Rescue and in vitro germination of immature embryos of black cedar (*Juglans neotropica* Diels)

Rescate y germinación in vitro de embriones inmaduros de cedro negro (*Juglans neotropica* Diels)

Oscar Darío Quintero-García[1*] and *Sonia Jaramillo-Villegas*[2†]

[1] Agricultural Engineer, Manager of Tissue Culture Laboratory. Ecosystem sub-division, Corporación Autónoma Regional del Centro de Antioquia (CORANTIOQUIA), A.A. 95400, Medellín, Colombia. Tel.: (+57-4) 4938888. [2] Agricultural Engineer M.Sc. Retired Profesor Universidad Nacional de Colombia, sede Medellín.
*Corresponding author: odquinte@unal.edu.co; †sjaramal@unal.edu.co

Rec.: 02.06.11 Acept.: 17.02.12

Abstract

Immature embryos of *Juglans neotropica* (Juglandaceae) with 16 and 20 weeks of development were aseptically removed from the fruits and placed in the following culture media for 60 days, without growth regulators: MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980) y DKW (Driver and Kuniyuki, 1984). It was observed that embryos with 16 weeks of development did not germinate in any of the three culture media. The embryos with 20 weeks of development germinated 100% in the three culture media. It was found an important effect of the culture media on the growth of the embryos, the MS medium was significantly better than the DKW and WPM medium ($P < 0.05$), due to the fact that in MS medium embryos had the highest height and a better proportion of stem/root ratio to obtain complete plantlets. After this, seedlings were adapted to environmental conditions (greenhouse).

Key words: Black cedar, culture media, germination, immature embryos, in vitro culture, *Juglans neotropica*, rescue, tissue culture, walnut.

Resumen

Embriones inmaduros de cedro negro (*Juglans neotropica*) (Juglandaceae) con 16 y 20 semanas de desarrollo fueron removidos asépticamente de los frutos y sembrados durante 60 días en los medios de cultivo MS (Murashige y Skoog, 1962), WPM (Lloyd y McCown, 1980) y DKW (Driver y Kuniyuki, 1984), sin reguladores de crecimiento. Se observó que embriones con 16 semanas de desarrollo no germinaron en los tres medios de cultivo; por el contrario, los embriones con 20 semanas de desarrollo presentaron una germinación de 100%. Se encontró un marcado efecto del medio en el crecimiento de los embriones, siendo el medio MS mejor ($P < 0.05$) que los medios DKW y WPM, debido a que en él los embriones presentaron mayor altura y proporción tallo/raíz para la obtención de plántulas completas.

Palabras clave: Cedro negro, cultivo de embriones, cultivo in vitro, embriones vegetales, germinación, *Juglans neotropica*, medio de cultivo, nogal, rescate.

Introduction

Black cedar (*Juglans neotropica* Diels), which is a forest species of high Andean forests of Colombia, has suffered the consequences of deforestation and progressive disappearance in some areas caused by the expansion of cattle ranching and agriculture (Humboldt Institute, 1998). Currently, natural populations of this species are in endangered due to intensive exploitation of wood, used in the construction of luxury furniture (Cardenas and Salinas, 2006).

Since 1999 *J. neotropica* species has been selected in the priority conservation list by the Regional Autonomous Corporation of Central Antioquia (Corantioquia). Then, a research and selection project of seed trees started allowing its propagation in nursery conditions, to promote their ex-situ recovery and use in the development of programs for reforestation in their jurisdiction (Gómez and Toro, 2007). However, the sexual propagation of the species has limitations under nursery conditions such as: low seed production per tree in natural conditions, caused by loss of fruit during development and the difficulty of preserving the few genotypes in the wild. Moreover, the seed has naturally latency, which produces low uniformity in germination under nursery conditions (Gomez and Toro, 2007).

From the statement above, Atwater (1980) mentioned that the main constrain in the germination of *J. neotropica* is associated with retention of inhibitors, which are contained in the seed coat due to its water permeability and semi or impermeable conditions to certain chemicals or gases, which also exert mechanical resistance to the developing embryo. Lopez (1997) mentions that the lignification degree in testa is associated with latency, limiting the amount of oxygen in the seed. Consequently, the production of growth-inhibiting substances happens in embryonic tissues. Moreover, Ospina *et al.* (2003) stated that the high oil content in the cotyledons can decrease the capacity and uniformity of germination, especially in high temperature conditions.

