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Callus Induction in *Caesalpinia echinata*, a Brazilian Endangered Tree

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ABSTRACT

Caesalpinia echinata (Brazilwood) is an endangered tree from Brazil. Its exploitation due to the extraction of its red dye (brazilin) is intimately connected with the history and colonization of Brazil. Tissue culture can be used as a potential method for vegetative multiplication, which can provide a high rate of selected plants. Leaf fragments, stem segments and seed primordia from small and large fruits were used as explants, and different balances of auxins (IAA, 2,4-D and NAA) and cytokinins (BA, zeatin, kinetin and thidiazuron) were used to induce organogenesis. Despite the various auxins, auxin/cytokinin balances and reduction in MS salts, only induction of callus was observed in the modified MS medium without any differentiation, with progressive oxidation after 40 days of experimentation. Seed primordia from small fruits formed a whitish callus, and the best results expressed as percentage of induced callus corresponded to 0.5 mg L^{-1} TDZ and 4 mg L^{-1} 2,4-D treatments. Microscopic analysis of the callus showed elongated cells, inadequate for multiplication and dedifferentiation as well as round cells with a large nucleus, appropriate to cell division and organogenesis. Callus induction occurred in most treatments with growth regulators, but the species showed recalcitrance to differentiation, an unsuitable characteristic for organogenesis.

Keywords: auxins, growth regulators, Leguminosae, organogenesis
Abbreviations: 2,4-D, 2,4-dichlorophenoxy-acetic acid; **B5**, Gamborg (1968) vitamins; **BA**, 6-benzylaminopurine; **FeEDTA**, ferric ethylenediaminetetraacetic acid; **LPm**, Von Arnold and Eriksson (1981) medium; **MS**, Murashige and Skoog (1962) medium; **NAA**, α-naphthaleneacetic acid; **PGR**, plant growth regulator; **PIC**, picloram; **TDZ**, thidiazuron

INTRODUCTION

Caesalpinia echinata Lam. (Leguminosae, subfamily Caesalpinioideae) is a slow-growing, medium-sized tree from the Brazilian Atlantic Forest. It was intensely explored since the discovery of Brazil in 1500 until 1835, because of a red pigment used as dye, known as brazilin, found in its trunk (Mayer 1996) and actually was included in Appendix II of CITES (CITES, 2007). The *in vitro* cultivation of woody species can be considered an effective multiplication alternative. This method presents many advantages allowing for high and steady plant propagation in a minimal cultivation area, fast selection and easy genetic transformation. However, the leguminous trees are usually recalcitrant with regard to plant growth regulator (PGR) responses, showing low regeneration rates; also, the presence of lignin makes it even more difficult to establish an *in vitro* culture (Somers *et al*. 2003). Thidiazuron (TDZ), a potent synthetic cytokinin, has been successfully used to regenerate herbaceous and tree species of many families (Huetteman and Preece 1993; Eapen *et al*. 1998; Murthy and Saxena 1998). The presence of 2,4-dichlorophenoxy-acetic acid (2,4-D) generally initiates the proliferation of undifferentiated cells from zygotic embryo cells, resulting in the formation of a potentially embryogenic tissue mass (callus) (Cuming *et al*. 1996).

The objective of this work was to optimize the production of *C. echinata* callus by using auxins, especially 2,4-D, and after the successful establishment of callus, to induce organogenesis by balancing growth regulators (TDZ and 2,4-D) in order to expand the possibilities for the cultivation and preservation of this species. The research also aimed to evaluate germination of immature zygotic embryos isolated under aseptic culture conditions and their potential to form callus and somatic embryos.

MATERIALS AND METHODS

Morphogenesis

C. echinata seeds were harvested from nineteen year-old trees from the Botanical Garden of São Paulo, Instituto de Botânica, Brazil (2002) and disinfected with 10% sodium hypochlorite (0.25% active chlorine) under agitation for 15 min. The testa was removed in a sterile environment and the seeds were dipped again into a sodium hypochlorite solution (1.25% active chlorine) under agitation for 10 min, followed by three washes with sterile distilled water.

