Y = 0.0001X^2 - 0.1476X + 84.225$	0.9824
CNHT	$Y = 0.0001X^2 - 0.1114X + 77.498$	0.5197
CNCCE	$Y = 0.0003X^2 - 0.1986X + 68.544$	0.7069
CNCCA	$Y = 0.0001X^2 - 0.1635X + 75.04$	0.9898
CNCT	$Y = 4*10^{-05}X^2 - 0.0693X + 70.894$	0.734
CRHCE	$Y = -0.0002X^2 + 0.1282X + 20.73$	0.4876
CRHCA	$Y = -0.0001X^2 + 0.1476X + 15.775$	0.9824
CRHT	$Y = -0.0001X^2 + 0.1114X + 22.502$	0.5197
CRCCE	$Y = -0.0003X^2 + 0.1986X + 31.456$	0.7069
CRCCA	$Y = -0.0001X^2 + 0.1635X + 24.96$	0.9898
CRCT	$Y = -4*10^{-05}X^2 + 0.0693X + 29.106$	0.734
BHCE	$Y = 3*10^{-05}X^2 - 0.02X + 4.0604$	0.9464
BHCA	$Y = 10^{-05}X^2 - 0.014X + 4.4818$	0.616
BHT	$Y = 10^{-05}X^2 - 0.0125X + 5.3929$	0.1688
BCCE	$Y = -10^{-06}X^2 - 0.0092X + 5.2476$	0.4786
BCCA	$Y = 4*10^{-05}X^2 - 0.0204X + 3.4405$	0.8397
BCT	$Y = -4*10^{-06}X^2 - 0.0024X + 4.4262$	0.4783

*Letters that follow these variables identify first the explant source, hypocotyl (H) and cotyledon (C), and lastly the antibiotics timentin (T), cefotaxime (CE) and carbenicillin (CA), respectively

Agrobacterium, although further studies on direct and indirect morphogenesis should be accomplished.

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Morfogênese in vitro de *Eucalyptus grandis*: efeitos de antibióticos sobre explantes

RESUMO - *Os efeitos de concentrações crescentes de cefotaxima, carbenicilina e timentin foram avaliados na morfogênese* in vitro *de explantes de* Eucalyptus grandis. *Foi observado que carbenicilina e timentin aumentaram a freqüência de explantes regenerando calos e reduziram a necrose até 600 mg L^{-1}, enquanto cefotaxima teve o mesmo comportamento até 300 mg L^{-1}, a partir da qual levou a um aumento da freqüência de explantes necrosados. Explantes de hipocótilos e cotiledonares apresentaram resultados semelhantes quanto à morfogênese. Nossos resultados indicam que carbenicilina ou timentin devem ser utilizados em protocolos de cultura de tecidos de* Eucalyptus *para controlar bactérias endofíticas ou em protocolos de transformação genética, considerando seu efeito positivo na calogênese.*

Palavras-chave: *Eucalyptus grandis*, morfogênese, antibióticos, cultura de tecidos vegetais.

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