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Full Length Research Paper

In vitro conservation and low cost micropropagation of *Cochlospermum regium* (Mart. Ex. Scharank)

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***Cochlospermum regium* (Mart. Ex. Scharank), a Cochlospermaceae is a Cerrado medicinal species showing antimicrobial activity on the female reproductive system. The species risks being genetically eroded since it is highly explored, the biome devastated and the plant roots are employed for phytotherapeutic preparations. This study aims to develop a micropropagation protocol for large scale production of plantlets and establish the species conservation in a germplasm bank, *in vitro*. Nodal segments were inoculated into Murashige and Skoog (MS) medium supplemented with different concentrations of sucrose, BAP, kinetin, zeatin, Phytagel® and activated charcoal. The bud position effect on the multiplication index was evaluated and the auxin IBA utilized in rooting experiments. The best multiplication response was with the apical bud in culture medium in absence of cytokinins. MS inoculated explants with different IBA concentrations did not root *in vitro*, but *ex vitro* rooting was satisfactory. Survival of explants, 47.62%, was for 4 months in germplasm bank conditions without culture medium transference.**

Key words: Cochlospermaceae, Cerrado, "algodãozinho do campo", medicinal plants, plant growth regulators.

INTRODUCTION

Cochlospermum regium, a Brazilian's Savanna (Cerrado) medicinal plant is known as "algodãozinho do campo" and mainly utilized in the treatment of female reproductive system infections (Nunes et al., 2003; Souza and Felfili, 2006; Tresvenzol et al., 2006). The use of roots in phytotherapeutic preparations exposes the plant to genetic erosion risks. The plant hydroalcoholic extracts and leaf essential oils show antibacterial activity and root aqueous extracts inhibit Walker carcinosarcoma (Honda et al., 1997; Oliveira et al., 1996; Sólon et al., 2009). Chemical assays demonstrated the presence of tannins, phenol derivatives, mucilage, saponins, steroidal triterpenes, flavonoids and essential oils (Honda et al., 1997; Lima et al., 1996). Chemical, pharmacognostic and toxicological studies partially warranted the efficacy and safety of *C. regium* phytotherapeutic extracts (Sólon et al., 2009). Nunes and Carvalho (2003) reported that lyophilized aqueous extract of *C. regium* roots did not show mutagenic effects on *Drosophila melanogaster* larvae and Andrade et al. (2008) verified that it did not modify the cellular DNA. Though several chemical and biological investigations have already been conducted with *C. regium*, agronomic studies with that species are still scarce. It is already known that *C. regium* seeds present tegumental dormancy that may be overcome with seed immersion in sulfuric acid and the germination speed index is correlated with seed size (Sales et al., 2002; Camillo et al., 2009; Inácio et al., 2010).

Moreover studies evidenced that *C. regium* shows high adaptation or phenotypic plasticity to both nutrient-poor soil and nutrient-rich soil, modifying its morphology what

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may explain its dispersion all over the Cerrado phyto-physiognomic (Inácio et al., 2010). Thus, the widespread use of *C. regium* roots in the proven efficient phytotherapeutic preparations justify agronomic studies aiming to establish high scale plant production methodologies. Thus, this report describes a protocol for the micropropagation and production of plantlets and the establishment of an *in vitro* germplasm bank for the species.

MATERIALS AND METHODS

General experimental conditions

C. regium explants, 0.5 cm long, obtained from *in vitro* germinated plantlets were inoculated into glass test tubes (8.5 × 2.5 cm) containing MS medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 100 mg/L of the antioxidant polyvinylpyrrolidone (PVP) and gellified with 2.5 g/L Phytagel®. Before inoculation the culture medium had the pH adjusted to 6.0 and was autoclaved for 15 min at 121°C. Explants were kept in a growing room under light intensity of 25 µMol $m^{-2}s^{-1}$ in cycled periods of 16:8 h at 25 ± 2°C. For the germplasm bank experiments room temperature was lowered to 15 ± 2°C.

