# Regeneration of coffee-shading Walnut (*Cordia alliodora* (Ruiz and Pav.) Oken) from indirect organogenesis

## Regeneración de nogal cafetero (*Cordia alliodora* (Ruiz y Pav.) Oken, a partir de organogénesis indirecta

*Lina María Londoño-Giraldo[1*], Luis Gonzaga Gutiérrez-López[2+]*

[1]Biologist, Master in Plant Biology student. Research group in Biodiversity and Biotechnology. Professor in the Environmental Management Program. Faculty of Environmental Sciences. Universidad Tecnológica de Pereira, Risaralda, Colombia. [2]Biologist, PhD in Plant Biotechnology. Director of the School on Environmental Management. Faculty of Environmental Sciences. Universidad Tecnológica de Pereira, Risaralda, Colombia. *Corresponding author: limalon@utp.edu.co; [1]uisgon@utp.edu.co

Rec.: 29.07.12 Acept.: 12.12.13

## Abstract

This study, using indirect organogenesis from cotyledonal leaf, evaluated *Cordia alliodora* (Ruiz and Pav) Oken regeneration, a tree native to Colombia with industrial, medical and aesthetic properties. The seeds were first treated with 3% sodium hypochlorite solution during 15 minutes supplemented with PPM® added to the culture medium to remove any bacteria and fungi. Three hormones were tested at the induction phase: BAP, Picloram and 2,4-D in concentrations from 0,5 to 12,5 mg/L under light and dark conditions; production of callus was obtained in all of them. After thirty days most of the callus were oxidized; some callus on BAP 10mg/L showed organogenic shoots. These results suggest a way for *in vitro* regeneration through indirect organogenesis from coffee-shading walnut cotyledonal leaves and suggest that more research is needed to evaluate this regeneration technique and other *in vitro* regeneration techniques.

**Key words:** BAP, callogenesis, indirect organogenesis, native forest, Picloram, 2-4,D.

## Resumen

En el estudio, utilizando organogénesis indirecta a partir de hoja cotiledonar se evaluó la regeneración de *Cordia alliodora* (Ruiz y Pav) Oken, un árbol nativo de uso industrial, medicinal y ornamental. Para la desinfección de las semillas se utilizó hipoclorito de sodio al 3% por 15 min acompañado de la adición del preservante para plantas PPM® al medio de cultivo. En la fase de formación de callo fueron evaluados tres reguladores de crecimiento: BAP, Picloram y 2,4-D en concentraciones desde 0.5 hasta 12.5 mg/lt en condiciones de luz (12 h) y oscuridad, obteniendo callo en todas ellos. Los callos fueron transferidos a los medios de cultivo (mg/lt): BAP (1), 2,4-D (7.5), Picloram (7.5) y a medio de cultivo sin reguladores de crecimiento. Después de 30 días de cultivo la mayoría de los callos se oxidaron, no obstante algunos cultivados en BAP (10 mg/lt) mostraron brotes organogénicos. Los resultados obtenidos sugieren una vía de regeneración *in vitro* posible mediante organogénesis indirecta a partir de hoja cotiledonar y permiten proponer investigaciones posteriores para avanzar en la regeneración exitosa a partir de esta u otras técnicas de regeneración *in vitro*.

**Palabras clave:** BAP, callogénesis, forestales nativos, organogénesis indirecta, Picloram, 2-4,D.

## Introduction

Diversification and growing demand tropical timber tree species for industrial reforestation, urban forestry, watershed protection and $CO_2$ capture selling to industrial countries, require the supply of new materials for sowing as well as the knowledge on the suitable techniques for its establishment and propagation (Farfán and Urrego, 2004). *Cordia alliodora* (Ruiz and Pav.) Oken (Boraginaceae) in a tropical deciduous tree between medium to large size (20 - 30 m), also known as coffee-shading walnut in Colombia, where it is find in several regions and is common to the coffee area, South of the Pacific coast, Middle Magdalena, Northeast of Choco, Caqueta and Arauca (Farfán and Urrego, 2004).