Glass flasks (400 ml) containing 60 ml of MS medium (Murashige and Skoog 1962) with 3.0% sucrose and solidified with 0.8% agar were used. Every medium pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Three seeds were placed in each flask and kept in a growth room under light intensity of 32 µmol m^{-2} s^{-1} of four fluorescent lamps, at 25-28°C and a 14-h photoperiod. For all other experiments, the culture room underwent the same basic conditions described above.

Leaves from *in vitro* germinated plants (6-month old) were excised in half and inoculated in MS medium supplemented with different balance of 1-naphthalene acetic acid (NAA) (0.5, 1.0 and 2.0 mg L^{-1}) and 6-benzylaminopurine (BA) (0.5, 1.0 and 2.0 mg L^{-1}). Each treatment had six replicates with three leaf segments, cultivated in the dark at 25-28°C.

For morphogenesis induction, callus fragments (approximately 0.5 cm in diameter) obtained in modified MS with 2 mg L^{-1} picloram (PIC) (Stella and Braga 2002) from leaf, stem, and plantlet were inducted to callus in half macro nutrients of MS medium, complete micro and vitamins, 3% sucrose, 0.8% agar and pH ad-

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Table 1 The treatments of auxins and cytokinins used to morphogenesis induction of callus.

Treatment	Auxins (mg L^{-1})				Cytokinins (mg L^{-1})		
	2,4-D	NAA	PIC	IAA	BA	Kinetin	Zeatin
T1	0.05						
T2	0.5						
T3	1.0						
T4	2.0						
T5	10.0						
T6	40.0						
T7		0.1					
T8		0.05			1.0		
T9		0.1			1.0		
T10		0.5					
T11		1.0			0.1		
T12		2.0				2.0	
T13			0.02				2.0
T14			2.0		5.0		
T15				0.1		10.0	
T16				0.2		4.0	
T17		10.0		10,0			
T18					5.0		

justed to 5.8 before autoclaving. The cultures (ten replicates, three explants per glass flask of 100 ml volume, 25 ml of medium) were maintained in the dark at 20-25°C. The treatments with growth regulators are described in **Table 1**.

Zygotic embryo- and organogenesis

Small green (3-4 cm in length, 30 to 45 days after anthesis) and large fruits (6-7 cm in length, 46 to 60 days after anthesis) were harvested and rinsed in sodium hypochlorite (2.5% active Cl^-) for 30 minutes, followed by three washes with sterile water. The fruits were opened in a sterile environment to remove seed primordia, measuring a maximum of 2.0 mm in length in both selected groups. These were cultivated in test tubes on LPm culture medium (Von Arnold and Eriksson 1981) supplemented with vitamin B5, 5 ml L^{-1} Fe EDTA, 0.07% glutamine, 3% sucrose, and 0.2% phytagel in six treatments containing different concentrations of TDZ and 2,4-D (A - 0.1 mg L^{-1} TDZ, B - 0.5 mg L^{-1} TDZ, C - 1.0 mg L^{-1} 2,4-D, D - 2.0 mg L^{-1} 2,4-D, E - 4.0 mg L^{-1} 2,4-D and F - 0.5 mg L^{-1} TDZ + 4.0 mg L^{-1} 2,4-D) at 15 ml medium per glass tube. The treatments were maintained in the dark at 20-25°C, with 24 replicates and two seed primordia per tube. For treatments A (0.1 mg L^{-1} TDZ), B (0.5 mg L^{-1} TDZ), C (1 mg L^{-1} 2,4-D), and F (0.5 mg L^{-1} TDZ and 4 mg L^{-1} 2,4-D), fruits were harvested in the morning and immediately taken to the laboratory, and disinfected for inoculation under aseptic conditions, without storage. Treatments D (2 mg L^{-1} 2,4-D) and E (4 mg L^{-1} 2,4-D) were stored in plastic bags with some sodium hypochlorite solution, and in the next day after harvesting, the fruits were disinfected with the same procedure and inoculated in the medium.