Micropropagation

Explants with apical, cotyledonary and hypocotyl buds inoculated in MS and MS/4 medium were used in evaluations of sprouting percentages, number of buds and callus presence, this last parameter determined by visual grades in a crescent incidence order (0<1<3<5<7). Apical buds were introduced in MS supplemented with 6-benzylaminepurine (BAP), kinetin and zeatin in the concentrations of 0, 0.5, 1.0 and 2.0 µM. In this experiment the parameters evaluated during 60 days were: Shoots percentages, number of buds per shoot, shoots per explant, callus percentages and incidence, vitrification and height of shoots. In another five experiments utilizing axilar buds, parameters evaluated were: shoots percentages, number of buds, height of shoots, percentage and incidence of callus and percentage and intensity of vitrification. Specifically the five experiments were characterized as follows:

1) Media were MS basal and MS supplemented with activated charcoal (3.5 g/L);
2) Different sucrose concentrations (Table 2);
3) Addition of Phytagel® in concentrations of 2.5 or 5.0 g/L;
4) Test tubes were covered with filter paper or with plastic caps (Figure 1A), aluminum foil, cotton or PVC film (Figure 1B); also used glass flasks with plastic caps (Figure 1C);
5) Buds localized at different positions in the stem (Figure 2).

For *in vitro* rooting explants had 1.5 cm in height and contained an apical bud and a nodal segment, which were inoculated into MS supplemented with indole butyric acid (IBA) in concentrations of 0, 1.0, 2.0, 4.0 and 6.0 µM. For acclimatizing and rooting, *ex vitro*, 3 cm. micro sticks produced *in vitro* were transferred to expanded polystyrene trays containing the commercial substrate Plantmax®, which were kept in green houses covered by glass during one week. In the following week, covers were removed at night and put back during the day and completely removed until the end of the experiment that lasted for 56 days. At this time, survival and rooting percentages and size of roots were evaluated.

Conservation in germplasm banks

Nodal segments of *C. regium* were inoculated into media MS and MS/2 supplemented with 2% sucrose and osmotic stress agents (Table 2) and kept in a germplasm bank room for 4 months when survival rates were evaluated.

Statistical analysis

The experimental design was completely random. Data were analyzed by the Scott Knott test, 5% confidence level, using the SISVAR program (Ferreira, 2005).

RESULTS AND DISCUSSION

Micropropagation

Hypocotyl buds produced the lowest callus incidence but the sprouting percentages were poor, while the cotyledonary buds were the most promising as to the higher number of bud/explant (5.16) (Figure 3A and B). This result confirms Moraes et al. (2007), who postulate that the cotyledonary bud, obtained after seed germination, as the most adequate to produce sprouts compared to other explant types. Vegetal regulators, BAP, kinetin and zeatin do not have any effect on the parameters evaluated during 30 or 60 days. Sprouting percentages at 60 days were 76.39% (BAP), 72.22% (kinetin) and 75.00% (zeatin) (Table 1). The greatest number of buds was found in a medium without regulators (2.96 at 60 days). There was no interaction between regulators and dosages employed. Although cytokinins are classical stimulators in multiplication processes *in vitro*, there are exceptions in species like *Calendula officinalis*, *Thymus vulgaris* and *Vernonia condensate*, which behave like *C. regium*, that is, they do not respond to this class of phyto regulators (Bertoni et al., 2006; Rubin et al., 2007; Vicente et al., 2009). Callus formation was stimulated by cytokinins, mainly BAP (Table 1 and Figure 4A) but did not intensify vitrification, which occurred in all elongated explants in the presence or absence of cytokinins. The high production of callus was lowered by activated charcoal, without decreasing the number of buds in comparison to values without charcoal (2.17 ± 0.38 and 1.93 ± 0.45, respectively).

Similar results were reported by Santos et al. (2006) in explants of *Caryocar brasiliense*, raised in culture medium supplemented with activated charcoal that was efficient in reducing the intense production of callus. Increasing sucrose concentration to 35 or 45 g/L eliminated vitrification in explants after 21 days and produced a higher number of buds. However, sucrose concentrations above 30 g/L produced red colored

Table 1. Effects of the growth regulators BAP, kinetin and zeatin on shoot proliferation rates, number of buds per shoot, number of shoots per stem, proportion of callus formation, vitrification ratios and shoot elongation after 60 days of culture.

