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In vitro seed germination and multiplication of *Calophyllum brasiliense*

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Abstract - *Calophyllum brasiliense* is a tree species with limited natural reproduction. *In vitro* germination may be an alternative for obtaining high-quality seedlings. Seeds were maintained in water before surface disinfestation and compared with control seeds (i.e. not immersed), without differences between treatments. $HgCl_2$ used during surface-disinfestation reduced contamination rates of cultures. Fungal contamination was reduced with fungicide added to culture medium (23 to 6.4%), although bacterial contamination increased (24 to 36%). In another experiment, seeds were immersed in plant preservative mixture (PPM™) prior to surface disinfestation. By combining immersion for 48 h and 2 mL L^{-1} in culture medium, contamination was only 6%. Seeds immersion in GA_3 prior to surface disinfestation reduced root formation as concentration increased. Germination rate and GSI were reduced, respectively, from 72% and 0.129 (24 h) to 60% and 0.092 (48 h) according to exposure time to GA_3. After 90 days in multiplication medium containing benzylaminopurine, average number of shoots per nodal segment was 3.4. In conclusion, *in vitro* germination of *C. brasiliense* seeds is feasible in sucrose-free WPM medium and reaches a high contamination-free rate (up to 93.3%).

Germinação *in vitro* de sementes e multiplicação de *Calophyllum brasiliense*

Resumo - *Calophyllum brasiliense* é uma espécie arbórea com sistema de propagação natural limitada. A germinação *in vitro* pode ser uma alternativa para obtenção de plântulas de qualidade. Sementes foram mantidas em água antes da desinfestação e comparadas com sementes controle (não imersas), sem diferença entre os tratamentos. $HgCl_2$ usado durante a desinfestação reduziu a contaminação das culturas. A contaminação fúngica foi reduzida com fungicida adicionado ao meio (23 para 6,4%), mas a porcentagem de bactérias foi aumentada (24 para 36%). Em outro experimento, as sementes foram imersas em *plant preservative mixture* (PPM™) antes da desinfestação. Combinando a imersão por 48 h e 2 mL L^{-1} no meio de cultura, a contaminação foi de 6%. A imersão das sementes em GA_3 antes da desinfestação reduziu a formação de raízes conforme a concentração foi aumentada. A germinação e o IVG foram reduzidos, respectivamente, de 72% e 0,129 (24 h) para 60% e 0,092 (48 h), de acordo com o tempo de exposição a GA_3. Após 90 dias em meio de multiplicação contendo benzilaminopurina, o número médio de brotações por segmento nodal foi 3,4. A germinação *in vitro* de *C. brasiliense* é viável em meio WPM sem sacarose, com até 93,3% de sobrevivência.

Introduction

Calophyllum brasiliense Cambess. (Clusiaceae), known in Brazil as guanandi, jacareuba or olandi, is a tree species native to tropical America, common in rainforests and humid soils, tolerant to hypoxia (Marques & Joly, 2000; Oliveira & Joly, 2010). This species is economically important due to the good quality of its wood (Cole et al., 2011), to its use for reforestation (Cole et al., 2011) and to its medicinal properties (Ito et al., 2002; Souza et al., 2009), including anti-HIV potential (Huerta-Reyes et al., 2004; Bernabé-Antonio et al., 2010).

Guanandi reproduction occurs at irregular intervals and its establishment rate is low, due to grazing and environmental conditions (Newstrom et al., 1994; Fischer & Santos, 2001; King, 2003; Cole et al., 2011). Their seeds are recalcitrant and therefore their storage time is limited (Cole et al., 2011).

Tissue culture is an alternative for producing high-quality seedlings and *in vitro* germination can be applied as in the case of *Calophyllum inophyllum* (Thengane et al., 2006), specially for selected seeds resulting from breeding programs, which usually produce few plant material which could be clonally propagated. However, contamination rates and specific culture conditions for multiplication of *C. brasiliense* are still unknown.

Contaminants such as fungi and bacteria often lead to losses during *in vitro* culture of plants, therefore surface disinfestation of seeds is necessary (Leifert et al., 1991; Mng'Omba et al., 2011). It can be performed by using a number of compounds such as calcium and sodium hypochlorite, mercury chloride, antibiotics and fungicides (Mng'Omba et al., 2011). Concentrations and exposure times for different chemicals varies depending on explants type (Leifert et al., 1991). A fungicide was used before disinfestation of *C. inophyllum* seeds (Thengane et al., 2006) and for the same species and *C. apetalum*, mercury chloride was applied to seeds (Nair & Seeni, 2003; Thengane et al., 2006), showing effective results.