Coffee-shading walnut is a promising specie in agroforestry systems because of its fast growth in diameter and length, wood quality, possibilities of planting together with agricultural crops and in campaigns for cushioning disasters (Farfán and Urrego, 2004). Additionally, it is a plant with high demand in industry, medicine and ornament, and it is found among the species with high interest in germplasm conservation programs (CONIF, 2005).

CONIF (2005) and Schuler *et al.* (2005) consider that this species should be used in detailed studies for *in vitro* propagation; however, in some tissue culture studies it was found that it maybe recalcitrant in this media, showing a low morphogenetic capacity (Marulanda *et al.*, 2000). The objective of this study was to evaluate aspects associated with the *in vitro* organogenic induction of *C. alliodora* using cotyledonary leaves, which required the standardization of protocols for seed and zygotic embryo disinfection, evaluation of the induction of callus and morphogenesis and, description of some aspects related to the conversion of organogenic structures to complete plants.

## Materials and methods

As plant material certified seeds of *C. alliodora* were used and as complete mineral solutions were used: MS (Murashige & Skoog, 1962), Gelrite® 2.7 g/l, 3% of sucrose and ascorbic acid (0.1 g/l) pH 5.8, and 2 ml/l of the mix for preservatives for plants PPM®.

Initially the seeds were submerged in water for 24 h and washed with water and soap for 15 min. For disinfection they were submerged in a solution of distilled water, 1% sodium hypochlorite (NaClO), 5 ml/l of the fungicide Agrodine® and 2 drops of Tween 20® for 10 min and then they were rinsed. Later they were submerged in 70% ethanol for 1 min followed for three times rinse, they were treated with 3% NaClO for 5, 10 and 15 min. For embryo isolation seeds were places on a laminar flow cabinet for a final rinse before extracting the embryo that was placed on media culture with 1 mg/l 6-benzylaminopurine (BAP).

### Callus formation

At this phase zygotic embryos were used, some were cut longitudinally and others transversally. Each treatment consisted on 10 flasks inoculated with 3, 4 or 5 embryos. For the callus induction a relatively wide range of growth regulators with or without BAP and auxins (Picloram and 2,4-D) were evaluated, each one at 0.5, 2.5, 5, 7.5, 10 and 12.5 mg/l concentrations, for a total of 18 treatments. Half of the treatments were subjected to a 12 h photoperiod and the other half was under dark conditions at 28 ± 1 °C; as controls inoculated explants in media without hormones were used. Thirty days later the callus formation and state was evaluated, including the number of induced callus and the ones with similar structures to the proembryogenic masses (PEM)

### Plant regeneration by organogenesis

For this regeneration only callus with bodies that look similar to proembryogenic masses (PEM) under stereoscope were used. They

had a weight close to 0.45 g and were inoculated in culture media as follows: a third part of the callus induced with auxins (Picloram and 2,4-D) were placed on media with BAP (1mg/l); the same number was multiplied on media with the original auxin concentration and, the rest were inoculated without growth regulators. In all the cases the treatments were placed under light or dark conditions.

One third of the callus generated with BAP were multiplied in media with Picloram (7.5 mg/l) and 2,4-D (7.5 mg/l), which are auxin concentrations that produced the highest number of callus with bodies similar to PEM. The remaining callus were multiplied in media with the with the original cytokinin concentrations. During the induction phase the temperature remained constant at 28 ± 1ºC. After 30 days of treatment observations on oxidation, organogenic structure presence in aerial and root parts were made. In each treatment were used 10 and 20 callus.

**Statistical analysis**

For the induction phase a random block design was used, where each flask contained between 3 and 5 explants with 10 replicated per treatment. Previously, the data were checked for the analysis of variance assumptions (normality, homogeneity and independence) and were subjected to analysis of variance (Anova) using the multiple range Tukey´s test ($P < 0.05$). The program used for this analysis was Statgraphics® Centurion XV.