After 20-day culture, the medium showed oxidation and the seed primordia were transferred to the same regulator-free medium. They remained there for another 15 days and then were transferred to the same medium in their corresponding treatments with growth regulators. Observations were made at 18, 25, and 85 days after seed inoculation. After three months, all seed primordia were transferred to the treatment containing 0.5 mg L^{-1} TDZ and 4 mg L^{-1} 2,4-D for maintenance and multiplication.

Hypocotyls of plantlets from *in vitro* germinated seeds were used for organogenesis induction after disinfection for 15 minutes in sodium hypochlorite at 2.5% active Cl^- and five washes with sterile distilled water. The hypocotyls were cultivated in test tubes in LPm medium containing vitamins, 5 ml L^{-1} Fe EDTA, 0.07% glutamine, 3% sucrose, 0.2% Phytagel® containing a balance between the following plant regulators: TDZ (0.1 mg L^{-1}), 2,4-D (2.0 mg L^{-1}), and TDZ (0.1 mg L^{-1}) + 2,4-D (2.0 mg L^{-1}), with five replicates and five explants per tube. Cultures were maintained in the dark at 20-25°C.

Leaf fragments (2.0-4.0 mm width) were excised from four-month plantlets treated with BA (2.0 mg L^{-1} via leaf spray, weekly) disinfected for 15 min. in sodium hypochlorite solution (1.25% active Cl^-) followed by five washes with sterilized distilled water and cultivated in LPm culture medium containing vitamin B5, 5 ml L^{-1} Fe EDTA, 0.07% glutamine, 3% sucrose, and 0.8% agar, with nine treatments containing different balances from 0 to 3 mg L^{-1} BA and 0.5 to 10 mg L^{-1} NAA. The treatments were maintained in the dark at 20-25°C, with 50 replicates and 1-4 leaf fragments per flask.

The fresh and dry mass of callus were obtained in analytical balance and microscopic analysis was done in fresh callus under optical binocular microscopy. The statistical analyses for all experiments were based on variance analysis (ANOVA) with 5% probability.

RESULTS AND DISCUSSION

Fresh and dry mass from leaf fragments of axenic plants (**Table 2**) did not present statistical differences among treat-

Table 2 Effects of NAA and BA on dry and fresh masses from leaf explants of *Caesalpinia echinata*.

Treatments	NAA (mg L^{-1})	BA (mg L^{-1})	Fresh weight (g)	Dry weight (g)
1	0.5	0.2	1.60 a*	0.71 a *
2	0.5	1.0	1.24 a	0.48 a
3	0.5	5.0	1.03 a	0.35 a
4	1.0	0.2	0.90 a	0.35 a
5	1.0	1.0	0.84 a	0.31 a
6	1.0	5.0	0.80 a	0.28 a
7	2.0	0.2	0.78 a	0.23 a
8	2.0	1.0	0.68 a	0.15 a
9	2.0	5.0	0.61 a	0.10 a
CV (%)			57.61	113.71

* Different lowercase letters in columns are statistically different (ANOVA, $p<0.05$)