Gibberellic acid plays a known role during seed germination, acting in breakage of aleurone layer and increasing oxygen uptake by embryos (Bewley et al., 2013). For species such as *Cicer arietinum* (Kaur et al., 1998), *Capparis ovata* (Soyler & Khawar, 2007) and *C. spinosa* (Arefi et al., 2012), the addition of this plant regulator increases germination rates.

A cytokinin is used to promote shoot multiplication in most cultures. For both *C. inophyllum* (Thengane et al., 2006) and *C. apetalum* (Nair & Seeni, 2003), 6-benzylaminopurine (BAP) was added to the culture medium resulting in multiple shoots formation in concentrations up to 44 μM.

The aim of this study was to determine the best surface disinfestation method for *in vitro* establishment and germination of guanandi seeds and *in vitro* multiplication from nodal segments.

Material and methods

Plant material and culture medium

Mature fruits of *Calophyllum brasiliense* were obtained from Instituto Brasileiro de Florestas (IBF) and were unpulped manually. After removing the tegument with a knife, the seeds were submitted to different disinfestation treatments and culture conditions (Figure 1a). The standard culture medium was WPM (Lloyd & McCown, 1980) without plant growth regulators (PGRs) supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar (Vetec®, Rio de Janeiro, Brazil). Culture medium was autoclaved at 121 °C, for 20 min, after pH adjusting to 5.8.

Seed disinfestation and aseptic culture establishment

a) Seed surface disinfestation with mercury chloride and water treatment

Seeds were kept in flasks containing autoclaved distilled water for 24 or 48 h, with a control treatment without water immersion. They were washed for 5 min in detergent and tap water and then rinsed 5 times in distilled water. In a laminar flow chamber, seeds were surface sterilized with ethanol 70% for 1 min and sodium hypochlorite (NaOCl) 10% w/v with 0.01% Tween 20® for 20 min. Finally, they were treated with mercury chloride ($HgCl_2$) solutions at 0.1 or 0.2%, for 4 or 8 min, and then rinsed 6 times in autoclaved distilled water.

Seeds were individually inserted into test tubes (15 cm high x 2.5 cm diameter), containing 10 mL of standard medium. The experimental design was completely randomized as a 3 x 5 factorial (water immersion x mercury treatments), with five replicates containing six seeds each. Contamination was evaluated one month after *in vitro* culture initiation.

b) Fungicide added to culture medium

The procedure was exactly the same as in the previous experiment. However, 0.5 g L^{-1} Cercobin® 700 WP (Iharabras S.A Indústrias Químicas, São Paulo State, Brazil), a systemic fungicide, was added to the culture medium.

c)PPM™ treatment and water immersion

Seeds were kept in Erlenmeyer flasks containing distilled autoclaved water or 2 mL L^{-1} plant preservative mixture (PPM™, Plant Cell Technology, Washington, DC., USA) solution for 24 or 48 h. The control seeds were not immerged in water.

After treatments, seeds were washed with detergent for 5 min and in a laminar flow chamber their surface were sterilized with 70% ethanol (1 min), followed by a 5% solution of NaOCl supplemented with 0.01% Tween 20® (Sigma-Aldrich, Saint Louis, Missouri, USA) for 20 min and finally 0.1% $HgCl_2$ (10 min). Seeds were rinsed six times in distilled autoclaved water and individually inserted into test tubes containing 10 mL of culture medium.

Standard medium was supplemented with 1.5 mL L^{-1} or 2 mL L^{-1} PPM™, plus a control treatment without this biocide. The experimental design was completely randomized as a 3 x 5 factorial (PPM™ concentration in culture medium x imbibition treatments), with six replicates containing five seeds each. The seeds were maintained in the growth room. Contamination and survival rates *in vitro* culture were evaluated after 30 days.

Effect of gibberellin on *in vitro* germination

Seeds were kept in water or gibberellin (GA_3) solution (2.89, 5.77, 14.43 or 28.89 µM) for 24 or 48 h. Seeds were surface-sterilized as in the experiment with PPM™ and introduced individually in test tubes containing sucrose-free standard medium. Seed germination was evaluated every two days for three months and data were utilized to determine the germination speed index (GSI).

The experimental design was completely randomized as a 2 x 5 factorial (exposure period x GA_3 concentration), with five replicates containing eight seeds each. The variables evaluated were: percentage of fungal and/or bacterial contamination, germination percentage, GSI and time required for the emergence of shoots. The GSI was calculated according to the equation proposed by Maguire (1962).

