Initial development of *in vitro* propagation protocols for Caracas walnut *Juglans venezuelensis*, a critically endangered tree endemic to El Ávila National Park, northern Venezuela

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SUMMARY

In order to design a micropropagation protocol for Caracas walnut *Juglans venezuelensis* (a critically endangered Venezuelan endemic), morphogenesis studies were performed based on different explants. The explants were cultivated in mediums with different combinations of growth regulators under various conditions of light and darkness. Using nodal and apical segments, aerial sprouts developed when using Thidiazuron (TDZ) with concentrations of 0.3, 1.2 and 3.0 mg/l, and Benzilamine Purine (BA) with concentrations ranging from 0.2 to 1.5 mg/l. Microshoots with a lateral bud, cultivated in a medium complemented with Indole Butyric acid (IBA) (0.01 and 0.05 mg/l) + BA (1.0 mg/l) and TDZ (0.01 and 0.02 mg/l), also developed aerial sprouts (later transferred to another cultivation medium to promote root growth). In addition, starting from foliar explants in a medium complemented with Kinetin (Kin) (1.0 mg/l) combined with Naphthaleneacetic acid (NAA) (0.1 mg/l), somatic embryos were developed in globular and torpedo stages, as well as thick radical structures and several radical hairs. The formation of new sprouts, followed by rooting and the conversion from embryos to plants is a key factor yet to be achieved in order to produce stock for incorporation into reforestation programmes.

BACKGROUND

The Caracas walnut *Juglans venezuelensis* is an endemic tree known only from a small area of montane forest within the 81,800 ha El Ávila National Park (10°32′N, 66°52′W) of the Cordillera de la Costa Mountains, northern Venezuela. The wild population is estimated to number only 100 adult individuals (Ortiz & Salazar 2004). Poor regeneration is attributed primarily to low seed germination and destruction of montane forest the main cause of the drastic reduction of the wild population. The Red Book of Venezuelan Flora 'Libro Rojo de la Flora Venezolana' (Llamozas *et al.* 2003) classifies the species as 'Critical Endangered (CR)'.

In mid-2006, a project to reforest an open mountainside area of the National Park was initiated (Martinez *et al.* 2007). However, due to low germination, alternatives to propagation from seed were required in order to radically increase the number of walnut saplings available for planting. *In vitro* cultivation is a technique sometimes used to achieve a rapid increase in the number of individuals of threatened plants (Toribio & Celestino 2000). This technique has proven useful for propagation of numerous tree species and it has been tested in other *Juglans* species (Pijut 1997, Artioli 2003, Fatima *et al*. 2004). The advantage of *in vitro* propagation is that it has the potential for mass propagation of plants from very small tissue sections in a short period of time. This present study focused on the *in vitro* morphogenic response of the Caracas walnut,

starting with different explants cultivated in several mediums so as to develop a micropropagation protocol. The work was undertaken in the years 2008-2009.

ACTION

Source plants: In order to establish a source stock for the *in vitro* trials, 20 Caracas walnut saplings (approximately 12 months old) were moved from the National Center for the Conservation of Phytogenetic Resources (Centro Nacional de Conservación de los Recursos Fitogenéticos; CNCRF) nursery and maintained in a greenhouse at the Agricultural Biotechnology Research Center (*Centro de Investigaciones en Biotecnología Agrícola*; CIBA). They were transplanted into polyethylene planting bags (5-l capacity) containing fertilized soil and watered on alternate days to maintain soil moisture. A systemic fungicide (Funlate 50 mg/l) and foliar fertilizer 20-20-20 (2.5 g/l) were applied to each plant by hand-sprayer once every three weeks. Plants were pruned to encourage growth of new sprouts, samples of which were taken for *in vitro* propagation.

Explants and preparation: Four types of explants were tested: a) leaf segments with central and lateral veins, b) nodal segments, c) apical segments, and d) microshoots. Bright green-coloured (i.e. healthiest) leaves were used when at a size of approximately 75% of total expansion, removing the stem and washing them with soapy water (blue biodegradable soap) to remove dirt and dust remnants. The leaves were then put in a solution of Funlate (2 g/l) for 15 min and washed three times with distilled water. Next, the leaves were put in a laminar flow hood with a solution of commercial chlorine 2.0% and constantly stirred for 10 min; then they were washed three times with sterile distilled water to eliminate chlorine remnants. Finally, using a sterile hole puncher, foliar segments were cut and immersed in an ascorbic acid solution (50 mg/l) so as to avoid the production of phenols; later, they were passed through ethanol 70% for 60 sec. In the case of apices and nodal segments, fragments of approximately 0.7 and 0.3 cm (respectively) were cut, and the same surface sterilizing procedure was followed for these and microshoots, except that the time of exposure to chlorine was 5 min plus ethanol for 20 sec.