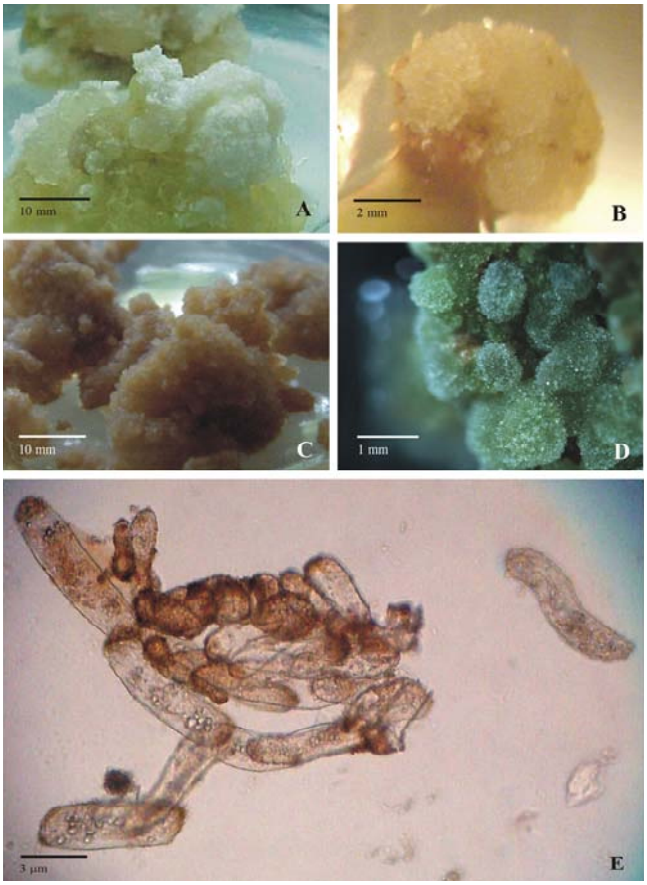

Fig. 1 Callus and cells of *Caesalpinia echinata*. (**A**) white friable callus; (**B**) callus seed primordial; (**C**) brownish oxidized callus; (**D**) green friable callus of seed primordial; (**E**) isolated cells with elongated (auxin effect) and round cell (potential viable).

Table 3 Percent oxidation or contamination after 85 days from seed primordia of large and small fruits of *Caesalpinia echinata*.

Treatments (mg L^{-1})	Lost by:	
	Contamination (%)	Oxidation (%)
A (0.1 mg L^{-1} TDZ)	43.8	62.5
B (0.5 mg L^{-1} TDZ)	60.0	60.0
C (1 mg L^{-1} 2,4-D)	60.9	54.4
D (2 mg L^{-1} 2,4-D)	97.5	82.5
E (4 mg L^{-1} 2,4-D)	90.9	84.1
F (0.5 mg L^{-1} TDZ + 4 mg L^{-1} 2,4-D)	77.5	72.5
General average (%)	71.8	69.3

ments. At 90 days, compact white or brown callus with no differentiation or organogenesis were obtained in all treatments; however the high values of variation coefficient, suggests that the responses are correlated to the organogenic potential from the different types of explants for callus production, without buds or roots induction.

Microscopical observation of the callus produced in modified MS showed elongated cells, inadequate for multiplication and differentiation, and round cells with a large nucleus, subjected to cell division and organogenesis (**Fig. 1E**).

Different types of auxins and auxin/cytokinin balances (T1 to T18), reduction in MS salts and consequent nitrogen concentration reduction (promotes cells multiplication in woody species) didn't induct organogenesis, only yellowish brown callus induction without differentiation was observed in most of the 18 treatments (after 40 days). Auxin shock treatments at 10 and 40 mg L-1 2,4-D (T5 and T6, respectively) also responded negatively with callus oxidation after approximately 40 days of experimentation (data not shown). Treatments 7 (0.1 mg L^{-1} NAA) and 14 (2.0 mg L^{-1} PIC and 5.0 mg L^{-1} BA) inducted callus formation after three months (**Fig. 1A**), followed by callus oxidation after four to six months. Development of visible cell masses occurred on two explants at 0.1 mg L^{-1} NAA and three explants at 2.0 mg L^{-1} PIC with 5.0 mg L^{-1} BA (data not shown) without induction of roots or buds. These results are possibly related to recalcitrance in this woody family, which calluses hardly differentiate for plant regeneration even in high growth regulator concentrations.

Explants from seed primordia of small and large fruits (**Table 3**) had high contamination rates (72% on average) and the sterile explants showed high oxidation (69% on average), especially after 85 days after inoculation.

Treatments D (2 mg L^{-1} 2,4-D) and E (4 mg L^{-1} 2,4-D) had higher contamination rates, probably due to overnight fruit storage in plastic bags that promoted contamination of this plant material, especially in seed primordia maintained for long experimentation periods.

Oxidation of excised seed primordia was observed after one day of inoculation, although it was removed almost without lesions from the vascular region of the immature fruit, indicating that exposure to the culture environment or to the MS medium already triggers this process (**Table 3**).