## Results and discussion

With the sodium hypochlorite (3% NaClO) treatment for 15 min the seed contamination was 36% and the survival was 70% (Figure 1), which provide enough clean material at the beginning of the experiment. The application of this disinfectant mixed with commercial additives such as PPM® at 2ml/l concentration is effective to control the

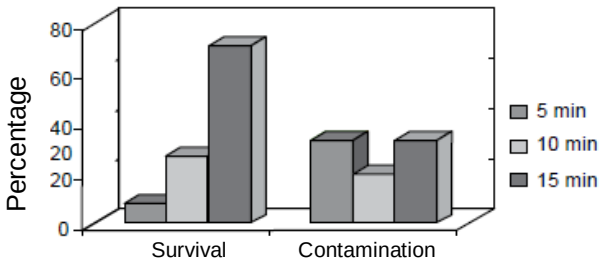



**Figure 1.** Percentages of contamination and survival of *Cordia alliodora* explants in three different expositions times to NaClO

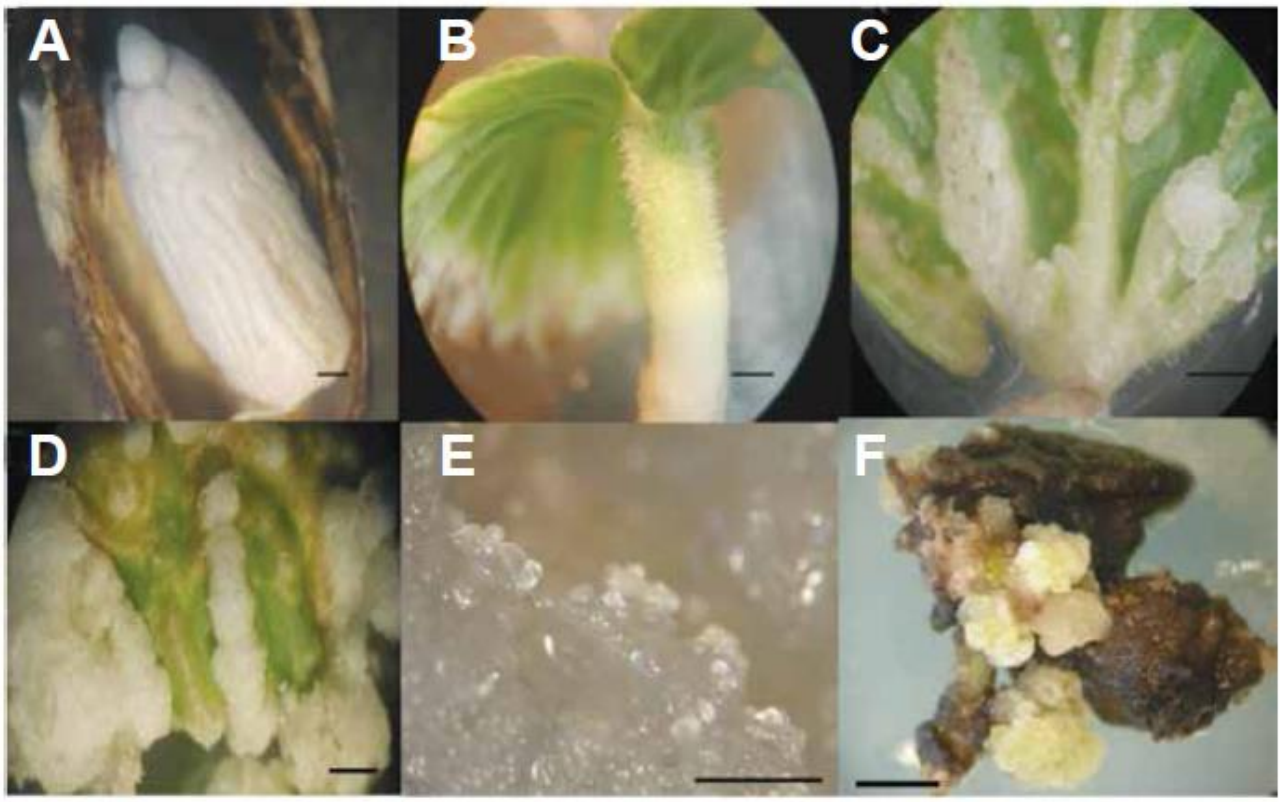
contamination for both, bacteria and fungi (Mroginski *et al.*, 2010).

