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Indirect somatic embryogenesis of *Swietenia macrophylla* King in semisolid culture medium

Raúl Collado*, Raúl Barbón, Daniel Agramonte, Felipe Jiménez-Terry, Martha Pérez, Odalys Gutiérrez *Autor para correspondencia

Instituto de Biotecnología de las Plantas, Universidad Central 'Marta Abreu' de Las Villas. Carretera a Camajuaní km 5.5. Santa Clara. Villa Clara. Cuba. CP 54 830. e-mail: raulc@ibp.co.cu

ABSTRACT
Biotechnological techniques are an alternative to propagate *Swietenia macrophylla* King. However, micropropagation by organogenesis in this species has been little or not developed due to big microbial contamination and low indexes of *in vitro* plants regeneration. Therefore, somatic embryogenesis plays an important role in the propagation of this woody species. Immature cotyledons were used as initial plant material to establish a protocol of indirect somatic embryogenesis. Different growth regulators and their concentrations were used to develop the indirect somatic embryogenesis stages. The use of a semisolid culture medium composed of MS salts with 2,4-dichlorophenoxyacetic acid (4.0 mg l^{-1}) favored the formation of callus in the explants. The highest percentage of high frequency somatic embryogenesis (59.01%) was obtained adding 1.0 mg l^{-1} 6-bencylaminopurine in the culture medium. Maturation of somatic embryos was increased using 6.0% sucrose. The greater percentage of somatic embryos germination (76.17%) was reached in the culture medium without growth regulator. A reproducible indirect somatic embryogenesis protocol from callus formation to somatic embryos germination in *Swietenia macrophylla* King was established.

Key words*:* callus, growth regulators, high frequency somatic embryogenesis, somatic embryos

RESUMEN
Las técnicas biotecnológicas son una alternativa para la propagación de *Swietenia macrophylla* King. Sin embargo, la micropropagación vía organogénesis de esta especies ha tenido muy poco o ningún desarrollo, debido a los grandes problemas de contaminación microbiana y a los bajos índices de regeneración *in vitro* de las plantas. Por tanto, la embriogénesis somática juega un rol importante en la propagación de esta especie maderable. Para establecer un protocolo de embriogénesis somática indirecta se emplearon cotiledones inmaduros como material vegetal inicial. Se emplearon diferentes reguladores de crecimiento y sus concentraciones para desarrollar las fases de la embriogénesis somática indirecta. El empleo de medio de cultivo semisólido compuesto por sales MS y ácido 2,4-diclorofenoxyacetic (4.0 mg l^{-1}) favoreció la formación de callos en los explantes. La formación y diferenciación de los embriones somáticos en *Swietenia macrophylla* King mostró buenos resultados. El mayor porcentaje de embriogénesis somática de alta frecuencia (59.01%) se obtuvo con la adición de 1.0 mg l^{-1} de 6-bencilaminopurina en el medio de cultivo. Con 6% de sacarosa se incrementó la maduración de los embriones somáticos. El mayor porcentaje de germinación (76.17%) de los embriones somáticos fue obtenido en el medio de cultivo sin reguladores de crecimiento. Se estableció un protocolo de embriogénesis somática indirecta reproducible desde la formación de callos hasta la germinación de los embriones somáticos en *Swietenia macrophylla* King.

Palabras clave: callo, embriogénesis somática de alta frecuencia, embriones somáticos, reguladores de crecimiento

INTRODUCTION

Swietenia macrophylla King (mahogany) is a tall tree that towers above the forest canopy. The bark has a sweet odor. It produces small white flowers in which the stamens characteristically fuse to form a tube. The fruit capsules release winged seeds that are dispersed by the wind (Haber *et al*., 2000).

Swietenia macrophylla King has a patchy distribution from southern Mexico through Central America and south to Brazil and Bolivia. There is little information on population numbers but in Bolivia and Brazil are still some. It is found in various forest types including Amazonian rainforest. *Swietenia macrophylla* King is the leading commercial timber of Latin America. Eventhough, many people in the industry are

not concerned that without enforced protection measures this species may also become commercially extinct within next years (Oldfield, 2003).