Shoot multiplication

Seedlings with 30 days after *in vitro* germination were used as donor of explants for multiplication phase. Nodal segments (1.25 ± 0.25 cm) from the epicotyls, with two nodes each and without visible contamination, were transferred to standard medium supplemented with 1 mL L^{-1} PPM™, 1 g L^{-1} polyvinylpyrrolidone (PVP) and 8.8 µM 6-benzylaminopurine (BA). Shoot apexes were discarded. The rest of the explant including the seed was transferred to glass bottles (6 cm diameter x 8.5 cm height) containing a fresh standard medium without PGRs. To evaluate the resprout capability of *in vitro* seedlings, the plantletS with one node were maintained until new shoots resprout at the base that could be cut to repeat the process. Two explants were placed in each bottle containing 40 mL of culture medium, each closed with a polypropylene cap.

Explants were subculture at 30 days intervals, being transferred to identical fresh medium. The necrotic edges were excised and discarded and shoots longer than 3 cm were individualized each month. The multiplication step had duration of three months and the number of shoots per explant was evaluated each month.

Culture conditions

Seed germination was performed in a germination chamber at 25 °C, under a long day photoperiod (16 h) and fluorescent light. During multiplication, explants were maintained in a growth chamber at 20 °C (night) and 26 ± 1 °C (day), under a 16 h photoperiod and fluorescent light with photosynthetic photon flux density of about 30 µmol m^{-2} s^{-1}.

Acclimatization

The plantlets originated from seeds after several subcultures were transferred to pots containing substrate (Plantmax®, Eucatex Agro-Florestal, São Paulo State, Brazil) and vermiculite (Dimy®, Paraná State, Brazil) at a ratio of 1:1, in a greenhouse at 24 °C and irrigated for 5 min every 6 h. Their survival was evaluated after 180 days.

Statistical analysis

Data were submitted to Bartlett's test and analysis of variance (ANOVA), then to mean comparison by Tukey's test at 5%. The statistical software used was Assistat 7.6 (Silva & Azevedo, 2009).

Results and discussion

Seed disinfestation and aseptic culture establishment

a) Seed surface disinfestation with mercury chloride and water treatment

After 30 days of culture, the mean contamination rate of the experiment was 52% (Table 1), approximately half by fungi and half by bacteria. Statistical analysis indicated that water immersion and $HgCl_2$ treatment did not affect fungal contamination or survival of seeds.

Table 1. Percentage of *Calophyllum brasiliense* contamination-free seeds, after a water immersion period and mercury chloride treatments ($HgCl_2$), 30 days after *in vitro* incubation into plant growth regulators-free culture medium.

$HgCl_2$ treatment	Contamination-free seeds (%)			
	0	24 h water	48 h water	Mean
Control	70.0	40.0	36.6	48.8 ^{ns}
0.1% 4 min	56.6	40.0	43.3	46.6 ^{ns}
0.1% 8 min	60.0	70.0	53.3	61.1 ^{ns}
0.2% 4 min	46.6	56.6	43.3	48.8 ^{ns}
0.2% 8 min	66.6	46.6	60.0	57.7 ^{ns}
Mean	60.0 ^{ns}	50.6 ^{ns}	47.3 ^{ns}	52.6

^{ns} Not significant (Tukey's test, $p > 0.05$).

Even though statistical analysis did not show significant differences, the number of contamination-free seeds after 60 days was higher in the control treatment, i.e. not imbibed. This may indicate that contamination increases as the seeds remain in water. Similar results were obtained for *Calophyllum inophyllum* (Thengane et al., 2006), indicating that keeping the seeds in water favors the appearance of contaminants. Thengane et al. (2006) showed that presoaking facilitated the leaching of phenolic compounds from seeds, preventing browning and hastening germination process. According to Bewley et al. (2013), as the seed absorbs water, the cell membranes lose the ability to retain solutes such as sugars, organic acids, proteins and ions and they are released. These may stimulate growth of fungi and bacteria, which can penetrate the seed, being a superficial disinfestation protocol unsuccessful.

There was no difference in bacterial contamination and survival between the two mercury chloride concentrations tested. Differences were observed only between the control and $HgCl_2$ treatments (Table 2). This indicates that the use of $HgCl_2$ is required to avoid bacterial contamination, and the lowest concentration is indicated. Fungal contamination was low and the incorporation of $HgCl_2$ did not reduce the number of affected explants.