**Callus regeneration**

The fast germination of *C. alliodora* seeds (Picture 1A) inhibit the direct response of the zygotic embryo at the callus induction, this was due to the explants starting their germination on a short time (5 to 6 days) (Picture 1B), therefore, callogenesis was induced from the colyledonary leaves, both in the complete embryo and the segmented one. In species multiplication the use of zygotic embryos is frequent, due to their favorable response in callus formation. Some wooden plants like *Eucalyptus* spp., among others, have shown responses that involved organogenic and embryogenic regeneration when cotyledon material is used (Glocke *et al.*, 2006).

When the *in vitro* induction of the *C. alliodora* cotyledons was done using BAP and 2,4-D and Picloram auxins, the formation of callus was observed in all the treatments in both conditions, light and dark (Table 1). Manjkhola *et al.* (2005) and Chithra *et al.* (2005) working with application of different growth regulators, like auxins (2,4-D, IBA and NAA) and cytokinins (BAP) found callus formation in *Arnebia euchroma* and *Rotula aquatica* from the Boraginaceae family.

Callus formation from *C. alliodora* segmented or complete cotyledonary leaves, happened 8 days after the establishment of the explants exposed to auxins (Picture 1C), these callus showed a friable consistency




**Picture 1.** Callus formation from *Cordia alliodora* cotyledons. **A**. explant (zygotic embryos), **B**. germinated seedling, **C**. initial callus induced by Picloram auxin, **D**. callus produced under BAP, **E**. recognizition of masses similar to proembryogenic masses PEM, **F**. Callus in necrosis process. Bar = 1 mm

and white to yellow color. Callus induced by BAP were originated 4 weeks after the establishment and appeared in both, segmented and complete leaves, and in light and dark conditions (Table 1), their consistency was friable and were predominantly white (Picture 1D). The morphological characteristics of the *C. alliodora* callus are similar to the ones produced by *Lithospermun erythrorhizon*, *A. euchroma* and *R. aquatica* (Hee-Ju *et al.*, 1997; Manjkhola *et al.*, 2005; Chithra *et al.*, 2005, respectively), which belong to the same botanical family.

The best response for callus induction was observed in the treatments containing 2,4-D (12.5 mg/l) and Picloram (75 mg/l) (Table 1), being in the first one better for the explants exposed to darkness, while in the second was for the explants under photoperiodical conditions (Table 1). The best BAP concentration for callus induction was 7.5 mg/l and 12 h photoperiod (Table 1).

In the control treatment there was callus formation in the segmented explants (Table 1) which agrees with the findings of Chithra *et al.* (2005) in somatic embryogenesis studies from cotyledonary leaves of *A. euchroma*. This phenomena is explained as a tissue cut effect and also, is probably the consequence of polyphenol production that causes oxidative stress in the explant (Bhojwani and Razdan, (1996), Radice (2010).

In the treatments that included auxins (2,4-D and Picloram) in all the concentrations (0.5 – 12.5 mg/l) were observed masses similar to PEM (see Pictures 1E and 1F). In the medium containing 7.5 mg/l of 2,4-D in the dark was found the highest number of PEM ($P < 0.05$) (Table 1). In this case is highlighted the function of these auxins at stimulating the cellular dedifferentiation and the consequently growth of callus, however, at high doses is possible that they interfere with the maturation of the somatic embryos

**Table 1.** Growth regulators, culture conditions, number of formed callus and with presence of proembryogenic masses (PEM) of *Cordia alliodora*.