Swietenia macrophylla was included on the Appendix II of the Convention on International Trade in Endangered Species (CITES) in 2002 as a vital move for the future protection of this majestic tree. This came into effect by the end of 2003 (Oldfield, 2003).

Traditional technologies of propagation in this species do not satisfy the demands of plantlets nowadays. It is necessary to reforest and for commercial goals. This problem aimed to the use of biotechnological techniques as an alternative for the propagation of the *Swietenia macrophylla* King (Tacoronte *et al*., 2004).

The micropropagation of mahogany by organogenesis has had little (Collado *et al.,* 2004) or non development, due to the big problems of contamination and the low indexes of *in vitro* regeneration of plants. The somatic embryogenesis will play an important role in the propagation of this species (Rodríguez *et al*., 2003).

Somatic embryogenesis, the ability of individual plant cells to produce embryos in response to appropriate stimuli, is a poorly understood physiological process. Somatic embryogenesis is induced by modulation of the auxin to cytokinin ratio in most culture systems (Denmastia *et al.,* 1996).

The role of auxins in the induction of somatic embryogenesis has been well documented (Michalczuk *et al.,* 1992) and specifically in peanut (*Arachis hypogaea*), the induction of somatic embryogenesis depends on the auxins content in the medium (Eapen and George, 1993). However, the role of cytokinins in the embryos induction and development is poorly understood due, in part, to a lack of cytokinin dependent experimental systems and effective inhibitors of cytokinin biosynthesis and metabolism (Hutchison *et al.,* 1996).

Regeneration by somatic embryogenesis has been described for several species of woody plants such as *Eucalyptus globulus* (Bandyopadhyay *et al*.,1999; Nugent *et al*., 2001) and *Eucalyptus nitens* (Bandyopadhyay and Hamill, 2000). *Cedrela odorata* L (Cameron, 2010) and *Quercus robur* L (San-José *et al*., 2010) reported this recently. There was also a report of somatic embryogenesis in *Swietenia macrophylla* from immature cotyledons (Collado *et al*., 2005; 2006; 2007). However, reports about indirect somatic embryogenesis have not been found yet in the scientific literature.

The objective of the present research was to determine the concentrations of growth regulators and sucrose for the indirect somatic embryogenesis establishment in *Swietenia macrophylla* King in semisolid culture medium.

MATERIALS AND METHODS

Plant materials

Cotyledons of seeds of immature fruits coming from elite plants of *Swietenia macrophylla* King from the Botanical Garden of UCLV were used as starting material.

Somatic embryogenesis induction

The fruits were disinfected with 3.0% Sodium Hypochlorite during 30 minutes. Then, these were rinsed twice with sterile water. The seeds were extracted after cutting the fruit into four sections. The cover of the seeds was eliminated and the cotyledons were separated. Each cotyledon was used as explants for the different assays.

Four explants were placed in culture jars with 10 ml of culture medium containing Murashige and Skoog salts (MS) (Murashige and Skoog, 1962) with 200 mg l^{-1} L-glutamine, 1.0 mg l^{-1} thiamine, nicotinic acid and pyridoxine, 200 mg l^{-1} malt extract, 4.0% sucrose, 3.0 g.l^{-1} Gelrite (SIGMA) to evaluate the effect of the combination of 2,4–dichlorophenoxyacetic acid (2,4 -D) with kinetin on the induction of indirect somatic embryogenesis. The medium pH was adjusted with KOH or HCl to 5.8 before autoclaving it. Three concentrations of 2,4-D (2.0, 4.0 and 6.0 mg l^{-1}) combined with 1.0 mg l^{-1} kinetin were tested. The experiment was developed in darkness at 27.0°C ± 2.0. The number of explants with callus was recorded (results as percentage explants with callus) after six weeks of culture. The repetitions were performed 80 times per treatment.

Somatic embryos formation and differentiation

The callus obtained in the previous experiment were subcultured in semisolid culture medium, containing half of the MS salts supplemented with 10.0 mg l^{-1} thiamine, 1.0 mg l^{-1} nicotinic acid, 1.0 mg l^{-1} pyridoxine, 50.0 mg l^{-1} L-Cysteine, 3.0% sucrose, 3.0 g.l^{-1} of Gelrite (SIGMA) to achieve the formation and differentiation of somatic embryos. The medium pH was adjusted with KOH or HCl to 5.8 before autoclaving it.