Table 2. Fungal and bacterial contamination in seeds of *Calophyllum brasiliense*, in function of the exposure time and mercury chloride ($HgCl_2$) concentration during surface disinfestation, 30 days after *in vitro* culture into WPM medium plus 0.5 g L^{-1} Cercobin®.

$HgCl_2$ concentration (%)	Fungal contamination (%)			Bacterial contamination (%)			Contamination-free seeds (%)		
	4 min	8 min	Mean	4 min	8 min	Mean	4 min	8 min	Mean
Control	-	-	7.8 ^{ns}	-	-	51.1 a	-	-	41.1 b
0.1	7.8	5.6	6.7 ^{ns}	41.1	27.8	34.4 b	51.1	66.7	58.9 a
0.2	5.6	5.6	5.6 ^{ns}	36.7	23.3	30.0 b	57.8	71.1	64.4 a
Mean	6.7 ^{ns}	5.6 ^{ns}	6.4	38.9 a	25.5 b	36.0	54.4 a	68.9 b	57.6

Means followed by the same letter do not differ to the same variable (Tukey's test, $p \leq 0.05$). ^{ns} Not significant ($p > 0.05$).

The longer the duration of exposure to mercury chloride for surface disinfestation, the lower the bacterial contamination and higher survival rates, although no differences were observed for fungal contamination.

The average germination rate was 12%, with no interference of treatments (data not shown). Germination was considered very low, and plumule emergence was observed in only one of the seeds, even after 120 days of culture. The abnormal seeds, emitting only roots, can be explained by the excess of solutes in the culture medium, as happened in the case of maize (Bradford, 1994; Bewley et al., 2013). The presence of sucrose in the culture medium may have reduced their water potential, resulting in poor absorption of water by the seed, reducing the germination as the embryo suffers water stress (Bradford, 1994). According to Bewley et al. (2013), if water uptake is too slow, germination is reduced due to seed deterioration.

Moreover, sucrose is one of the main products of protein and lipid storage breakdown during seed germination and it is considered a regulatory agent, with signalizing functions (Rolland et al., 2006; Borek et al., 2012, 2013). A study of To et al. (2002) showed that mobilization of seed storage lipid by *Arabidopsis thaliana* was delayed in the presence of exogenous sugars, resulting in almost complete elimination of lipid breakdown, without interference of osmotic stress. Thus, important enzymes for storage lipid breakdown are more active in organs cultured in absence of sucrose (Borek et al., 2013).

a)Fungicide added to culture medium

Cercobin® is a systemic fungicide. Its active compound is thiophanate methyl which combats fungal contamination due to the recognized property of its group, benzimidazoles, of preventing tubulin polymerization during metaphase, thereby causing inhibition of mitotic divisions and cell proliferation (García-Cela et al., 2012).

Average contamination of seeds in culture medium plus Cercobin® was 42% after 30 days, similar to that obtained in previous experiment without this product. Fungal contamination was only 6.4%, showing the efficiency of this fungicide (Table 3). On the other hand, fungal contamination was increased when seeds were maintained in water for 48 h.

Table 3. Fungal and bacterial contamination in seeds of *Calophyllum brasiliense*, after a water immersion period and surface disinfestation in ethanol, sodium hypochlorite and mercury chloride, 30 days after *in vitro* insertion into WPM medium plus 0.5 g L^{-1} Cercobin®.

Immersion period (hours)	Fungal contamination (%)	Bacterial contamination (%)	Contamination-free seeds (%)
Control	3.3 a	32.7 ^{ns}	64.0 ^{ns}
24	3.3 a	40.7 ^{ns}	56.0 ^{ns}
48	12.7 b	34.7 ^{ns}	52.7 ^{ns}
Mean	6.4%	36.0%	57.5%

Means followed by the same letter do not differ to the same variable (Tukey's test, $p \leq 0.05$). $^{ns.}$ Not significant ($p > 0.05$).

Bacterial contamination, however, was not affected by water immersion, but it increased if compared with experiment without Cercobin® (36% compared to 24%). This increase suggests that fungi might repress bacteria growth and, in the absence or reduction of fungal population, bacteria tend to multiply more easily in the culture medium.

Immersion period in water favored contamination of seeds through microorganism proliferation. Growth of fungi and bacteria can be stimulated by leakage of solutes that occurs during imbibition, such as sugars, organic acids, ions, amino acids, and proteins (Bewley et al., 2013).

