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Influence of antibiotics on indirect organogenesis of Teak

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Abstract. *Agrobacterium* is the largest method employed to transform woody plants. The bacterium is required to introduce the transgene into the plant nuclear genome. After transferring T-DNA to the plant cell, the bacteria affect plant growth negatively and have to be eliminated from plant tissue culture medium through the use of antibiotics. The effect of different antibiotics (timentin, cefotaxime and carbenicillin) on in vitro shoot regeneration of teak (*Tectona grandis* L. f.) was compared in hypocotyl, mature cotyledon and cotyledonary segments explants. Timentin and cefotaxime (100-300 mg l^{-1}) did not affect shoot regeneration and the number of shoots per explant. Moreover, at these concentrations, the two antibiotics seem to stimulate shoot regeneration. Carbenicillin at a dosage of 300 mg l^{-1} as well as cefotaxime and timentin at a dosage of 500 mg l^{-1} induced abundant calli formation and inhibited regeneration. Our data show that cefotaxime and timentin (300 mg l^{-1}) can be harmless to teak regeneration and can be used as bactericide agents during *Agrobacterium*-mediated transformation of *Tectona grandis*. Furthermore, we discuss the effect of antibiotic degradation on plant morphogenesis and its effect on regeneration from different explants.
Keywords *Tectona grandis*, co-cultivation, plant tissue culture, woody plant.

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Introduction

Teak (*Tectona grandis* L. - Lamiaceae) is a native forest species from India, Myanmar, Thailand and Laos and it is very famous worldwide for its high timber value (Fofana et al. 2009). Although a number of authors have propagated clonally teak by tissue culture techniques

(Tiwari et al. 2002, Shirin et al. 2005, Gyves et al. 2007), some efficient protocols for regeneration via organogenesis or somatic embryogenesis have been developed (Baghel et al. 2008). Breeding forest species via conventional procedures is a difficult and expensive process due to heterozygosity, long breeding time and large field trial procedures (Giri et al. 2004). Genetic transformation is a useful alternative tool to add characteristics in forest trees to those already present in the selected genotype or variety. However, the production of transgenic plants is entirely depended on an efficient regeneration system.

Agrobacterium-mediated genetic transformation is the largest method used to generate transgenic woody plants (Poupin & Arce-Johnson 2005). After transfer of T-DNA to the host cell it has to be eliminated from the tissue culture system by using antibiotics, affecting the potential of the transformed cells to regenerate (Pollock et al. 1983). During the process of genetic transformation plant cells and/or tissues have different responses to the antibiotics used in order to eliminate the Agrobacterium (Bosela 2009). To reach high rates of efficiency in this process it is indispensable to optimize the conditions for each species (Zhi-neng et al. 2007). The elimination of *Agrobacterium* is required to allow proper plant regeneration (Tereso et al. 2006). Carbenicillin, cefotaxime and timentin are the most common antibiotics used to suppress the growth of *Agrobacterium*. These antibiotics specifically suppress prokaryotic cell wall synthesis and eliminate bacteria, with almost none negative effect on plant tissues (Nauerby et al. 1997, Recht et al. 1999, Katayama et al. 2003).

However, the effect of these antibiotics seems to be highly depended on plant genotype and/or species. Their effect is controversial, once some of them are toxic to plant cells, and consequently, decrease the regeneration rates affecting the efficiency of transformation. On the other hand, many antibiotics can increase the ability of explants to produce buds, shoots and somatic embryos (Costa et al. 2000, Tereso et al. 2006). Antibiotics used to eliminate *Agrobacterium* should have a negligible effect on plant tissues and regeneration (Pollock et al. 1983, Bosela 2009).

Information concerning the influence of antibiotics on teak in vitro organogenesis is completely unknown. Regeneration via organogenesis and/or somatic embryogenesis is a prerequisite for successful genetic transformation. We report here the effect of carbenicillin, cefotaxime and timentin on in vitro indirect organogenesis from different explants of teak. We show, for first time, that cefotaxime and timentin can be successfully used as selection agents for co-cultivation during teak regeneration and genetic transformation. These two antibiotics do not affect the in vitro organogenesis of teak. Moreover, we developed an efficient regeneration protocol from different explants, which can be used in further transformation experiments.

Materials and methods

Plant material and antibiotics

The experiments were conducted with seeds of open pollinated populations of teak, courtesy from *Proteca Biotecnologia Florestal LTDA,* State of Mato Grosso, Brazil. Three sources of antibiotics were used in this study, carbenicillin (carboxypenicillin), cefotaxime (cefotaxime sodium) and timentin (3g of Ticarcillin + 0,1 g of potassium clavulanate).