Three concentrations of 6-bencylaminopurine (6-BAP) were studied (0.5, 1.0 and 2.0 mg l^{-1}). The results were compared with a culture medium without growth regulators. Five explants were placed in glass jar with 30 ml of culture medium carrying out 10 repetitions (glass jar) per treatment.

The experiment was developed in darkness at 27.0°C ± 2.0. The number of callus with high and low frequency of somatic embryogenesis after 60 days of culture were recorded. Besides, the average number of somatic embryos per callus and the number of somatic embryos that reached the cotyledonary stage after 80 days of culture were quantified.

Somatic embryos maturation

Somatic embryos at the cotyledonary stage were placed on the surface of MS semisolid culture medium (0.25% Gelrite) which contained several sucrose concentrations (4.0%, 6.0% or 8.0%). Glass jars of 250 ml with 30 ml of culture medium were used. Each jar contained seven embryos. After 30 days, somatic embryos cultured in MS medium with sucrose were transferred to culture medium containing MS salts, supplemented with MS vitamins and 2.0% sucrose. Somatic embryos in cotyledonary stage, that were cultured without sucrose in the medium, were used as control. Each jar contained seven somatic embryos too. Five replicates were used per treatment, and the experiment was repeated four times. The experiment was developed in sun light growth chambers with 13 h photoperiod and light intensity of 52–68 ì mol m^{-2} s^{-1} at 25.0°C ± 2.0. Survival and germination of the somatic embryos were evaluated after 60 days.

Data were analyzed by analysis of variance and significant differences among treatment means were determined by the Multiple Range Test (Duncan) at the 5.0% level of significance. The statistical package Statgraphics Plus version 5.0 for Windows was used.

RESULTS AND DISCUSSION

Somatic embryogenesis induction

A high percentage of the explants showed callus in the three combinations of 2,4–D using kinetin but with differences among these treatments during the six weeks of culture. The calli were developed in all the surface of the explants. These presented dark brown color and nodular texture in all the treatments (Figure 1a).

The greater number of explants with callus was observed when the culture medium with 4.0 mg l^{-1} and 6.0 with 1.0 mg l^{-1} of kinetin were used. There were significant differences with the treatment using 2.0 mg l^{-1} of 2,4-D (Table 1).

Table 1. Effect of different combinations of 2,4-D and kinetin in the callus formation from cotyledonal sections in *Swietenia macrophylla* King after six weeks of culture.

Concentrations of 2,4-D (mg l^{-1}) combined with 1.0 mg l^{-1} of kinetin	N°. of explants with callus/jar	Callus formation (%)
2.0	3.75 b	93.70
4.0	3.87 a	96.98
6.0	3.88 a	97.02
S. E.	± 0.03	

Means within a column with unequal letters differ significantly according to Duncan test (p <0.05). S.E Standard error

The results obtained demonstrated the positive effect of the 2,4-D and kinetin combination on indirect somatic embryogenesis induction. Similar results were informed in the hybrid (*Swietenia macrophylla* King X *Swietenia mahogani* Jacq) by Medina and Sotolongo (2004). This authors indicated that the greater number of explants with callus was obtained when 2,4-D (4.0 – 6.0 mg l^{-1}) and kinetin (1.0 mg l^{-1}) were combined in the culture medium.

The use of 2,4-D (1.0 – 5.0 mg l^{-1}) combined with kinetin (0.5 – 2.0 mg l^{-1}) in the genus *Swietenia*, has had success for the formation of calli in investigations about indirect somatic embryogenesis induction (Peña and Lezcano, 2001; Cruz de Rocha and Quoirin, 2004). The effect of 2,4-D and 6-BAP combination on induction of indirect somatic embryogenesis in *Cedrela odorata* L. has been currently described (Cameron, 2010). This author explains that the somatic embryogenesis induction in the mentioned species was much related with exogenous ratio of auxin/ citokinin and added into the culture medium.

