Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section E: Plant Biotechnology

CODEN (USA): JCBPAT Research Article

Total Phenolic Content Present in Tissue Cultures and Commercial Plantations of *Cedrela odorata* Linnaeus

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Abstract: Plant- tissue *in vitro* culture is a method for massive propagation that can be used in forest species with high commercial values such as *C. odorata* L. The major problem during this process is the tissue phenolization known as browning. To overcome this, the relationship between explant age and total phenol content (TPC) of *in vitro* cultures (hypocotyls, cotyledons and callus masses), and commercial plantations (leaves and meristematic shoot tips) was evaluated. TPC was evaluated by the Folin-Ciocalteu method and results were expressed as µg of gallic acid equivalents (GAE)/g tissue. TPC values found in explants of commercial plantations were significantly higher than those found in *in vitro* culture. Leaves from 3-year-old trees showed the highest TPC values (455.87 ± 0.08 GAE/g tissue), while the highest values obtained from *in vitro* cotyledons were 36.18 ± 6.05 GAE/g tissue. TPC low values found in hypocotyls (22.4 ± 4 – 11.1±0.9 GAE/g tissue) did not affect the induction of organogenesis.

Key words: Browning, phenols, *Cedrela odorata*, *in vitro* culture, commercial plantations.

INTRODUCTION

Spanish red cedar (*Cedrela odorata* L.) is an economically important timber species native to the American tropics, with a natural distribution from southern México to northern Argentina [1]. The success in establishment of commercial plantations has been limited due to a broad genetic diversity, the lack of domesticated varieties, and pest damage (*Hypsipyla grandella*). To overcome this difficulty, *in vitro* mass propagation has been

proposed as an efficient method to multiply elite individuals with characteristics that allow them to be successful on commercial plantations. However, the major problem in tissue culture has been the oxidation (browning) and subsequent death of cultured explants. It is well known that browning of the plant tissues during *in vitro* culture is correlated with accumulation of phenolic compounds [2-4]. Phenolic compounds constitute a wide range of plant substances which possess an aromatic ring with one or more hydroxyl groups [5]. The oxidation of the hydroxyl group by polyphenol oxidases (PPOs) and peroxidases (POXs) is involved in organogenic processes and stress responses [6-8]. POXs catalyze the oxidation of phenolic compounds, and have been implicated in the processes of plant growth, development, defense, and cell wall formation [9]. PPOs catalyze the oxidation of phenols to quinones, which are highly reactive and toxic at tissue level [10]. However, at low concentration, they play a role in pigment formation, oxygen scavenging, and the defense mechanism against insects and plant pathogens [11].

The aim of this study was to determine the total phenolic content of *C. odorata in vitro* cultures as well as commercial plantations of the same species.

METHODS

Plant material: Fruits, leaves and meristemic shoot tips (MSTs) from *C. odorata* were collected from commercial plantations present at Acayucan, Veracruz, Mexico (18° 02' 40.97'' N; 94° 51' 14.82'' W). Leaves and meristematic shoot tips (MSTs) were excised from adult trees at different ages (1, 2, 6, and 20 years old). Fruits were collected from elite adult trees (20-year-old trees or older resistant to *H. grandella* attack).

Disinfection: *C. odorata* seeds were obtained from collected fruits. Briefly, they were washed in tap water for 5 min followed by immersion in an antifungal/antibacterial solution (2 g/L Captan®, 2 g/L Manzate®, and 2 g/L Agromicina®) for 24 h , then rinsed 3 times with sterile distilled water. The seeds were disinfected by 15-min immersion in a sodium hypochlorite solution [NaOCl 5% (v/v)], which contained 50 L/L of polyoxyethylene sorbitan monolaurate (Tween 20®). Immediately afterwards the seeds were immersed in 70% (v/v) ethanol for 5 min; after that, three rinses of 5 min each with sterile distilled water were performed. Embryos were isolated aseptically from the seeds. As for the leaves and MSTs, they were washed with commercial detergent and then rinsed 4 times with sterile distilled water to eliminate any trace of detergent.

Tissue culture and callus Induction: Embryos from above were cultured on semisolid MS medium supplemented with 30 g/L sucrose, and 0.7% (w/v) agar. Cultures were kept at 25°C under 140 $\mu M/m^2s$ over a 16 h photoperiod. After 30 days, seedlings were obtained. The resulting hypocotyls and cotyledons were dissected, and used for callus induction on semisolid MS medium supplemented with 5 mg/L 2, 4-dichlorophenoxyacetic acid (2,4D). Hypocotyls and cotyledons used in this study were 40, 50 and 60 days-old while callus masses were 30, 60, 90, 120, 150 and 180 days-old. *C. odorata* seedlings at different ages (40, 50, and 60 days old) were dissected in order to obtain hypocotyls. These were cultured on induction medium (MS medium supplemented with 30 g/L sucrose, 0.7% (w/v) agar, and 3.0 mg/L spermidine) to induce organogenesis.