Callus induction and development at 18, 25, and 85 days after inoculation of the seed primordia was obtained from small and large fruits of *C. echinata* (**Table 4**). There was no statistical difference on callus induction among embryos primordia size in small and large fruits (0.5 and 2.0 mm in length), but the results indicates that fruit development stage, expressed by fruit length, may induce callus formation. It is possible that seed primordia originated from longer maturation period of larger fruits induced and deve-loped a higher percentage of callus. Treatment F (0.5 mg L^{-1} TDZ and 4 mg L^{-1} 2,4-D) followed by treatment C (1 mg L^{-1} 2,4-D) presented higher percentage of induced callus form seed primordia of small fruits. The callus showed a whitish coloration and after 85 days of culture high rates of oxida-tion and contaminations. After 18 days of culture treatment A (0.1 mg L^{-1} TDZ) showed highest ratio of callus forma-tion and production of darker callus in seed primordia from large fruits. Same results were observed in treatment F (0.5 mg L^{-1} TDZ and 4 mg L^{-1} 2,4-D) in the 25-85-day period. However, differentiation into roots or stem buds did not oc-cur, besides callus production in all treatments followed by oxidation.

Seed primordia from both types of fruits (**Table 5**), grown in treatments containing only TDZ [treatments A (0.1 mg L^{-1} TDZ) and B (0.5 mg L^{-1} TDZ)], showed smaller and grayish callus formation, while seed primordia cultured in treatments containing 2,4-D only [treatments C (1 mg L^{-1} 2,4-D), D (2 mg L^{-1} 2,4-D), E (4 mg L^{-1} 2,4-D)] and 2,4-D in association with TDZ (Treatment F), showed a more compact and yellowish callus formation. Data relative to callus fresh weight in the various treatments could not be obtained, due to their small mass and high contamination ratio. The contamination occurred over 20 days inoculation, possibly endogenous from the explants.

Under natural conditions *C. echinata* seed germination usually occurs in less than two weeks (Borges *et al*., 2006). After 63 days of inoculation, few explants from seed primordia developed the epigeal segment and an etiolated stem with one slow growing apical bud (**Table 6, Fig. 1B**). This epicotyl with the presence or absence of non-expanded leaves, without callus formation and showing fast senescence (**Fig. 1C**), probably corresponds to an anomalous growth related to the long period of explants exposure to TDZ or 2,4-D regulators treatments. Abnormal bud development were observed in explants of leaf fragment cultures in peanut (*Arachis hypogaea* L.) that remained for an extended period in contact with TDZ, and plant regeneration was not possible due to absence of a typical apical meristem and the fusion of disorganized vascular bundles (Akasaka *et al*. 2000).

Development of the seed primordia stem in treatments A (0.1 mg L^{-1} TDZ), B (0.5 mg L^{-1} TDZ), and D (2 mg L^{-1} 2,4-D) is possibly related to the presence of growth regulators, which induced the development of etiolated buds without expansion of primary leaves, mainly observed seeds from large fruits (data not shown).

Fratini and Ruiz (2002) considered cytokinins TDZ (2 mg L^{-1}) plus BA (2 mg L^{-1}) the most effective plant growth regulators for bud induction in lentils, while Das *et al.*

Table 4 Callus induction (%) from seed primordial of large and small fruits of *Caesalpinia echinata* 18, 25 and 85 days after inoculation.

Treatments (mg L^{-1})	Callus induction (%)					
	Small fruits			Large fruits		
	18 days	25 days	85 days	18 days	25 days	85 days
A (0.1 mg L^{-1} TDZ)	20.8	38.9	33.3	66.7	75.00	76.5
B (0.5 mg L^{-1} TDZ)	12.5	50.0	0.0*	9.5	55.6	72.2
C (1.0 mg L^{-1} 2,4-D)	11.1	66.7	50.0	14.3	47.62	65.0
D (2.0 mg L^{-1} 2,4-D)	0.0	0.0	0.0	26.5	45.5	60.0
E (4.0 mg L^{-1} 2,4-D)	10.0	50.0	0.0*	8.8	50.0	66.7
F (0.5 mg L^{-1} TDZ + 4.0 mg L^{-1} 2,4-D)	22.2	64.3	90.0	13.6	64.3	88.9
Callus formation's means (%)	15.3[a]	54.0[a]	57.8[b]	23.2	56.3	71.5