Due to this problem in germination, different pre-germinative treatments have been evaluated. Treatments were the primer and stratification as Lopez and Piedrahíta (1999) emphasized. The combination of both treatments, using primer for 15 days followed by 60 days of stratification, lead to 30.8% germination and a germination rate of 40 days (beginning of germination). In contrast, the control treatment reached 18% germination and 95 days to reach germination in 180 days of evaluation. In another study, a beneficial effect of stratification for 40 days at 20-33°C was found. Here, the germination achieved 80% and 24 days before the start of germination (Gomez, 2002).

Rescue techniques and multiplication of embryos in vitro are a complementary alternative solution for the forestry sector in the short term. These techniques have demonstrated to be important in the protection and conservation programs for species that have difficulties with sexual propagation, viability, seed shortages, interspecific crosses, long latency, and recalcitrance problems (Shibu and Gillespie, 1998; Benson, 2000; Azofeifa, 2009).

The technique was also been used to avoid the presence of endogenous contaminants and release phenolic compounds, including juglona alelochemical that interferes with cell growth of *Juglans* spp. (Rietveld, 1983). Evidence of the establishment and micropropagation difficulties of this genus are the work of Cornu and Jay-Allemand (1989), Revilla *et al.* (1989), Leslie and McGranahan (1992), Pijut (1997), Cruz and Cruz (2003), Rivers *et al.* (2007). Moreover, the in vitro culture of *Juglans* embryos has facilitated the establishment and subsequent micropropagation of *J. nigra* (-Sudholt Heile *et al.*, 1986), *J. regia* and interspecific crosses (Jay-Allemand and Cornu, 1986; Scaltsoyiannes *et al.,* 1997, Lopez, 2004; Bosela and Michler 2008).

An important aspect of several studies conducted in vitro with immature and mature embryos of the genus *Juglans*, is the effect of the culture medium and plant growth regulators on germination. *J. regia* has shown different responses. Thus, Rodriguez (1982) first used K(h) medium, while in another cultivar of the same species used MS medium (Rodriguez *et al.,* 1989). Allemand Jay (1982) initially used Knop medium in the same species to half concentrations and subsequently the medium Miller (MI). Cossio and Minotta

(1983), after comparing eight different combinations of salt nutritious in *J. regia*, concluded that MS medium was the most suitable for the development and growth of the species. Fernandez *et al.* (2000) stimulated the growth of *J. regia* embryos in liquid MS medium, supplemented with 5 mg/l of BA for 7 days, and then the same medium but without hormone. In parallel, Kaur *et al.* (2006) evaluated different growth regulators (BAP, Kinetin and GA3) on MS medium with five cultivars of *J. regia*. They only obtained 66% germination with the best treatments (MS, Kin 0.5 mg/l, BAP 0.5 mg/l and GA3 2 mg/l). Sánchez *et al.* (2006) found significant differences in the percentage of germination of embryos from *J. regia* cultivar after evaluating the medium WPM, MS, DKW and NGE, free of growth regulators. The highest percentage of germination was obtained with WPM (81%), followed by NGE (62%), DKW (54%) and MS (27%). Besides, the release of phenolics and darkening of the embryos was observed in DKW medium.

Given the diversity of results in the propagation of the genus *Juglans*, is necessary to standardize the in vitro culture conditions of black cedar (*J. neotropica*). Therefore, the aim of this study was to evaluate the response of embryos extracted from immature fruits of this species by growing them in vitro in three different media, trying to reduce the problems in its propagation.

Materials and methods

Obtaining the embryos

Black cedar embryos (*J. neotropica*) came from a seed bank previously identified by Corantioquia (Gómez, 2003), in the municipality of Olaya, located at 2000 MASL (06° 38' N, 75° 45' W), with a rainfall > 1900 mm and average temperature of 17°C (Gómez, 2003). The research was conducted at the Laboratory of Plant Tissue Culture of the Biodiversity Station Corantioquia, located in the village of Santa Elena, east of Medellin, which is located at 2400 MASL and an average annual temperature of 17°C, belonging to the area of lower montane wet forest life (bh-MB) (Holdridge, 1967). Immature fruits of the tree were collected every 4 weeks until the 24th week, after flower pollination. Fruit surfaces of 4, 8 and 12 weeks old, were disinfected with sodium hypochlorite at 2.5% (v/v) for 20 min, and washed thoroughly with sterile water during 10 min. In parallel, fruits of 16, 20 and 24 weeks old were removed from the epicarp and mesocarp with a knife, before proceeding to wash the endocarp (testa) under running water. Then, the endocarp was disinfected in laminar flow by immersing the seeds in 90% ethanol (v/v) and flamed three times with fire.