Cultivation medium and growth regulators: MS salts (Murashige & Skoog 1962), in glass Petri capsules, were used as the cultivation medium. This was complemented with growth regulators at the following concentrations: 6-Benzilamine Purine (BA) (0.05, 0.02, 0.1, 0.11, 0.2, 0.23, 0.4, 0.5, 0.68, 0.9, 1, 1.13, 1.5, 1.58, 2, 2.25, 3, 3.38, 4, 4.51, 5.63, 8 and 12 mg/l); Thidiazuron (TDZ) (0.01, 0.02, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2, 1.5, 1.8, 2.4 and 3.0 mg/l); Indolebutyric acid (IBA) (0.01, 0.05, 0.1 and 0.2 mg/l); Indoleacetic acid (IAA) (1, 2 and 3 mg/l); Kinetin (KIN) (1 and 2 mg/l); Naphthaleneacetic acid (NAA) (0.1, 0.2, 0.3, 1 and 2 mg/l); Copper (II) sulfate (0.5 mg/l); Picloram (10, 15 and 20 mg/l); Hydrolyzed Casein (250 and 1000 mg/l); 2.4-Dichlorophenoxyacetic acid (2.4-D) (0.5, 1 and 1.5 mg/l), alone or combined. Also, B5 salts (Gamborg et al. 1968) and Casein (500 mg/l) were used, complemented with 2.4-D (1, 2.5, 3.5 and 10 mg/l) + KIN (0.2 mg/l). In all cases, the pH was adjusted to 5.8 (± 0.2) and solidified with Phytagel (3 g/l).

The capsules containing the explants were incubated at 23 (±2) ºC, 70% humidity in different growth chambers programmed to provide both a photoperiod of 12-h with a light intensity of 32.5 μmol m^{-2} s^{-1} (white fluorescent lamps), and complete darkness.

When leaf segments were used, the experiment was carried out using four explants per Petri capsule; in the case of apices, nodal segments and microshoots, three explants per capsule were used. In all cases, each treatment was repeated 10 times (a total of 40 and 30 explants per treatment, respectively).

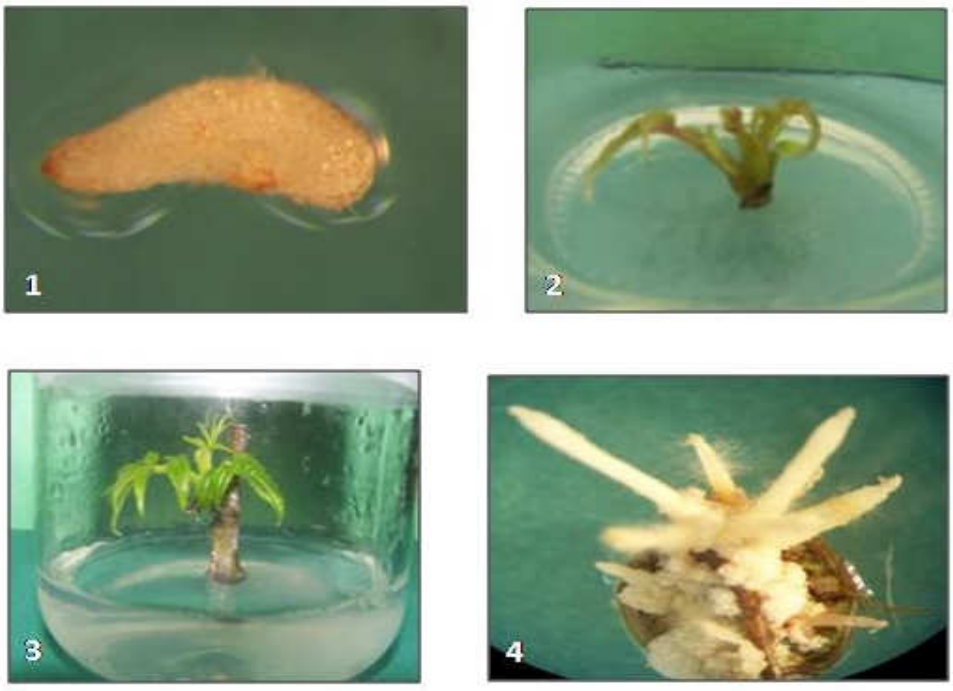
Sub-cultivation: Monthly sub-cultivations were performed in cultivation mediums in order to induce embryogenesis and/or organogenesis. In order to induct somatic embryos, the calluses obtained were dispersed and put in a MS medium, Hydrolyzed Casein (1.0 g/l) complemented with 2.4-D (1.0 mg/l) + BA (0.2 mg/l), BA (0.9 mg/l) + NAA (0.3 mg/l), and BA alone (1.5 mg/l). Then, they were transferred into an embryo development medium, which contained MS salts with Hydrolyzed Casein (250 mg/l) complemented with the growth regulators NAA (0.1 mg/l) and BA (0.05 mg/l). In addition, for root induction, aerial sprouts were transferred to a medium with MS salts and Copper (II) sulfate (0.5 mg/l) complemented with Picloram (10, 15 and 20 mg/l), with KIN (1 and 2 mg/l) + NAA (0.1 and 0.2 mg/l), KIN (1 and 2 mg/l) + IBA (0.1 and 0.2 mg/l) and IBA alone (4.0 and 8.0 mg/l).