| Growth regulator | Concentration (mg/lt) | Culture conditions[a] | Callus formed (± S.E.) | Callus with PEM (± S.E.) |
|---|---|---|---|---|
| 2.4-D | 0.5 | Light | 2.2 ± 0.7 | 1.6 ± 0.5 a* |
| | | Dark | 1.0 ± 0.7 | 0.5 ± 0.2 a |
| | 2.5 | Light | 2.3 ± 0.4 | 1.4 ± 0.4 a |
| | | Dark | 0.7 ± 0.2 | 0.5 ± 0.3 a |
| | 5.0 | Light | 0.2 ± 0.2 | 0 a |
| | | Dark | 1.0 | 0.5 ± 0.5 a |
| | 7.5 | Light | 2.6 ± 0.3 | 2.6 ± 0.3 b |
| | | Dark | 4.6 ± 0.4 | 4.2 ± 0.5 b |
| | 10 | Light | 2.6 ± 0.6 | 3.0 ± 0.6 b |
| | | Dark | 2.7 ± 1.2 | 2.7 ± 1.2 b |
| | 12.5 | Light | 3.2 ± 0.6 | 2.0 ± 0.4 b |
| | | Dark | 4.5 ± 0.6 | 4.0 ± 0.3 b |
| Picloram | 0.5 | Light | 2.3 ± 0.4 | 1.6 ± 0.6 a |
| | | Dark | 2.6 ± 0.3 | 1.6 ± 0.6 a |
| | 2.5 | Light | 2.6 ± 0.3 | 2 ± 0.5 a |
| | | Dark | 0.6 ± 0.6 | 1.6 ± 0.8 a |
| | 5 | Light | 2.6 ± 0.8 | 1.3 ± 0.8 b |
| | | Dark | 2.5 ± 0.6 | 2.5 ± 0.5 ab |
| | 7.5 | Light | 5.5 ± 0.4 | 4.5 ± 0.3 c |
| | | Dark | 3 ± 0.4 | 2.6 ± 0.4 c |
| | 10.0 | Light | 4.3 ± 0.3 | 3.3 ± 0.3 bc |
| | | Dark | 2.5 ± 0.9 | 2.7 ± 0.2 bc |
| | 12.5 | Light | 5 ± 0.5 | 4.3 ± 0.8 c |
| | | Dark | 4.3 ± 0.6 | 3.6 ± 0.6 c |
| BAP | 0.5 | Light | 2.0 | 2.0 a |
| | | Dark | 0.5 ± 0.5 | 1.0 ± 1 a |
| | 2.5 | Light | 2.0 | 2.0 ab |
| | | Dark | 3.0 | 2.0 ab |
| | 5 | Light | 1 ± 1 | 1.0 ± 1 a |
| | | Dark | 0.4 ± 0.2 | 0.4 ± 0.4 a |
| | 7.5 | Light | 2.1 ± 0.4 | 2.1 ± 0.4 c |
| | | Dark | 0.8 ± 0.2 | 0.8 ± 0.2 c |
| | 10.0 | Light | 1.3 ± 0.5 | 1.5 ± 0.5 ab |
| | | Dark | 0.4 ± 0.3 | 0.6 ± 0.3 ab |
| | 12.5 | Light | 0.6 ± 0.4 | 1 ± 0.4 a |
| | | Dark | 0.2 ± 0.2 | 0.6 ± 0.4 a |
| Control | | Light | 2.5 ± 0.5 | 0 |
| | | Dark | 1.5 ± 0.5 | 0 |

a = Light: 12 h photoperiod. Dark= dark conditions.
S.E. = Standard error.
*Values in the same regulator doses followed by different letters differ statistically ($P < 0.05$), according to Tukey´s test.

or plant organs generated by organogenesis and in the mutagenic effect or somaclonal variation. With Picloram in similar concentrations and conditions as the ones stated above, the PEM´s formation was also significant ($P < 0.05$) (Table 1). With the use of BAP, callus formed PEM (Picture 1E) in all the concentrations, nonetheless the highest number was found at 7.5 mg/l concentration under light conditions.