Using semisolid culture medium containing MS salts with 200 mg l^{-1} L-glutamine, 1.0 mg l^{-1} thiamine, nicotinic acid and pyridoxine, 200 mg l^{-1} malt extract, 1 mg l^{-1} kinetin, 4% sucrose and 2,4- D (4.0 mg l^{-1}) the callus in *Swietenia macrophylla* King were formed.

Somatic embryos formation and differentiation

Somatic embryos were obtained from calli after 45 days of culture (Figure 1b). Indirect somatic embryogenesis was observed in callus cultured on medium with all concentrations of 6-BAP tested (0.5–2.0 mg l^{-1}), and also in the culture medium without growth regulator. The highest number of calluses with high and low frequency of somatic embryogenesis was obtained with a 1.0 mg l^{-1} 6-BAP (Table 2).

Results demonstrated that the addition of 6-BAP to the culture medium increases significantly the somatic embryos formation in calli obtained from cotyledons sections. It also showed that this process may be produced without growth regulators in the culture medium. Similar results were described in other species such as *Azadirachta excelsa* and *Azadirachta indica*. These other results indicated that the calli cultured with 1.0 mg l^{-1} 6-BAP showed a superior somatic embryos formation compared to those cultured in the culture medium without growth regulators (Giagnacovo *et al*., 2001; Salvi *et al*., 2001).

The maximum average number of somatic embryos per callus was obtained in the calluses that were cultured in the culture medium with 1.0 mg l^{-1} 6-BAP, with differences to the other treatments (Table 3).

A high percentage of somatic embryos reached the cotyledonary stage in all the treatments. This was observed when evaluating the effect of different concentrations of 6-BAP on the development of the somatic embryos formed from calli after 80 days of culture. The highest number of somatic embryos per callus in this stage of development was achieved by adding 1.0 mg l^{-1} 6-BAP to the culture medium, with differences to the other treatments (Table 3). Somatic embryos reached the cotyledonal stage in the culture medium for somatic embryos formation and differentiation with 1.0 mg l^{-1} 6-BAP after 80 days of culture (Figure 1c).

Table 2. Effect of 6-BAP on the *Swietenia macrophylla* King indirect somatic embryogenesis after 60 days of culture.

6-BAP (mg l^{-1})	Callus with high frequency somatic embryogenesis		Callus with low frequency somatic embryogenesis	
	No. callus/jar	(%)	No. callus/jar	(%)
0.0	1.43 c	23.81	0.67 d	11.13
0.5	2.77 b	46.15	1.71 c	28.56
1.0	3.18 a	53.01	2.04 a	33.92
2.0	2.82 b	47.00	1.83 b	30.61
S. E.	± 0.04		± 0.02	

Means within a column with unequal letters differ significantly according to Duncan test (p <0.05). S.E Standard error

Table 3. Effect of different concentrations of 6-BAP on the number of somatic embryos per callus and the development of the somatic embryos in *Swietenia macrophylla* King after 80 days of culture.

6-BAP (mg l^{-1})	No. of somatic embryos per callus	No. of somatic embryos in cotyledonary stage per callus	Somatic embryos in cotyledonary stage (%)
0.0	18.73 d	15.58 d	83.21
0.5	31.62 c	27.58 c	87.23
1.0	43.35 a	40.37 a	93.12
2.0	36.18 b	32.83 b	90.74
S. E.	± 0.36	± 0.34	

Means within a column with unequal letters differ significantly according to Duncan test (p <0.05). S.E Standard error