Extraction and analysis of total phenolic content: Hypocotyls, cotyledons and callus masses from *in vitro* culture as well as MSTs and leaves from commercial plantations were used for determination of TPC, using a modified protocol according to Singleton [12]. Briefly, phenolic compounds were extracted from 250 mg of fresh tissue (leaves, MSTs, hypocotyls, cotyledons and callus masses) by using 500 µL of methanol and shaking for 15 min at room temperature. Right after, each sample was centrifuged at 13000 rpm for 15 min.

After the supernatant was recovered (200 µL), 300 µL of distilled water, and 250 µL of Folin-Ciocalteu reagent were added. This solution was vortex-mixed thoroughly, and it was allowed to stand for 8 min. Subsequently, an amount of 250 µL of 20% (w/v) sodium carbonate was added so the reaction could take place for 2 h in the dark. Absorbance was measured at 760 nm. A standard curve was developed using gallic acid as a reference phenolic compound. TPC for each sample was expressed as µg of gallic acid equivalents (GAE)/g tissue.

RESULTS

C. odorata seeds were germinated on MS medium as described in Methods. The resulting seedlings **(Fig. 1A)** were used to evaluate TPC as well as starting material for the formation of callus masses *in vitro* **(Fig. 1B)**. On the other hand, tissue samples from commercial plantations (Fig. 1C) were employed for the evaluation of TPC.

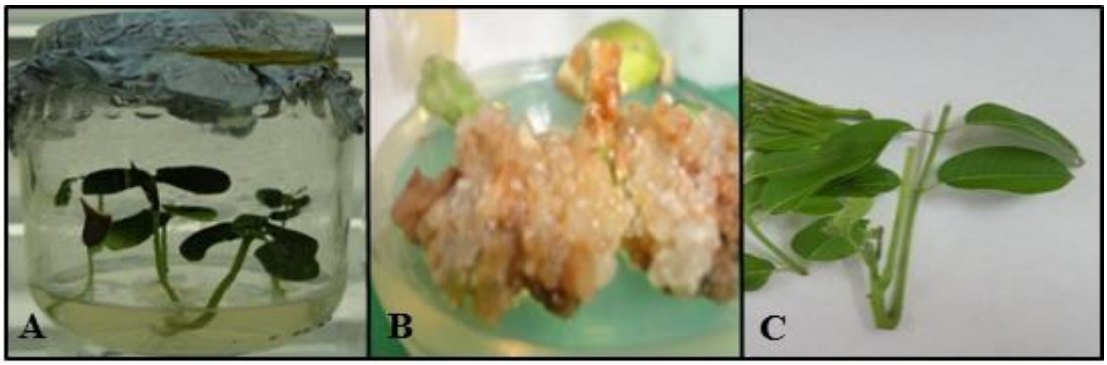

Fig.1: Material used for TPC analysis. A) Seedlings. B) Callus masses. C) Meristemic shoot tips.

When hypocotyls and cotyledons were subjected to the TPC assay at different ages, hypocotyl TPC values were significantly higher than those ones reported for cotyledons **(Fig. 2)**. The higher TPC values for both cotyledons (36.18 ± 6.05 GAE/g tissue) and hypocotyls (22.36 ± 4.01 GAE/g tissue) were found when these were 40 days old. As the tissues age, TPC values decreased significantly **(Fig. 2)**. A similar behavior has been reported for other species such as *Scutellaria baicalensis Georgi*, *Gossypiu hirsutum* L, *Saccharum officinarum, Ipomoea batatas* and *Pinus sylvestris* L. [13-17]. As it is known, the phenolic compounds in plants are synthesized in leaves and transported to other organs; this may be the reason why the TPC values in cotyledons are higher than in hypocotyls.

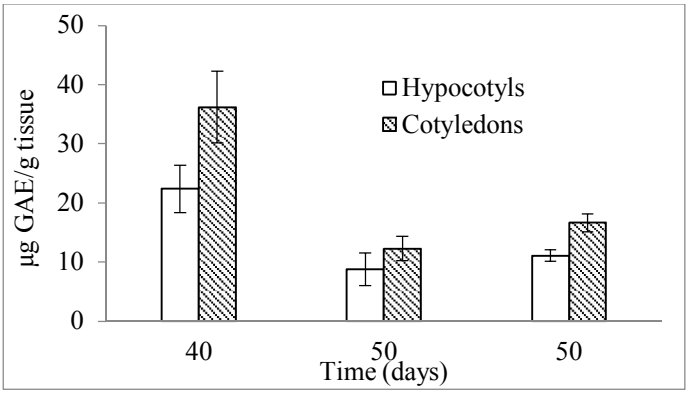

Fig.2: Total phenol content of hypocotyls and cotyledons found in seedlings at different time of *in vitro* culture.