The average germination rate of *C. brasiliense* seeds observed was 16% and it was not affected by the treatment used in surface disinfestation or by water immersion (data not shown). Only two seeds developed shoots, while the others only emitted roots, indicating abnormal seedling development, possibly due to water potential reduction (Bradford, 1994) or regulatory effects mediated by sucrose (Rolland et al., 2006; Borek et al., 2012, 2013)

b) PPM™ treatment and water immersion

After 30 days of culture, the mean contamination rate of seeds was 27%, nearly half of that observed in the previous experiments with or without Cercobin®. The use of PPM™ in culture medium reduced contamination (Table 4). Contamination rates were also affected by the solution in which the seeds were soaked (water or PPM™).

Table 4. Percentage of fungal and bacterial contamination, and contamination-free seeds of *Calophyllum brasiliense*, after immersion in 2 mL L^{-1} of plant preservative mixture (PPM™) or water, and addition of different concentrations of PPM™ to plant growth regulator-free culture medium prior to autoclaving, 30 days after *in vitro* incubation.

Imbibition treatment	**Contamination-free seeds (%)**			
	0	**1.5 mL L^{-1} PPM™**	**2 mL L^{-1} PPM™**	**Mean**
Control	53.3	66.7	73.3	64.4 b
24h water	63.3	86.7	76.7	75.6 ab
24h PPM™	60.0	76.7	73.3	70.0 ab
48h water	50.0	66.7	76.7	64.4 b
48h PPM™	80.0	86.7	93.3	86.7 a
Mean	61.3 b	76.7 a	78.7 a	72.2

Means followed by the same letter do not differ to the same variable (Tukey's test, $p \leq 0.05$). $^{ns.}$ Not significant ($p > 0.05$).

The combination of fungicides and antibiotics to eliminate bacteria as well as fungi in the culture medium should be avoided because it tends to be inefficient and phytotoxic (Leifert et al., 1991). Thus, the use of PPM™ was effective and it may be recommended instead of addition of antibiotics to the culture medium.

Although germination rate reached almost 35%, exceeding the results of the experiments using only mercury chloride or with Cercobin®, the plumule emission was still low and occurred in less than 1% of the seeds (Fig. 1b), even 120 days after the *in vitro* introduction. This indicates that some aspects of the culture is not suitable for seeds germination and initial development of plantlets.

Effect of gibberellin on *in vitro* germination

Water or GA_3 exposure for 48 h was harmful to seeds, reducing germination rate to 60% if compared with 72% for a 24 h immersion (Table 5). This exposure time negatively affected plumule emergence.

Table 5. Germination speed index (GSI), GSI for plumule emergence and germination percentage of *Calophyllum brasiliense* seeds, exposed to different concentrations of gibberellic acid (GA_3) or maintained in water for 24 or 48 h after *in vitro* culture in free-sucrose WPM medium.

GA_3 concentration (µM)	GSI (up to 60 days)		Germination after 60 days (%)		GSI (plumule emergence)		
	24 h	48 h	24 h	48 h	24 h	48 h	Mean
Control	0.217 aA	0.160 aA	77.5 aA	57.5 abB	0.265	0.184	0.224 ab
2.89	0.084 bcA	0.153 aA	75.0 aA	42.5 bB	0.248	0.184	0.216 ab
5.77	0.167 abA	0.069 abB	77.5 aA	70.0 aA	0.283	0.258	0.270 a
14.47	0.150 abA	0.069 abB	80.0 aA	67.5 aA	0.294	0.244	0.269 a
28.89	0.027 cA	0.011 bA	50.0 bA	62.5 abA	0.183	0.167	0.175 b
Mean	0.129	0.092	72	60	0.254 a	0.207 b	0.231

Means followed by the same lower letter in column, and capital letter in row, do not differ to the same variable (Tukey's test, $p \leq 0.05$). $^{ns.}$ Not significant ($p > 0.05$).

Plumule emergence started at day 6 after treatment with 5.77 µM GA_3. However, no radicle was observed in several cases, particularly with the higher doses of gibberellin, even 90 days after *in vitro* introduction. This suggests some type of inhibition caused by an increase in regulator concentration (Figure 1c-d).

The GSI was reduced as the duration of GA_3 treatment increased ($p < 0.05$), and the treatment of 28.89 µM showed statistical difference if compared to other treatments. At this concentration, there was inhibition of root protrusion and plumule emergence, and seed germination rate was reduced. This fact can indicate that this regulator causes phytotoxic effects in cases of prolonged exposure of seeds or high concentrations, as observed for seeds of *Capparis ovata* (Soyler & Khawar, 2007), *Securidaca longipedunculata* (Zulu et al., 2011) and *Capparis spinosa* (Arefi et al., 2012). For *Cicer arietinum*, the optimal concentration of gibberellin for germination was 6 µM (Kaur et al., 1998).