Plant regulator	Concen-tration (µM)	Shoot proliferation (%)	Number of buds per shoot	Number of shoots per stem	Callus formation (%)	Incidence of callus	Vitrification (%)	Shoot length (cm)
BAP	0.5	75.00[a]	1.58[a]	1.38[a]	100.00[a]	4.17[a]	16.67[b]	1.29[a]
	1.0	85.50[a]	2.75[a]	1.60[a]	100.00[a]	4.33[a]	41.67[a]	1.53[a]
	2.0	66.67[a]	1.49[a]	1.42[a]	95.83[a]	4.54[a]	45.83[a]	1.32[a]
	CV%**	14.43	32.23	30.00	4.23	14.96	20.78	10.08
Kinetin	0.5	87.50[a]	3.00[a]	1.62[a]	100.00[a]	2.92[b]	12.50[a]	1.56[a]
	1.0	70.83[a]	2.25[a]	1.48[a]	100.00[a]	3.67[a]	29.17[a]	1.35[a]
	2.0	58.33[a]	1.29[a]	1.34[a]	100.00[a]	3.83[a]	25.00[a]	1.13[a]
	CV%**	16.32	38.08	24.74	0.00	9.30	40.10	17.10
Zeatin	0.5	70.83[a]	2.17[a]	1.47[a]	87.50[a]	2.46[a]	29.17[a]	1.72[a]
	1.0	87.50[a]	3.08[a]	1.57[a]	95.83[a]	2.96[a]	62.50[a]	1.86[a]
	2.0	66.67[b]	2.33[a]	1.43[a]	91.67[a]	2.75[a]	45.83[a]	1.76[a]
	CV%**	7.86	29.12	21.65	12.86	42.04	20.33	20.21

*Means followed by the same letters within each column did not significantly differ by Scott–Knott test at P < 0.05. **CV - coefficient of variation.

Table 2. Effects of adding sucrose to the culture medium on shoot proliferation rates, number of buds per explant, proportion and intensity of vitrification, shoot elongation and survival rates after 30 days of culture.

Concentration of sucrose (g/L)	Shoot proliferation (%)	Number of buds per explant	Proportion of vitrification (%)	Intensity of vitrification	Shoot length (cm)
20	94.44[a]	4.19[b]	94.44[a]	4.61[a]	1.14[a]
30	71.11[b]	3.39[b]	65.56[b]	2.83[b]	0.56[b]
35	100.00[a]	7.08[a]	58.33[b]	0.92[c]	0.92[a]
40	88.89[a]	5.86[a]	58.59[b]	0.92[c]	1.03[a]
45	100.00[a]	4.67[b]	40.00[c]	0.40[c]	0.67[b]
50	100.00[a]	5.00[b]	33.33[c]	0.33[c]	0.87[a]
60	94.44[a]	3.18[b]	16.67[c]	0.17[c]	0.75[b]
CV%**	10.11	21.15	33.86	39.07	17.64

*Means followed by the same letters within each column did not significantly differ by Scott–Knott test at P < 0.05. **CV - coefficient of variation.

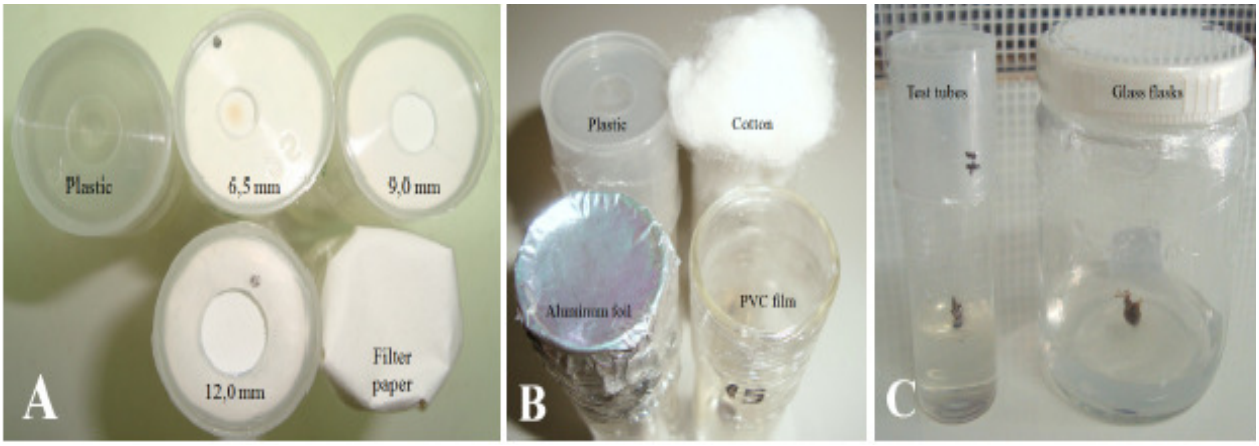

Figure 1. (A) Different types of closures (plastic caps and filter paper) used to seal the flasks, (B) plastic caps, cotton, aluminum foil and PVC film, (C) different types of flasks used in the vitrification experiments.