Subsequently, the hardened endocarp fruits (16 - 24 weeks old) were opened by exerting pressure with a hydraulic press in the binding scar stem to extract the embryos. Then, those embryos were grown on MS medium (Murashige and Skoog, 1962), WPM medium (Lloyd and McCown, 1980) and DKW medium (Driver and Kuniyuki, 1984) (Table 1). Each medium was supplemented with 0.1 mg/l thiamine HCl, 100 mg/l myo-inositol, 2 mg/l glycine, 0.5 mg/l pyridoxine and nicotinic acid, sucrose 3% (w/v) (Table 1). The pH on the media was adjusted to 5.7 and 0.2% (w/v) and, Phytagel was added before dispensing them in glass bottles of 128 cc, which were sterilized at 121°C for 30 min. All cultures were incubated in darkness for 15 days to continue its growth for 45 days. Light was supplied by white fluorescent light bulbs of 20 Watts, with 12/12 h photoperiod and a temperature of 22 ± 1°C.

Data analysis

After 60 days of immature embryos growth, in vitro germination rates were determined together with the lengths of the stem and root. Furthermore, the shoot/root ratio of each plantlet was calculated in the three culture media. The treatments (culture medium) were placed in a completely randomized design with 10 replications (embryos) and analysis of variance were performed for each variable, followed by a Tukey´s test, when differences were detected ($P < .05$). We calculated Pearson coefficients to determine the effect of total nitrogen content and the relationship NO_3^-/NH_4^+ on stem length and shoot/root ratio. All analyzes were performed with the program Statistica version 7.1 (StatSoft, 2005).

Table 1. Chemical composition of the culture media.

Compound	MS	WPM	DKW
	mg/lt (mM)	mg/lt (mM)	mg/lt (mM)
Macro nutrients			
NH_4NO_3	1650 (20.6)	400 (4.9)	1416 (17.68)
KNO_3	1900 (18.8)	—	—
$MgSO_4.7H_2O$	370 (1.5)	370 (1.5)	740 (3.0)
$CaCl_2.2H_2O$	440 (2.99)	96 (0.65)	149 (1.01)
KH_2PO_4	170 (1.25)	170 (1.25)	265 (1.95)
$Ca(NO_3)_2.4H_2O$	—	556 (2.35)	1967 (8.33)
K_2SO_4	—	—	1559 (8.96)
Micro nutrients			
KI	0.83 (0.005)	—	—
H_3BO_3	6.2 (0.1)	6.2 (0.19	4.8 (0.078)
$MnSO_4.4H_2O$	22.3 (0.13)	22.3 (0.13)	33.5 (0.195)
$ZnSO_4.7H_2O$	8.6 (0.029)	8.6 (0.029)	---
$Na_2MoO_4.2H_2O$	0.25 (0.01)	0.25 ((0.01)	0.39 (0.0156)
$CuSO_4.5H_2O$	0.025 (0.0001)	0.25 (0.001)	0.25 (0.001)
$CoCl_2.6H_2O$	0.025 (0.0001)	—	—
Na_2 EDTA	37.3 (0.1)	37.3 (0.1)	45.4 (0.12)
$FeSO_4.7H_2O$	27.8 (0.1)	27.8 (0.1)	33.8 (0.12)
$NiSO_4.\ 6H_2O$	—	—	0.005 (0.00002)
$Zn(NO_3)_2.\ 6H_2O$	—	—	17 (0.057)

Seedlings adaptation

After 60 days of in vitro growth, the seedlings were removed from the culture media and roots were washed with distilled water. Then, each seedling was planted individually in 12-ounce plastic cups, containing a mixture of sterile soil and sand in proportion (2:1). The plastic cups were covered with similar plastic cups but transparent to avoid dehydration. Once sown, seedlings were sprayed weekly with MS medium, diluted to 25% of the original concentration, to complete four applications. This stage was conducted in a greenhouse covered with bleak of 65% and laterals made of glass. The seedlings were kept for 1 month under these conditions with a natural light regime and temperature of 23 ± 1°C. After 30 days, perforations were made at the top of the vessel to facilitate the adaptation of the plants. The finalization of the adaptation process was determined when the seedlings formed one or two leafs (90 days).