The seeds were mechanically removed and washed with distilled water. Thereafter, the seeds were sterilized with ethanol 70% (v/v) for one min and NaOCl solution 75% (v/v) for 25 minutes (commercial bleach from 2.0 to 2.5% active chlorine). Finally, seeds were rinsed 3 times in distilled autoclaved water.

Seeds were germinated in MS (Murashige and Skoog 1962) medium supplemented with 0.1 mg l^{-1} thidiazuron (TDZ), 30 g l^{-1} sucrose

and solidified with 2.3 g.l^{-1} Phytagel. Cultures were kept at 25 ± 2°C, irradiance of 30 μmol. m^{-2}. s^{-1} under a 16 h light period using cool white fluorescent bulbs (Philips Eco MASTER).

Hypocotyl (5mm ± 1mm) and cotyledonary segment (7mm ± 1mm) explants were removed from seedlings 20 days after germination. They were placed in MS medium with plus 1mg l^{-1} BAP (6-benzyl amino purine), 0.5 mg l^{-1} GA_3 (Gibberellic acid) and the following concentrations of cefotaxime, carbenicillin and timentin (0, 100, 300 and 500 mg l^{-1}). Mature cotyledons (15mm ± 5mm) were placed in MS medium with plus 3 mg l^{-1} BAP, 0.5 mg l^{-1} GA_3 and the concentrations of antibiotics described above. Explants were kept under complete darkness at 25 ± 2ºC for 1 week. Cultures were then transferred to the same conditions described above for seed germination.

Statistical analysis

Three antibiotics (cefotaxime, carbenicillin and timentin) and different concentrations were evaluated (0, 100, 300 and 500 mg l^{-1}), as for the effect on explant type, on callus induction, frequency of adventitious shoots induction (%), number of shoots per explant and length of shoots. The trial was a factorial in a randomized design, with ten replications. Each replicate contained five explants in a flask. Data were transformed to $((x+0.5)/100)^{0.5}$; where x is the rate of regeneration, number of plants per responsive explant and elongation explant. After six weeks the variables cited above were analyzed. Before variances analyses (ANOVA), data were submitted to Hartley test ($P < 0.05$) to check homogeneity of variance between treatments. The variance analysis (ANOVA) procedures were used to test significant effects of the treatments for the variables measured ($P < 0.05$ and $P < 0.01$). Means were compared using Tukey test ($P < 0.05$).

Results

Antibiotics have been extensively used in plant tissue cultures due to their ability to inhibit or prevent bacteria growth as well as to stimulate in vitro development of shoots and roots (Costa et al. 2000, Mamidala & Nanna 2009). In this study, we tested the effect of three antibiotics (carbenicillin, cefotaxime and timentin) at different concentrations (0, 100, 300 and 500 mg l^{-1}) on in vitro regeneration of different teak explants (hypocotyls, cotyledonary segments and mature cotyledons).

Hypocotyl (H) and cotyledonary segment (CS) explants were placed in a MS medium supplemented with 1mg l^{-1} BAP and 0.5 mg l^{-1} GA_3. Mature cotyledons (MC) were placed in MS medium supplemented with 3 mg l^{-1} BAP and 0.5 mg l^{-1} GA_3. The optimal medium was previously defined for each teak explant (data not shown). Germination of teak seeds on thidiazuron-containing medium is essential for acquisition of competence for regeneration (data not shown, Murthy et al. 1998, Kong, 2009, Kucharska & Orlikowska 2009).

It was observed an inoffensive or positive effect of timentin and cefotaxime at 100 to 300 mg l^{-1} on shoot regeneration, whereas carbinicillin clearly inhibited shoot organogenesis in both explants and concentrations used (Figure 1a, d, g and Table 1). The percentage of regeneration, shoot number and length of shoots were statically significantly affected by explant type, concentration and antibiotic type (Table 1). Regeneration frequencies of hypocotyl, cotyledonary segments and mature cotyledon explants were not significantly affected in the medium with 100 to 300 mg l^{-1} timentin or cefatoxime. However, carbenicillin at concentrations above of 300 mg l^{-1} and timentim and cefotaxime at 500 mg l^{-1} induced abundant calli formation and inhibited shoot regeneration (Figure 1).

The number of shoots regenerated, although not significantly different, was higher in the regeneration medium supplemented with 300