CONSEQUENCES

By using segments of leaves in MS medium complemented with KIN (1.0 mg/l) combined with NAA (0.1 mg/l), somatic embryos were developed from a callus in globular and torpedo stage (Fig. 1). Once transferred to a medium for development of embryos to promote their growth, embryos turned brown (a rusty-looking tone), later adopting a creamy colour with presence of a secondary callus in the apical portion. To date, calluses continue to develop thus augmenting the calluses' mass, but somatic embryos appear not to be present.

Aerial sprouts and roots were obtained through organogenesis. Aerial sprouts were developed from nodal and apical segments. Of those tested, the regulator TDZ produced the highest number of sprouts (concentrations of 0.3, 1.2 and 3.0 mg/l). The regulator that was least efficient in terms of number of sprouts produced was BA, in concentrations of 0.23 and 1.5 mg/l (Fig. 2). These sprouts were transferred to another cultivation medium in order to promote the development of roots. Aerial sprouts were also obtained from microshoots in a MS medium complemented with IBA (0.01 and 0.05 mg/l) and TDZ (0.01 and 0.02 mg/l) (Fig. 3). We observed thick radical structures of creamy colouration and several radical hairs from foliar explants cultivated in MS complemented with KIN (1.0 mg/l) combined with NAA (0.1 mg/l) (Fig. 4). In some dispersed calluses, we observed the presence of a single root, somewhat globby in appearance and dark in colour.

Further protocol development: With regard to organogenesis, a protocol to promote a higher number of sprouts followed by a rooting stage must be established in order to obtain the whole seedling. Obtaining somatic embryos in globular and torpedo stages constitutes one of the initial developments which has been achieved and necessary for successful growth. At present, somatic embryos have not developed further. However, we continue to test other cultivation mediums in order to promote their development, based on protocols that have been effective in other tropical tree cultivations (Michelangeli *et al*. 2002, Rahman *et al*. 2003, Feitosa *et al*. 2007), particularly of the *Juglans* genus (Fatima *et al*. 2005, Rios *et al*. 2007). The possible formation of new sprouts followed by a rooting stage and the conversion from somatic embryos to plants is a key factor in a plan for accelerated production of plants in reforestation programs of *J. venezuelensis*.



Figures 1-4. *In vitro* morphogenic response of Caracas walnut in MS medium: 1) somatic embryo obtained from foliar segments with 1 mg/l KIN + 0.1 mg/l NAA; 2) sprouts obtained from apical segments with 0.23 mg/l BA; 3) sprouts obtained from microshoots in medium with 0.05 mg/l IBA + 1 mg/l BA; and 4) radical structures from foliar explants with 2 mg/l KIN + 0.2 mg/l NAA.

Propagation difficulties: During the trials, a great number of electrical failures in growth chambers were experienced. Effects of culture temperature, gaseous environment, contaminants and physical condition of the medium all influence *in vitro* culture success. Manipulation of the physical environment has dramatic effects on how explants respond *in vitro*; light treatments were also compromised. In general, in growth chambers best conditions for micropropagation are constant temperatures (20 to 24ºC) and 50 to 200 μmol m^{-2} s^{-1} photosynthetic photon flux density. Since temperature control in growth chambers is of great importance, extreme care must be taken in order to avoid overheating, an aspect that could not always be controlled in this present experiment, leading to an undesirable environment under which the explants, calluses, sprouts and embryos were cultured. As well as recurrent elevated temperatures, there were uncontrolled light-darkness cycles and periods of very high humidity (causing the presence of water drops inside the Petri capsules). We strongly consider that these aspects greatly affected the conditions for explants development and reduced the success of the trial, especially given that successful micropropagation has been achieved in other species of the genus *Juglans*, where reliable growth chambers have been used.

Conclusions and discussion: The micropropagation trials were based on protocols that have proven effective in other tropical cultivations, particularly of the genus *Juglans*, woody plants and forest trees; as regards explants, culture media and combinations of growth regulators. One of the major problems associated with *in vitro* introduction of Caracas walnut mature leaf material was the endogenous bacterial contamination (i.e. that from within the leaf tissue) and phenolic compound exudation. This aspect was overcome by encouraging the growth of new material by repeated pruning of the young trees, allowing development of shoot tips with high growth rates. The use of annual growth shoot tips, appear to be one of the best alternatives for introduction of explants from field to *in vitro* conditions. Previous work has indicated that propagation of the genus *Juglans* has been possible using nodal segments from different cultivars.

Although a suitable micropropagation protocol for Caracas walnut has only been partly developed, two important results have been obtained so far: formation of somatic embryos in globular and torpedo stage, and aerial sprouts and roots via organogenesis. Once the formation of new sprouts followed by a rooting stage is obtained or the conversion from somatic embryos to plants is achieved, a micropropagation protocol to produce plants suitable for out-planting will be established. Work is currently ongoing.

ACKNOWLEDGEMENTS

This study was financed by PROVITA (Venezuela) and CIBA (Facultad de Agronomía, Universidad Central de Venezuela).

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