According to Bhojwani and Razdan (1996) and Glocke *et al.* (2006), in several species once considered recalcitrant ones,

the success of plant regeneration by tissue culture is due, in high measure, to changes in the media manipulation and selection of type of explant, since most of the plants with subtle morphogenic response express their cellular totipotency only in embryogenic explants culture. This response could be associated with important factors such as origin, age and quality of the donor plant. In this study, the initial explant of coffee-shade walnut was obtained from conserved seeds handle under controlled humidity and storage conditions, which, together with the use of cotyledons, probably affected positively the callus induction with the type and concentration of regulators used.

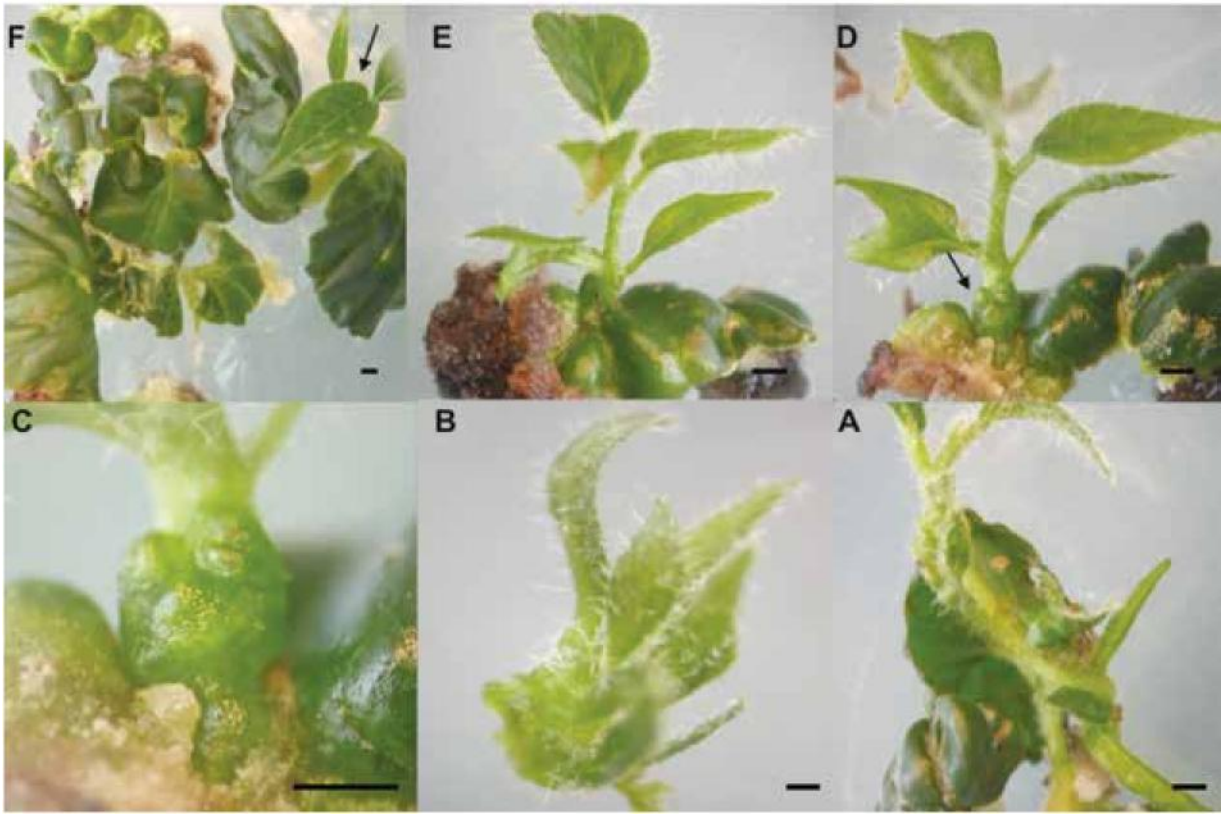
**Plant regeneration by organogenesis**

Callus proliferation was affected by oxidation in all the treatments (Table 2), in addition to dehydration that affected their weight (Picture 1F). Callus in all the original concentrations of the regulators, both under light and dark, showed oxidation, being 100% for the ones formed in 2,4-D, which agrees with the results of Hee-Ju *et al.* (1997), Manjkhola *et al.* (2005) and Chithra et al. (2005) who suggest that the darkening is a previous process to the somatic embryo appearance. On the other hand, Glocke *et al.* (2006) on *in vitro* studies with eucalyptus consider that callus darkening is associated with several factors including media composition, light exposure, growth area temperature; among others, which are not always associated with a later regeneration of somatic embryos.