Results and discussion

Obtaining embryos

Following the development of *J. neotropica* fruits for obtaining embryos, it was observed that the immature ones collected with4, 8 and 12 weeks of development, had not yet formed the embryo. From the week 16, fruits had formed embryo which were used for the experiments, as well as those with 20 weeks of development. Embryos of these immature fruits had a pearly white color. According to Lopez and Piedrahita (1999), and Gomez (2002), 24 weeks old seeds come from fruits that have reached physiological maturity and are suitable for to start pregerminative treatments for conventional sexual propagation.

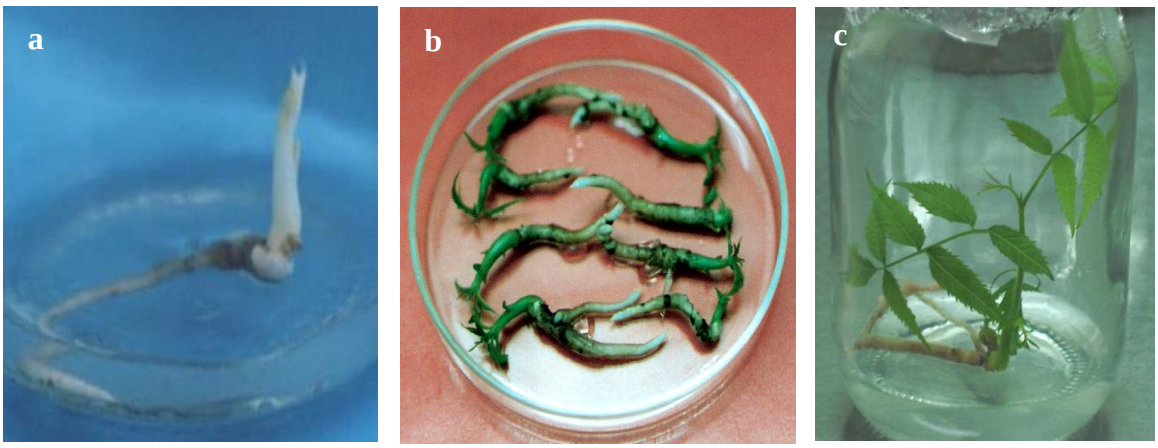
Sowing and germination

Immature embryos from 16 weeks old fruits did not develop in the in vitro media. Some of them became necrotic or remained quiescent

after 30 days of culture. In contrast, embryos with 20 weeks of development initiated germination between day 5 and 7 after sowing in all culture media, reaching 100% germination after 15 days of culture (Figure 1). These values are higher than the results of Lopez and Piedrahita (1999), and Gomez (2002) for power and speed of germination. These results should be taken into account, mainly because they are suitable for propagating a vulnerable extinction species, which requires prolonged seed pre-germination treatments (40-60 days) to break naturally dormancy, showing that the main restriction for germination is associated with the seed coat.

No browning was observed in the culture medium, which is caused by the release of toxic exudates by the explant, or by callus formation in seedlings, as found in *J. nigra* (Heile-Sudholt *et al.*, 1986), in *J. regia* cultivars (Jay-Allemand and Cornu, 1986, Sanchez *et al.*, 2003, Sanchez *et al.*, 2006), and hybrids of these species (Cornu and Jay-Allemand, 1989). The studied media presented 100% germination of immature embryos of *J. neotropica* (Picture 1b), opposite to other studies with *J. regia* (Sanchez *et al.*, 2003, Sanchez *et al.*, 2006; Toosi and Dilmagani, 2010).

The culture media results showed significant differences ($P < 0.05$) of growth and development of embryos after 60 days (Table 2). Embryos grown in MS medium showed the highest stem length and the best value or seedling conformation (shoot/root), compared to DKW and WPM media. Moreover, root length between DKW and MS medium was different ($P < 0.05$) in relation to WPM medium. Embryos that grew in DKW and WPM medium presented higher root length (2



Picture 1. Sequence of *J. neotropica* embryo growth under in vitro conditions. From left to right: **(a)** Initial embryo growth with 10 days of culture medium, **(b)** growth at 20 days of culture medium, and **(c)** 45 days seedling cultivation on MS medium.

Table 2. Mean values for each variable (± SD) in embryos at 60 days, total N and NO_3^-/NH_4^+ ratios in each culture medium.