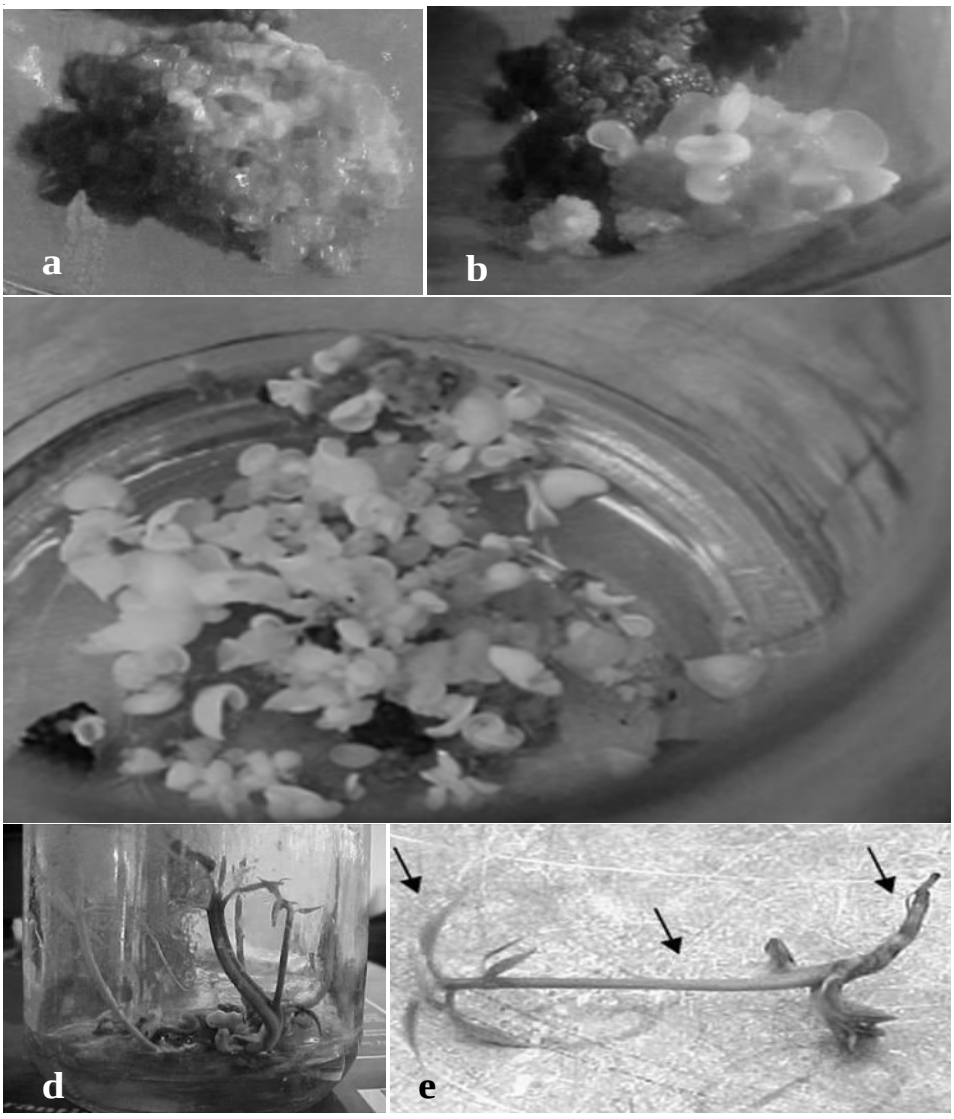

Figure 1a-e. Indirect somatic embryogenesis in *Swietenia macrophylla*. **a** Callus formed from cotyledon after six weeks of culture, **b** Callus with high frequency of somatic embryogenesis after 45 days of culture, **c** Somatic embryos at the cotyledonary stage in the culture medium for somatic embryos differentiation with 1.0 mg l^{-1} 6-BAP after 80 days of culture, **d** and **e** Somatic embryos germinated on MS medium after 60 days of culture.

Results demonstrated the effect of the 6-BAP in the development of *Swietenia macrophylla* King somatic embryos which were formed from calli. The addition of different concentrations of 6-BAP in the range of 0.5–1.5 mg l^{-1} in the culture medium increased the number of somatic embryos that reached the cotyledonary stage in woody plant species such as *Eucalyptus globulus* Labill and *Quercus robur* L, (Pinto *et al*., 2002; San-José *et al*., 2010).

Similar results were obtained using low concentrations of 6-BAP (0.5-1.0 mg l^{-1}) for the differentiation of somatic embryos in other woody plant species such as *Psidium guajava* L. cv. EEA 18-40 and *Coffea arabica* L. cv. Caturra rojo (Vilches *et al*., 2001; Barbón *et al*., 2002).