Callus masses also exhibited total phenolic content **(Fig. 3)**, which was in the same order of magnitude as the content found in hypocotyls and cotyledons **(Fig. 2)**. 30-day-old callus masses showed TPC values of 26.10 ± 0.94 GAE/g tissue. During the subsequent 90 days, these values decreased up to 13.24 ± 3.30 GAE/g tissue **(Fig. 3)**. After 90 days, they increased, reaching similar values to those ones reported for 30 days (28.87 ± 4.12 GAE/g tissue).

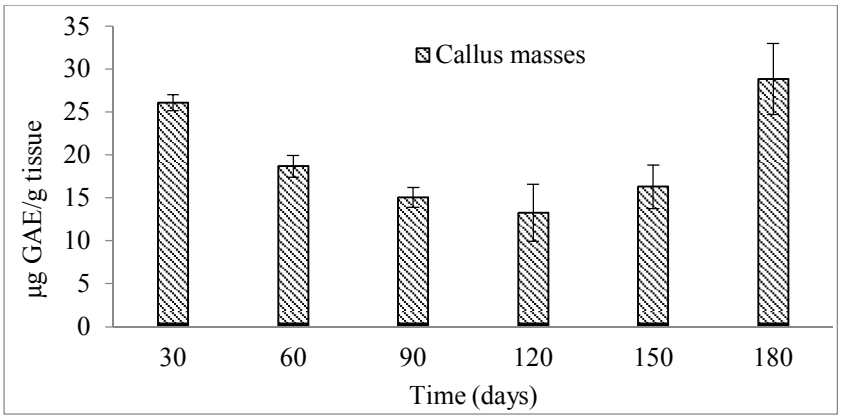

Fig.3: Total phenol content in callus masses at different culture time.

On the other hand, leaves and MSTs from commercial plantation showed higher TPC values than those ones reported *in vitro* (Fig. 4). The highest values were found in leaves collected from 3-year-old trees (455.87 ± 0.08 GAE/g tissue). These were found to be 12 times higher than those reported for cotyledons grown *in vitro* (36.18 ± 6.05 GAE/g tissue). An apparent correlation between age of the tree and TPC values was not observed (Fig. 4). This might be due to other factors affecting the TPC values rather than age. Among these factors *H. grandella* attack/resistance and hydric stress might be mentioned. Interestingly, leaves and MSTs from 3-year-old trees exhibited the highest TPC values, but it is worthy to mention that these trees showed the highest rate of *H. grandella* attacks.

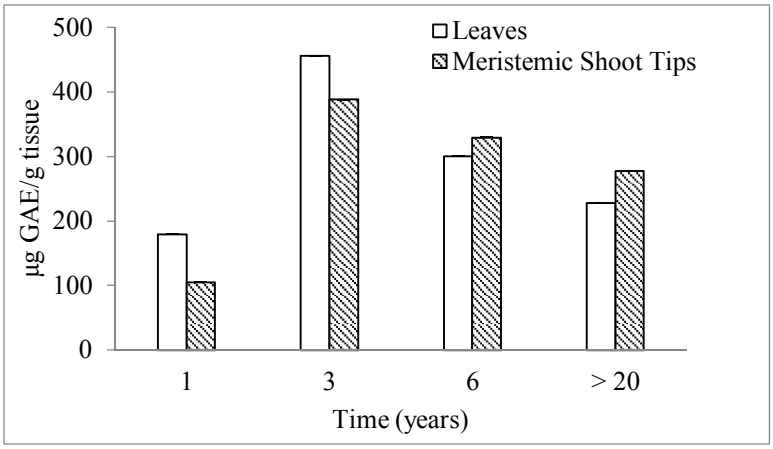

Fig.4: Total phenol content in leaves and meristemic shoot tips of adult trees from commercial plantations.

Finally, explants from *C. odorata* seedlings at different ages (40, 50 and 60 days old) were cultured on induction medium to favor organogenesis. Though there is a significant difference in the TPC values according to the age of the explant, shoot formation rate was no significantly different (Table 1). In order to evaluate the effect of TPC on organogenesis, it would be interesting to add an antioxidant to the induction medium, and evaluate both TPC values and shoot formation rate. Results should be compared to what was obtained in **Table 1.**

Table 1: TPC and organogenesis from hypocotyl segments at different ages.

Days old	µg GAE/ g tissue	Number of shoots
40	22.4 ± 4.0a	3
50	11.7 ± 2.7b	1
60	11.1 ± 0.9b	2

CONCLUSIONS

Total phenolic content of explants grown *in vitro* is significantly different to that of explants obtained from commercial plantations. Callus masses were generated from hypocotyls and they were grown up to 180 days. At this final time, TPC values were similar to those found initially. However, during the intervening period, a depletion was detected. Organogenesis did not correlate with TPC values, probably due to the fact that they were low for this species under these tested conditions.

ACKNOWLEDGEMENTS

This work was supported by grants from CONACyT, through the projects FOMIX-2008-01 and CB-106172.

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