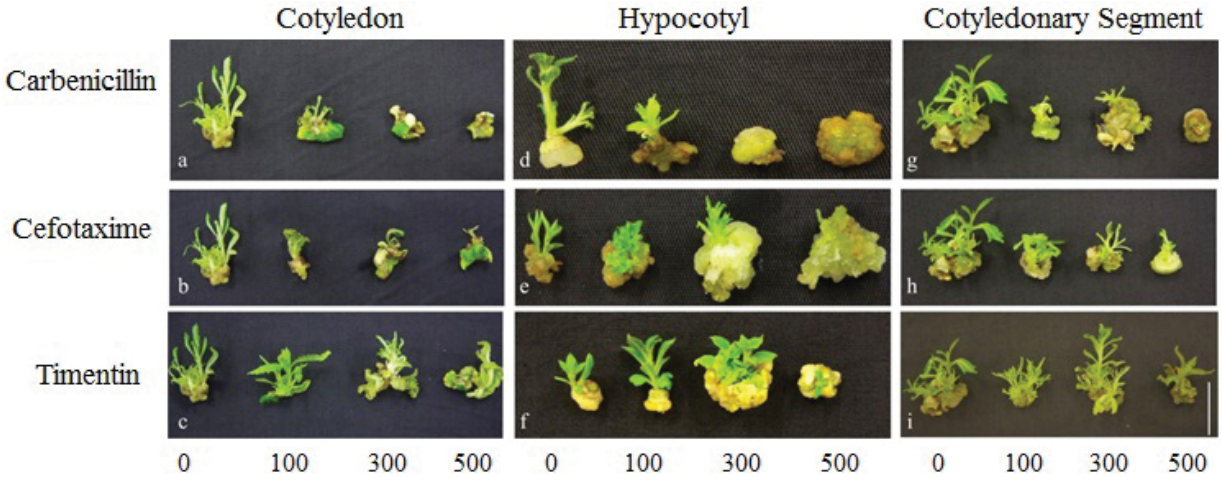

Figure 1 Effects of carbenicillin, cefotaxime and timentin antibiotics (0, 100, 300 and 500 mg.L^{-1}) on shoot regeneration of mature cotyledon, hypocotyls and cotyledonary segments of *Tectona grandis*. (a) Mature cotyledon in carbenicillin-containing medium; (b) mature cotyledon in cefotaxime-containing medium; (c) mature cotyledon in timentin-containing medium; (d) hypocotyls in carbenicillin-containing medium; (e) hypocotyls in cefotaxime-containing medium; (f) hypocotyls in timentin-containing medium; (g) cotyledonary segments in carbenicillin-containing medium; (h) cotyledonary segments in cefotaxime-containing medium; (i) cotyledonary segments in timentin-containing medium

Table 1 Effects of different concentrations of timentin (Ti), cefotaxime (Ce) and carbenicillin (Ca) on the in vitro regeneration of different explants of *Tectona grandis*

Antibiotic (mg.l^{-1})	Frequency of adventitious shoot induction (%)			Number of shoots per explant			Length of shoots (cm explant^{-1})		
	H	CS	MC	H	CS	MC	H	CS	MC
Control	50^{Aab}	70^{Aa}	30^{Ab}	2.0^{BCb}	2.0^{Bb}	4.0^{Aa}	1.5^{ABCb}	3.0^{Aa}	3.0^{Aa}
Mean	50^{Aa}	70^{Aa}	30^{Aa}	2.0^{Aa}	2.0^{Aa}	4.0^{Aa}	1.5^{Aa}	3.0^{Aa}	3.0^{Aa}
100 Ti	40^{ABa}	60^{Aa}	30^{Aa}	2.8^{ABa}	2.8^{Ba}	3.0^{ABa}	2.0^{Ab}	1.7^{BCc}	3.0^{Aa}
300 Ti	50^{Aa}	70^{Aa}	35^{Aa}	3.2^{Ab}	4.0^{Aa}	2.8^{Bb}	1.7^{ABc}	3.0^{Aa}	2.0^{Bb}
500 Ti	16^{ABCab}	45^{Aa}	5^{Ab}	1.4^{CDa}	1.0^{Ca}	1.0^{CDa}	0.5^{DEc}	1.2^{Cb}	2.0^{Ba}
Mean	35^{ABa}	58^{Aa}	23^{Aa}	2.5^{Aa}	2.6^{Aa}	2.3^{Ba}	1.4^{Aa}	2.0^{Ba}	2.3^{Aa}
100 Ce	50^{Aa}	70^{Aa}	35^{Aa}	2.0^{BCa}	2.0^{Ba}	2.0^{BCa}	1.2^{BCc}	1.8^{Ba}	1.5^{BCb}
300 Ce	45^{Aa}	57^{Aa}	25^{Aa}	3.0^{Aa}	2.6^{Ba}	2.6^{Ba}	1.5^{ABCa}	1.6^{BCa}	1.6^{BCa}
500 Ce	5^{BCb}	45^{Aa}	10^{Aab}	1.0^{Db}	2.0^{Ba}	2.0^{BCa}	0.5^{DEc}	1.6^{BCa}	1.0^{CDb}
Mean	33^{ABa}	57^{Aa}	23^{Aa}	2.0^{Aa}	2.2^{Aa}	2.2^{Ba}	1.0^{ABa}	1.7^{Ba}	1.3^{Ba}
100 Ca	25^{ABCa}	40^{Aa}	10^{Aa}	1.0^{Da}	1.0^{Ca}	0.6^{Da}	1.0^{CDa}	0.6^{Db}	0.6^{DEb}
300 Ca	0.0^{Ca}	0.0^{Ba}	0.0^{Aa}	0.0^{Ea}	0.0^{Da}	0.0^{Da}	0.0^{Ea}	0.0^{Ea}	0.0^{Ea}
500 Ca	0.0^{Ca}	0.0^{Ba}	0.0^{Aa}	0.0^{Ea}	0.0^{Da}	0.0^{Da}	0.0^{Ea}	0.0^{Ea}	0.0^{Ea}
Mean	8^{Ba}	13^{Ba}	3^{Aa}	0.3^{Ba}	0.3^{Ba}	0.2^{Ca}	0.3^{Ba}	0.2^{Ca}	0.2^{Ca}