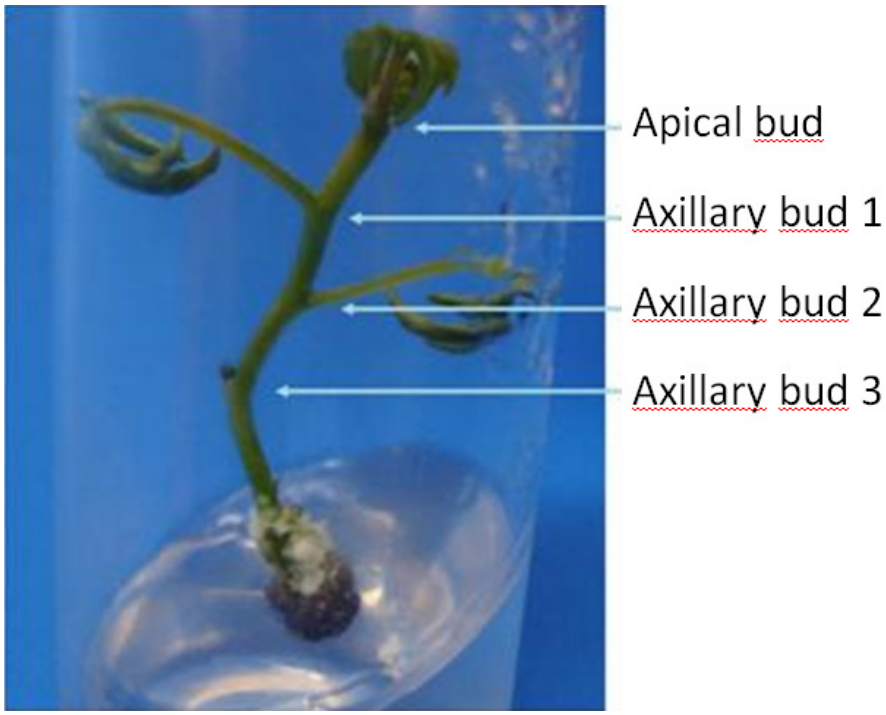

Figure 2. Position of buds on *C. regium* plants.

leaves, leaf abscission and later explant necrosis (Table 2). According to Braidot et al. (2008), senescence responsible hormones, like abscisic acid and ethylene may influence biosynthesis of phenolic compounds like flavonoids, antocyanins and tannins. This could explain the change in leaf color and explant necrosis. Increased Phytagel® concentrations from 2.5 to 5.0 g/L did not solve the vitrification problem and it decreased the number of buds in explants (2.37±0.57 and 2.23±0.42, respectively). Even though high levels of gelling agents may be efficient to reduce vitrification in other species, in most cases the multiplication index is lowered (Brand, 1993; Cuzzuol et al., 1995; Leite et al., 1993). With the objective of controlling vitrification different covers like, aluminum foil, PVC film and cotton have been used for *in vitro* cultures. Satisfactory results depended on the species in study