Medium	Stem length (cm)	Root length (cm)	Shoot/root ratio	Total nitrogen (mM)	NO_3^-/NH_4^+	Time of in vitro conditions (days)
MS	7. 22 (±0.81) a	6.41 (±0.71) a	1 (±0.08) a	60.00	1.91	60
DKW	3.3 (±0.69) b	7.5 (±0.45) a	0.4 (±0.08) b	52.14	1.95	120
WPM	1.42 (±0.69) c	5.54 (±0.75) b	0.2 (±0.12) c	14.50	1.96	150

* Different letters show significant differences ($P < 0.05$) between the culture media.

Values in parentheses represent the standard deviation (± SD) for each variable (n = 10).

and 5 times, respectively) with respect to the stem (Table 2).

Acclimation was done 60 days after for seedlings that came from MS medium. Embryos that came from WPM and DKW medium had to be sown again once or twice in their culture medium to reach a larger size after 120-150 days in order to have an ex vitro adaptation. This means a reduction of 2 to 3 months in the process of in vitro establishment and less expenditure of culture media by using MS medium. Macronutrients are essential for growth *Juglans* explants (Amiri, 2004), which was confirmed in this study with *J. neotropica* on MS medium, which is rich in high salt concentrations, followed by DKW medium and contrasting with WPM medium that has no sufficient nitrogen concentration to maintain growth of embryos (see Table 2). Similar results were found with MS and DKW media, in *J. regia* (Revilla *et al.,* 1989, Fernandez *et al.*, 2000; Saadat and Hennerty 2002; Kaur *et al.,* 2006) and *J. nigra* (Bosela and Michler, 2008).

One of the most important nutrients for in vitro culture is the amount of total nitrogen in the culture medium as well as the oxidized nitrogen (NO_3-)/reduced nitrogen (NH_4+) ratio. Results of several studies suggest that the culture media is more important than the ratio of the total nitrogen in the medium. Calculations of Pearson correlation coefficient (r) were performed in this study for each of the variables: stem length, root length and shoot/root ratio, with total nitrogen content and the ionic (NO_3-)/(NH_4+) ratio (Table 3).

Table 3. Correlation coefficients matrix between growth and nitrogen variables.

Variable	(NO_3^-)/(NH_4^+)	N total
Shoot/root	-0.9986*	0.8007
Stem length	-0.9912	0.8463
Root length	0.1251	0.7312

* $P < 0.05$.

Correlation values were close to 1 for the shoot/root ratio and length of the stem, compared to ionic (NO_3-)/(NH_4+) ratio than the obtained with total nitrogen content of the culture media, but only causality was found with $P < 0.05$ for seedlings that showed better growth (shoot/root) on MS medium, followed by DKW and WPM medium in the negative sense (Figure 1). This is similar to observations made by George *et al.* (2007), who argue that in vitro plantlets respond better to the relationship of both types of ions (NO_3)/(NH_4+) than to the total content of total nitrogen.

Ex vitro acclimatization of seedlings

Seedlings adaptation to ex vitro conditions was evident when new leaves appeared together with root growth (creamy white color). After seedlings transplanting, they remained covered for 30 days to prevent dehydration. Then, 120 days were needed to decrease moisture to nursery conditions. A feature of plant material produced by the embryo rescue technique is the homogeneity of growth under greenhouse conditions (Picture 2).

Picture 2 Ex vitro adaptation ex vitro of black cedar (Juglans neotropica) seedling obtained from in vitro immature embryos. **(a)** Growth of new roots after 30 days in the greenhouse. **(b)** Black cedar plants adapted to greenhouse conditions with 120 days (20 to 25 cm).