Formation and differentiation of somatic embryos in *Swietenia macrophylla* show good results using a semisolid culture medium composed by half of MS salts supplemented with 10.0 mg l^{-1} thiamine, 1.0 mg l^{-1} nicotinic acid, 1.0 mg l^{-1} pyridoxine, 50.0 mg l^{-1} L-Cysteine, 3.0 % sucrose and 1.0 mg l^{-1} 6-BAP.

Results demonstrated that the formation and differentiation of somatic embryos from calli in *Swietenia macrophylla* King was favored with the addition of 6-BAP to the culture medium. The greater percentages of high and low frequency somatic embryogenesis, as well as the greater number of somatic embryos per callus and somatic embryos that reached the cotyledonary stage were obtained with 1.0 mg l^{-1} 6-BAP.

Somatic embryos maturation

The survival level of somatic embryos transferred from culture medium with 8.0% sucrose to a culture medium containing half MS salts, MS vitamins and 2.0% sucrose was affected after 60 days of culture due to a high sucrose concentration in the maturation culture medium (Table 4).

Treatments: 1-Somatic embryos cultured in 4.0% sucrose, 2-Somatic embryos cultured in 6.0 % sucrose, 3-Somatic embryos cultured in 8.0% sucrose, Control: somatic embryos in cotiledonary stage cultured without different sucrose concentrations.

Influence of active osmotic agent in somatic embryos maturation has been observed by Márquez *et al*. (2003) in *Persea americana* L. These authors demonstrated that the survival of somatic embryos cultured during 15 and 30 days with 7.5% sucrose or superior concentrations was affected.

The addition of sucrose (4.0-8.0%) in the culture medium increased the somatic embryos maturation. Somatic embryos cultivated with sucrose presented greater percentages of germination than the control, but the highest results were obtained in the treatment two (Table 4). However, somatic embryos germination was diminished due to the high sucrose concentration in the culture medium (8.0%). This high concentration produced big necrotic zones in somatic embryos tissue causing death to the somatic embryos. Similar results were obtained by Abedini *et al*. (2000). A somatic embryos maturation procedure was described in *Acacia caven*. Results showed that addinga sucrose concentration superior to 7.5% to the maturation culture medium affected somatic embryo germination. The same authors demonstrated that decreasing of somatic embryos germination was much related to cellular death.

Table 4. Effect of different sucrose concentrations on the survival and germination of somatic embryos of *Swietenia macrophylla* King, after 60 days of culture.

Treatments	*Somatic embryos survival*		*Somatic embryos germination*	
	No. SE/jar	(%)	No. SE/jar	(%)
1	7.00 a	100.00	4.59 b	65.64
2	7.00 a	100.00	5.33 a	76.17
3	6.51 b	93.00	3.77 c	53.83
Control	7.00 a	100.00	2.74 d	39.14
S. E.	± 0.08		± 0.11	

Means within a column with unequal letters differ significantly according to Duncan test (p <0.05). S.E Standard error

Somatic embryos germinated on MS culture medium. Root formation occurred simultaneously with shoot elongation. This presented normal development, similar to a plant in nature conditions (Figure 1d-e).

The results reported in this research showed that the addition of sucrose to the culture medium increases the somatic embryos maturation and germination. The use of sucrose in the maturation of somatic embryos has already been reported for other woody plant species such as *Acacia caven* (Abedini *et al*., 2000) and *Gossypium hirsutum* (Kunria *et al*., 2003).

The results also demonstrated that the addition of a high sucrose concentration in the culture medium stimulates the somatic embryos maturation, with 6% of this osmotic agent, somatic embryos survival was not affected and the highest values of germination were obtained.

CONCLUSION

A reproducible indirect somatic embryogenesis protocol was established from callus formation to somatic embryos germination in *Swietenia macrophylla* King. The best combination of 2,4-D and kinetin (4.0 and 1.0 mg l^{-1}) for callus formation and somatic embryogenesis induction was defined. The somatic embryos formation and somatic embryos differentiation increased twice when 6-BAP was added in the culture medium. A high sucrose concentration (8.0%) affected somatic embryos survival, however, the addition of a sucrose concentration (6.0%) in the culture medium favored somatic embryos maturation. The presence of this osmotic agent in the culture medium increased more than twice the somatic embryos germination.

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