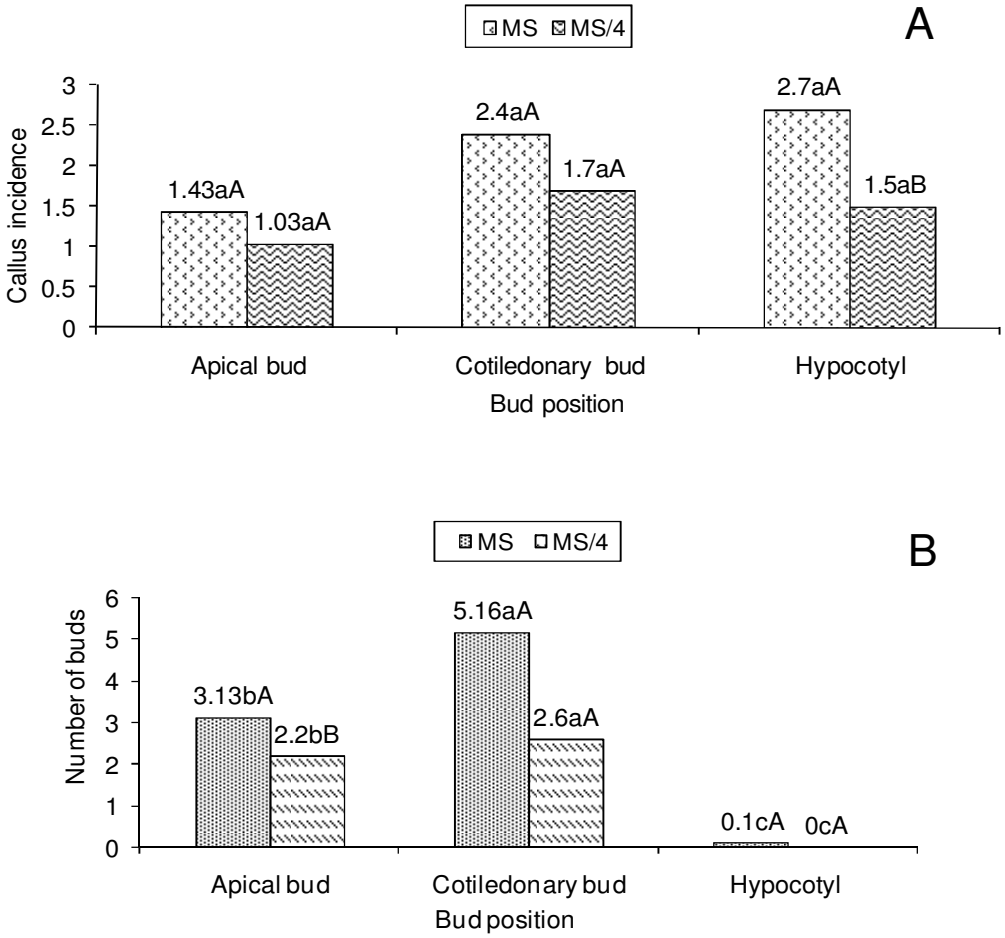

Figure 3. (A) Callus incidence and (B) number of buds produced in MS and MS/4 medium according the bud position: apical, cotiledonary and hypocotyl. Capital letters compare different buds in the same medium and small letters compare the same bud in both media MS and MS/4.

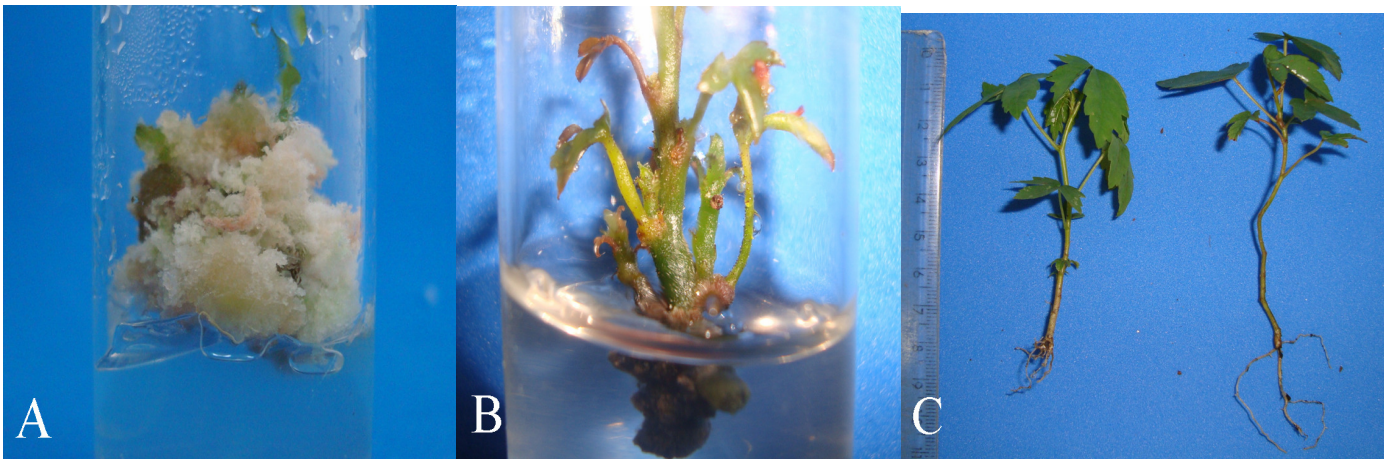

Figure 4. (A) Callus formation in *C. regium* explants cultured in medium supplemented with BAP. (B) Shoot proliferation induced by the addition of 2 µM of IBA in the culture medium. (C) *Ex vitro* rooting of *C. regium* plants.