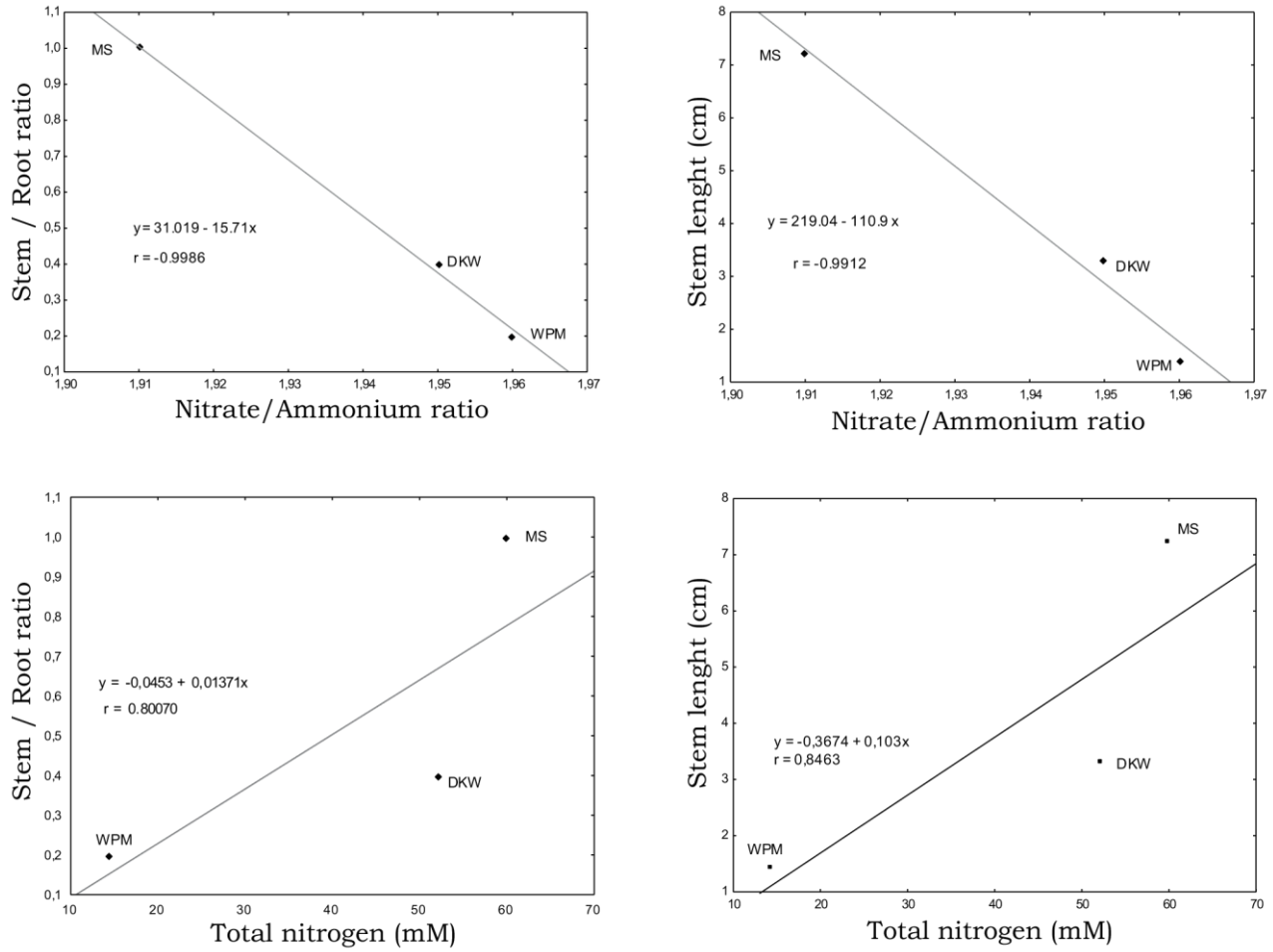


Figure 1. Correlations between (NO_3-) / (NH_4+) ratio and total nitrogen of the three culture media with respect to the shoot/root ratio and stem length variables, of seedlings that came from immature embryos of *Junglans neotropica..*

Conclusions

- This study reports the first *J. neotropica* plants developed from embryos that came from 20 weeks old immature fruits. MS medium (Murashige and Skoog, 1962) provided the best support for the in vitro growth and development of seedlings to finally be ready for ex vitro adaptation process, reducing a time equivalent of 2 to 3 months with respect to the means of the DKW and WPM media. Therefore, MS medium can be regarded as the standard medium for the cultivation of immature embryos and to the propagation of this species.
- The in vitro planting of immature embryos prevent latency issues and pre-germinative issues that seeds normally present in natural conditions for nursery propagation.

Acknowledgements

Thanks to Juan Lazaro Murillo Toro (Forestal Engineer), Project Manager of Conservation and Sustainable Management of Forests, Flora and Fauna, assigned to the sub-direction of Ecosystems of the Regional Autonomous Corporation of Central Antioquia (CORANTIOQUIA) for the funding and the opportunity to participate in his working group. This article is part of a Master's Thesis

of in vitro multiplication of Cedar Black (*Juglans neotropica* Diels).

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