Callus kept in 0.5 mg/l of 2,4-D and Picloram, under light or dark, were characterized by the formation of roots, probably as a complementary response to callus formation. As it is known, endogenous auxins or in low concentrations are used in micropropagation to induce the root formation in non-differentiated callus as well as to stimulate the cell division.

**Table 2**. Oxidation degree and morphogenic response to *Cordia alliodora* callus in different growth regulators and culture conditions.

| Original auxin[a] | Growth regulator | Culture conditions[b] | Oxidation | Morphogenic response | |
|---|---|---|---|---|---|
| | | | | Roots | Aerial part |
| Picloram | BAP 1mg/l | Light | 97 | YES | NO |
| | | Dark | 100 | NO | NO |
| | Picloram | Light | 96 | YES | NO |
| | | Dark | 100 | NO | NO |
| | Without hormones | Light | 97 | YES | NO |
| | | Dark | 90 | YES | NO |
| 2,4-D | BAP 1 mg/l | Light | 100 | NO | NO |
| | | Dark | 100 | NO | NO |
| | 2,4-D | Light | 100 | NO | NO |
| | | Dark | 100 | NO | NO |
| | Without hormones | Light | 87 | YES | NO |
| | | Dark | 83 | YES | NO |
| BAP | Picloram 7.5 mg/l | Light | 50 | NO | NO |
| | | Dark | 66 | NO | NO |
| | 2,4-D 7.5 mg/l | Light | 90 | NO | NO |
| | | Dark | 100 | NO | NO |
| | BAP | Light | 28 | NO | YES |
| | | Dark | 100 | NO | NO |




**Picture 2.** *in vitro* regeneration of *C. alliodora* from callus obtained and kept on 10 mg/l BAP media. **A, B, C** = seedlings developed from adventitious buds generated from callus. **D** = close up to an adventitious bud at the base of an organogenic bud (arrow, picture 3C). **E** and **F** = separated seedlings isolated from callus. Bar = 2 mm.

The indirect organogenic response was observed in callus kept in BAP (10 mg/l), that showed none or low oxidation (Picture 2) and low formation of adventitious buds (Pictures 2C and 2D) and aerial buds (Table 2 and Pictures 2A, 2B, 2E and 2F) which were induced from callus. These results agree with the ones of Hee-Ju *et al.* (1997) in *L. erythrorhizon* using ANA and kinetin, Manjkhola *et al.* (2005) in *A. euchroma* using IBA plus BAP, and Chithra *et al.* (2005) in *R. aquatica*.

In coffee-shading walnut is has been found direct organogenesis from apical buds, nodes and buds, with favorable results till acclimations at nursery (Schuler *et al.*, 2005); therefore, the indirect organogenesis observed in this study is the first report for this species.

## Conclusions

- The hypochlorite treatment (3%) for 15 min gave the best results for *C. alliodora* seed disinfection for in vitro culture. With this treatment 64% disinfection of the explants was reached.
- With the use of MS and 2,4-D, Picloram and BAP growth regulators was possible, under light and dark conditions, to produce callus from cotyledonary leaves of *C. alliodora*.
- In the callus obtained from the treatments that included 7.5 mg/l of 2,4-D and dark conditions, Picloram in the dark and BAP under light, were observed the formation of similar structures to proembryogenic masses (PEM).
- From the *C. alliodora* callus cultures in MS media supplemented with 10 mg/l BAP, adventitious buds and aerial buds

were regenerated, demonstrating regeneration by indirect organogenesis.