Table 3. Effects of different the test tubes covers on shoot proliferation rates, number of buds per explant, proportion and intensity of vitrification, shoot elongation and proportion of explants necrosis after 30 days of culture.

Type of cover/type of flask	Shoot proliferation (%)	Number of buds per explant	Proportion of vitrification (%)	Intensity of vitrification	Shoot length (cm)	Explant necrosis
Plastic caps/test tubes.	57.14[a]	1.71[a]	28.57[a]	-	0.98[a]	0.00[b]
Plastic caps with a 6.5 mm hole/test tubes.	28.57[a]	0.61[b]	0.00[b]	-	0.75[a]	19.04[b]
Plastic caps with a 9.0 mm hole/test tubes.	28.56[a]	0.66[b]	0.00[b]	-	0.71[a]	23.81[b]
Plastic caps with a 12.0 mm hole/test tubes.	28.57[a]	0.42[b]	0.00[b]	-	0.77[a]	4.76[b]
Filter paper.	9.52[a]	0.09[b]	0.00[b]	-	0.54[a]	52.38[a]
CV %**.	67.39	35.42	50.35	-	14.77	47.65
Plastic caps/test tubes.	90.48[a]	5.24[a]	88.57[a]	4.06[a]	0.97[a]	-
Aluminum foil/test tubes.	47.62[b]	5.33[a]	93.33[a]	3.67[a]	1.21[a]	-
PVC film/test tubes.	82.71[a]	6.56[a]	48.09[b]	0.88[b]	1.14[a]	-
Cotton/test tubes.	68.33[b]	3.72[a]	0.00[c]	0.00[b]	0.93[a]	-
PVC film/flasks.	95.24[a]	5.72[a]	95.24[a]	3.79[a]	1.36[a]	-
CV%**.	20.64	28.03	25.63	35.80	18.42	-

*Means followed by the same letters within each column did not significantly differ by Scott–Knott test at $P < 0.05$, **CV - coefficient of variation, (-) not evaluated.

(Bandeira et al., 2007; Ribeiro et al., 2007; Santana et al., 2008; Souza et al., 1999). *C. regium* explants kept in test tubes covered with filter paper and perforated plastic caps (perforations in several sizes) were devitrified but the number of buds/explant decreased when compared to samples kept in test tubes covered with plastic film without the filter paper. After 30 days evaporation of the culture medium was high, explants necrosis was intense especially were filter paper was the only cover. In test tubes sealed by PVC film, necrosis and vitrification intensity was lower after the same period of time (48.09 and 0.88%, respectively), while controls (plastic caps) showed values significantly superior respectively (Table 4).

In contrast, Faria et al. (2007), Malosso et al. (2008) and Pereira et al. (1995) observed an improved vegetative development of plants starting from axilar buds instead of apical ones. *In vitro* rooting of *C. regium* did not occur in any of the IBA concentrations evaluated. However, the auxin promoted multiplied sprouting mainly at 2 µM (Figure 4B and Table 5). It probably occurs due to auxin/cytokinin phyhormone balance that induce root or aerial part formation (Taiz and Zeiger, 2004), though *C. regium*, differently from most species, when cultured with higher cytokinin concentration showed callus proliferation and with higher auxin concentration presented enhance shoot formation. That may be explained by the high concentration of endogenous cytokinin present in *C. regium*. During these experiments, samples that remained in IBA-containing media, after some time started showing necrosis suggesting that after 30 days or more, *C. regium* explants were intoxicated by the presence of the regulator. In general, Cerrado endemic plants do not easily produce roots under *in vitro* conditions or conventional rooting protocols, as happens with *Anemopaegma arvense*, *Mandevilla illustris* e M. *velutina* (Biondo et al., 2004, 2007; Moraes et al. 2007; Pereira et al., 2003). Survival of plantlets during the acclimatization period was 45.85±18.93% and of these 77.5±15.00% rooted *ex vitro* and after 56 days the average final root size was 1.57±0.63 cm (Figure 4C).