Gibberellin action during embryo development is linked to increased oxygen uptake by seeds and stimulated production of hydrolytic enzymes that degrade the aleurone layer present in the seeds, releasing sugars and peptides that allow germination (Paleg, 1960; Briggs, 1963; Bewley et al., 2013). With increase in respiration caused by gibberellin, it is likely that immersion in water reduces the availability of oxygen to the point at which the seeds are irretrievably damaged and lose the ability to germinate. Excess water under anaerobic conditions is harmful to the seed and, under anoxia conditions, seeds accumulate ethanol and lactic acid produced during fermentation (Bewley et al., 2013). Furthermore, GA_3 tends to inhibit the formation of roots (Brian et al., 1960; Moshkov et al., 2008) as observed in the present study (data not shown).

Sucrose, absent in the culture medium but present in previous experiments, may affect germination. It reduces the water potential of culture medium, The total amount of water provided to the seeds is restricted, hindering imbibition and subsequent germination (Bewley et al., 2013). For *Cereus jamacaru* seed germination the concentration of 2.5 mg L^{-1} in the medium was more effective than higher concentrations (Rêgo et al., 2009).

Germination rates were up to 80%, with no effect caused by treatment with gibberellic acid. This value is much higher than those obtained in previous experiments

and is comparable to the germination rates of the species in nursery (Marques & Joly, 2000; Silva et al., 2014) and forest environment (King, 2003). However, *in vitro* germination is advantageous for seedling rescue, as mean survival and establishment of seedlings in natural conditions is low (37%) and affected by light, flooding and high herbivory pressure (King, 2003). *In vitro* germination can be associated to a breeding program for propagation of elite seeds, which could be further clonally propagated by *in vitro* multiplication. The plantlets originated from seeds were acclimatized in a greenhouse with approximately 90% survival.

Shoot multiplication

Seedlings maintained in the culture medium were sectioned and used as explants for multiplication phase. They quickly resprouted and the nodal segments could be individualized at intervals of 2 weeks to one month (Figure 1e). From a single germinated seed, new nodal segments were cut up to 3 times without loss of regrowth capacity, and then the size of seedlings prevented their culture *in vitro*. This resprout capacity was also reported in natural environment by King (2003), with up to 18 resprouts in a single plant after suffering grazing. For *C. inophyllum* cultured in a medium containing plant growth regulators, up to 20 shoots were obtained from decapitated seedling after 60 days (Thengane et al., 2006).

After 30 days in culture medium containing 8.8 µM BA, the average number of shoots per segment nodal was 2. After 60 and 90 days, it was 2.7 and 3.4, respectively. Shoots developed directly, without callus formation (Figure 1f). These multiplication rates are close to those found for *C. apetalum*. For this species, the maximum number of shoots obtained from nodal segments of a mature tree was 3.2 after 6 weeks (Nair & Seeni, 2003).

Figure 1. Seeds of *Calophyllum brasiliense*, respectively, with endocarp; endocarp-free and with tegument; tegument-free (as introduced *in vitro*) (a); germinated seed in WPM with 30 g L^{-1} sucrose, forming only roots (b); in sucrose-free medium, without GA_3 application (c); in sucrose-free medium with 5.77 µm GA_3, rootless (d); showing three resprouts that were used as explants for multiplication, the tallest one with 30 days (e); nodal segment after 30 days in culture medium containing 8.8 µM BA (f). Bar: 1 cm.

Conclusions

Germination of guanandi seeds *in vitro* is possible although time consuming and expensive. It is recommended the immersion of seeds in 0.2% solution of plant preservative mixture (PPM™) for 24 h before surface disinfestation. The use of PGR-free and sucrose-free WPM is indicated for seeds introduction, with addition of 1.5 mL L^{-1} PPM™.

Gibberellic acid accelerates the emission of shoots up to a concentration of 14 µM but inhibits it above that concentration. The higher the concentration of GA_3

used, the smaller the number of plants that emit roots at this stage. GA_3 may therefore be added in a higher concentration if the aim of the study is to obtain only shoots.

Multiplication rates of shoots excised from seedlings were close to those obtained for other species of *Calophyllum* genus. Moreover, seedlings maintained *in vitro* had a high ability to sprout again, and this may be an alternative for obtaining plants on a large scale.

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