Note. Means followed by the same capital letters within column and by the same lowercase letters within rows do not differ by the Tukey test ($P < 0.05$). H, CN and MC - Hypocotyls, Cotyledonary nodes and Mature cotyledons, respectively.

mg l^{-1} timentin (hypocotyl and cotiledonary node explants) or 300 mg l^{-1} cefotaxime (hypocotyl explants), as compared to the control (antibiotic-free regeneration medium). This antibiotic effect was not observed for mature cotyledons suggesting that the organogenetic response to the antibiotic may be determined by the explant (Table 1). Shoot elongation was

also not affected by low concentrations of timentin and cefotaxime (100 to 300 mg l^{-1}), whereas carbenicillin and 500 mg l^{-1} of timemtin or cefotaxime reduced the shoot growth (Table 1).

Discussion

The co-cultivation is a procedure required for T-DNA transfer to the plant cell. After this process, *Agrobacterium* is eliminated from plant tissue culture media by using antibiotics. The most commonly used antibiotics include the β-lactam families such as cefotaxime, timentin and carbenicillin (Tereso et al. 2006). Antibiotics that inhibit proteins biosynthesis should be avoided because they can affect gene expression in both cytoplasm and organelles inhibiting the growth and the regeneration ability (Recht et al. 1999, Katayama et al. 2003)

Timentin, carbenicillin and cefotaxime are types of β-lactam antibiotics and belong to the penicillin group. These substances has a similar structure to that of penicillin G, and it can produces phenylacetic acid (PAA), a naturally occurring auxin (Wightman & Lighty 1982, Lin et al. 1995, Nauerby et al. 1997, Ling et al. 1998). This suggests that an increase of auxin concentration may account for detrimental effects of 300-500 mg l^{-1} carbenicillin and 500 mg l^{-1} timentin or cefotaxime on shoot regeneration of teak.

The presence of auxins in the regeneration medium has an inhibitory effect on teak shoot organogenesis (data not shown, Baghel et al. 2008). Under these conditions a high calli induction was observed. Similarly to the literature, our results suggested that carbenicillin (300-500 mg l^{-1}), timentin (500 mg l^{-1}) and cefotaxime (500 mg l^{-1}) induce high calli formation inhibiting shoot regeneration (Figure 1).
In general, hypocoty and cotyledonary segments demonstrate higher frequencies of regeneration and higher shoot number per explant than mature cotyledons (Table 1). The different potential of totipotency of these explants might be explained through tissue morphology. Cotyledonary segments contain apical meristematic cells (lateral buds), which are more responsive to organogenic events than parenchyma cells of mature cotyledons (Ascough et al. 2009). Hypocotyls contain procambium-associated tissues, therefore they are high responsive explants. Usually, shoots or somatic embryos start from the tissue around procambium (Miranda et al. 1999, Paiva Neto et al. 2003).

Moreover, in this research we observed that the response of explants differ significantly to the antibiotics. These distinct plant organs may contain different levels of endogenous plant growth regulators, which can influence the tissue ability to produce new organs in the presence of antibiotics (Souza et al. 2003). This endogenous hormonal balance may interact with the breakdown product of β-lactam antibiotics affecting regeneration positively or negatively.

It seems that the effect of antibiotics during the co-cultivation is highly dependent on plant species and/or genotype. Type and concentration of antibiotic affected regeneration of *Platanus acerifolia* (Zhi-neng et al. 2007), *Citrus cinensis* (Mendes et al. 2009) and tomato (Costa et al. 2000). However, regeneration of *Picea glauca* and *Pinus pinaster* was not affected by presence of these three antibiotics or by their different concentrations (Le et al. 2001, Tereso et al. 2006).

Conclusions

In conclusion, the data showed that timentin and cefotaxime can be used as selection agents while cabicillin showed to interfere negatively on in vitro organogenesis during co-cultivation in *Agrobacterium*-mediated transformation experiments of *Tectona grandis*.

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