Conservation in the germplasm bank

It was possible to keep *C. regium* explants in germplasm bank conditions, under minimal growth in MS supplemented with 2% sucrose, 4% mannitol and 2 mg/L of calcium pantothenate. In these conditions 47.62% of explants survived (Table 6), showing the effectivity of these compounds. There was no vegetative development in all treatments during the period evaluated. As a rule, Cerrado plants like for example *A. arvense* and *M. velutina* have responded well to germplam bank *in vitro* conditions when protective agents are used to

Table 4. Effects of the apical and axillary bud positions on shoot proliferation, number of buds per explant, callus formation, incidence of callus, shoot elongation and survival rates after 30 days of culture.

Bud position	Shoot proliferation (%)	Number of buds per explant	Callus formation (%)	Incidence of callus	Shoot length (cm)	Survival rate (%)
Apical	88.89[a]	4.12[a]	57.14[b]	1.76c	2.25[a]	90.47[a]
Axillary 1	58.41[a]	1.72[b]	83.01[a]	3.61[a]	0.85[b]	85.71[a]
Axillary 2	56.67[a]	1.82[b]	87.78[a]	2.79[b]	0.85[b]	76.19[a]
Axillary 3	53.33[a]	1.13[b]	100.00[a]	3.80[a]	0.72[b]	71.43[a]
CV%**	28.79	29.23	10.13	17.55	32.86	11.39

*Means followed by the same letters within each column did not significantly differ by Scott–Knott test at $P < 0.05$. **CV - coefficient of variation.

Table 5. Effects of adding IBA to the culture medium on rates, number of shoots and buds per explant and shoot elongation on the 30th day of culture and survival rates after 50 days of *C. regium in vitro* culture.

IBA (µM)	Rooting (%)	Number of buds per explant	Number of shoots per explant	Shoot length (cm)	Survival rate after 50 days of *in vitro* culture (%)
0.0	0.00	6.76[a]	2.07[a]	2.96[a]	81.90[a]
1.0	0.00	4.99[b]	1.24[b]	1.78[b]	47.62[a]
2.0	0.00	5.24[b]	1.62[a]	1.47[b]	55.56[a]
4.0	0.00	4.70[b]	1.20[b]	1.42[b]	46.62[a]
6.0	0.00	3.70[b]	1.21[b]	1.34[b]	47.60[a]
CV%**	0.00	16.04	20.64	22.62	37.31

*Means followed by the same letters within each column did not significantly differ by Scott–Knott test at $P < 0.05$. **CV - coefficient of variation.

Table 6. Effects of mannitol, sorbitol and calcium pantothenate on minimal growth of *C. regium* explants after 30, 60, 90 and 120 days of *in vitro* culture.

Culture medium	% of plant survival (days of culture)			
	30	60	90	120
MS + 2% sucrose	9.52[b]	9.52[b]	4.76[c]	0.00[c]
MS/2 + 2% sucrose	47.62[a]	19.04[b]	4.76[c]	4.76[c]
MS + 2% sucrose + 4% sorbitol	9.52[b]	9.52[b]	4.76[c]	4.76[c]
MS/2 + 2% sucrose + 4% sorbitol	19.04[b]	0.00[b]	0.00[c]	0.00[c]
MS + 2% sucrose + 4% sorbitol + 2 mg L^{-1} calcium pantothenate	33.33[b]	0.00[b]	0.00[c]	0.00[c]
MS/2 + 2% sucrose + 4% sorbitol + 2 mg L^{-1} calcium pantothenate	19.04[b]	0.00[b]	0.00[c]	0.00[c]
MS + 2% sucrose + 4% mannitol	42.86[a]	19.04[b]	19.04[c]	9.52[b]
MS/2 + 2% sucrose + 4% mannitol	57.14[a]	42.86[a]	33.33[b]	14.28[b]

Table 6. Contd.

MS + 2% sucrose + 4% mannitol + 2 mg L^{-1} calcium pantothenate	61.90[a]	47.62[a]	47.62[a]	47.62[a]
MS/2 + 2% sucrose + 4% mannitol + 2 mg L^{-1} calcium pantothenate	61.90[a]	42.86[a]	28.57[b]	19.04[b]
CV%**	40.77	65.68	54.78	61.57

*Means followed by the same letters within each column did not significantly differ by Scott–Knott test at $P < 0.05$. **CV - coefficient of variation.

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