- Both, the callus cultured in MS media without growth regulators (control) and those coming from 2,4-D and Picloram (0.5 mg/l), originated rhizogenesis under light and dark conditions.

## Acknowledgements

To Marta Marulanda, PhD, director of the Research Group on Biodiversity and Biotechnology of the Universidad Tecnológica de Pereira, to the members of the group and the lab member of the Plant Biotechnology lab UTP.

## References

Bhojwani, S. and Razdam, M. 1996. Plant tissue culture: theory and practice, a revised edition. Elsevier. Holanda. 767 p.

Chithra, M.; Martin, K. P.; Sunandakumari, C.; and Madhusoodanan, P. V. 2005. Somatic embryogenesis, encapsulation and plant regeneration of *Rotula aquatic* Lour. A rare rhoeophytic woody medicinal plant. In Vitro Cell. Dev. Biol. Plant 41:28 - 31.

CONIF (Corporación Nacional de Investigación y Fomento Forestal). 2005. Informe Anual 2004. Bogotá D.C. 120 p.

Farfán, V. F. and Urrego, J. B. 2004. Comportamiento de las especies forestales *Cordia alliodora, Pinus oocarpa* y *Eucaliptus grandis* como sombrío e influencia en la productividad del café. Rev. Cenicafé 55(4):317 - 329.

Glocke, P.; Collins, G; and Sedgley, M. 2006.Efects of auxins on organogenesis and somatic embryogenesis from juvenile explants of *Eucalyptus erytronema, E. stricklandii,* and two inter-specific hybrids. J. Hort. Sci. Biot. 81(6):1009 - 1014.

Hee-Ju, Y.; Soo Kyung, O.; Man-Ho, O.; Dong-Woog, C.; Young Myung, K., and Sang-Gu, K. 1997. Plant regeneration from callus cultures of *Lithospermum erythrorhizon.* Plant Cell Reports 16:5:261 - 266.

Manjkhola, S.; Dhar, U.; and Joshi, M. 2005. Organogenesis, embryogenesis and synthetic seed production in *Arnebia euchroma* – A critically endangered medicinal plant of the Himalaya. In Vitro Cell. Dev. Biol. Plant. 41:244 - 248.

Marulanda, M. L.; Gutiérrez, L. G.; and Vallejo, A. 2000. Selección y propagación de nogal cafetero (*Cordia alliodora*), Aliso (*Alnus acuminata*) y Mora (*Rubus glaucus*) por cultivo de tejidos in vitro. In: Colciencias. Publicaciones y Formación de Personal en Proyectos Cofinanciados por Colciencias en el Periodo 1991 - 2000. 50 p

Mroginski, L.; Sansberro P.; and Flaschland, E. 2010. Capítulo 1: Establecimiento de cultivos de tejidos vegetales Pag: 17-25. En: Levitus, G.; Echenique, V.; Rubinstein, C.; Hopp, E.; and Mroginski L. (eds.). Biotecnología y Mejoramiento Vegetal 2. Instituto Nacional de Tecnología Agropecuaria (INTA). Buenos Aires. 644 p.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473 – 497.

Radice, S.2010. Morfogénesis. In: Levitus, G.; Echenique, V.; Rubinstein, C.; Hopp, E.; and Mroginski, L. (eds.). Biotecnología y Mejoramiento Vegetal. Cap. 2. Instituto Nacional de Tecnología Agropecuaria (INTA). Buenos Aires. 644 p.

Schuler, G. I.; Baquero, O. S.; Gaona, T. D.; Vega, G. E.; Rodríguez, R. J.; Ramírez, S. C.; Nieto R. V.; and Hodson de Jaramillo, E. 2005. Propagación in vitro de material seleccionado de *Tabebuia rosea* (Bertol.) DC. (Ocobo) y *Cordia alliodora* (Ruiz y Pav.) Oken (nogal cafetero*).* Rev. Colombiana de Biotecnología 7(1):39 – 50.