* Percent decrease of callus due to oxidation or contamination losts
[a] Treatment D was not considered;
[b] Treatments B, D and E were not considered

Table 5 Total percentage of callus induction from seed primordial of large and small fruits of *Caesalpinia echinata* 18, 25 and 85 days after inoculation.

Treatment (mg L^{-1})	Callus formation (%)		
	18 days	25 days	85 days
A (0.1 mg L^{-1} TDZ)	43.8	57.9	58.6
B (0.5 mg L^{-1} TDZ)	10.0	55.0	65.0
C (1.0 mg L^{-1} 2,4-D)	13.0	50.0	63.6
D (2.0 mg L^{-1} 2,4-D)	22.5	41.7	54.6
E (4.0 mg L^{-1} 2,4-D)	9.1	50.0	60.0
F (0.5 mg L^{-1} TDZ + 4 mg L^{-1} 2,4-D)	17.5	64.3	89.5

Table 6 Callus production and leaf development (%) from seed primordia of large and small fruits of *Caesalpinia echinata* 85 days after inoculation.

Treatments (mg L^{-1})	Development (%)	
	Callus	Leaf
A (0.1 mg L^{-1} TDZ)	41.4	17.2
B (0.5 mg L^{-1} TDZ)	50.0	15.0
C (1 mg L^{-1} 2,4-D)	59.1	4.6
D (2 mg L^{-1} 2,4-D)	36.4	18.2
E (4 mg L^{-1} 2,4-D)	60.0	0.0
F (0.5 mg L-1 TDZ + 4 mg L-1 2,4-D)	89.5	0.0

(2002) obtained *Vigna mungo* callus subjected to regeneration germinating seeds with 2 mg L^{-1} BA solution. High rates of shoot induction in *Caesalpinia pulcherrima* node buds was obtained by Rahman *et al*. (1993) using 1 mg L^{-1} BA plus 1 mg L^{-1} NAA treatment.

Hypocotyls from axenic plants (15 days after germination) grown in LPm medium supplemented with 0.1 mg L^{-1} TDZ, 2.0 mg L^{-1} 2,4-D, or 0.1 mg L^{-1} TDZ + 2.0 mg L^{-1} 2,4-D did not induct callus or organogenesis of buds or roots, again demonstrating recalcitrance character for growth regulators and concentrations used on this specie. Best results in callus induction in *Eucalyptus grandis* x *E. urophylla* were recorded for treatment with TDZ (0.5 mg L^{-1}) plus NAA (0.1 mg L^{-1}), achieving callus formation of all stem explants, mainly in the extremities of these, regenerating adventitious buds (Alves *et al*. 2004).

Leaf fragments of from young plants pretreated with BA had a low contamination rate, but without induction of callus, buds or roots in any of the nine treatments tested, after 60 culturing days leaf fragments still green without oxidation or senescence (**Fig. 1D**).

Despite the use of several growth regulators, auxin shocks, auxin/cytokinin balances and *C. echinata* explants (callus, seed primordia, plantlet hypocotyls or young plants treated with cytokinins), organogenesis of roots or buds was not obtained, indicating the recalcitrance of this legume tree in tissue culture, as many other legumes are not subjected to initiation, multiplication, and regeneration (Somers *et al*. 2003).

It is well known that the loss of regenerative ability of recalcitrant plants affects its organogenesis. This feature can be due to the atypical biochemical cycles from which they are apparently unable to escape (Gaspar *et al*., 2000), with the production of somatic mutations and the epigenetic changes that cause aberrant regulation of the cell cycle machinery. Other explants, the selection of suitable developmental stages, and new growth regulators combinations will possibly allow this species to be propagated *in vitro*.

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