



Successive grafting confers juvenility traits to adult Spanish red cedar (*Cedrela odorata* Linnaeus): a tool for the rescue of selected materials

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Abstract

Spanish red cedar, *Cedrela odorata* L. (Meliaceae), is a valuable timber tree in tropical American forests. Existing demand for elite individuals endangers the conservation of interesting germplasm, prompting the development of efficient protocols for the establishment of orchards for tree breeding. Based on previous work regarding grafting adult individuals onto young rootstocks, superior trees from Calakmul Biosphere Reserve in Campeche, Mexico were employed as a source of explants for plant regeneration after successive rounds of grafting and micrografting onto juvenile rootstocks. The results showed that root length and appearance, internode distance, leaf length and number, and plant height values for trees derived from successive rounds of grafting and micrografting were similar to those obtained from juvenile trees derived from seeds, suggesting that developmental traits associated with reinvigoration were partially induced following the reported procedure. This protocol may be useful for the propagation of mature elite trees belonging to the Meliaceae family.

Keywords Micrografting · Superior elite tree · Reinvigoration · Phase change · Tropical forest · Tree biotechnology

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Introduction

Clonal forestry is widely recognized as one of the most effective strategies for rapid genetic gain in tree-breeding processes (Thorpe et al. 1991). However, a clear limitation to clonal forestry is the recalcitrance of mature materials to producing vegetative tissues with acceptable multiplication rates. As the phenotypic traits linked to economic benefits are expressed only when superior trees reach maturity, ineffective vegetative propagation limits the mass propagation of such materials (Bonga 1987). Phase change from juvenility to maturity in trees, appears to affect this recalcitrance through changes presumably controlled by epigenetic modifications (von Aderkas and Bonga 2000). To cope with this problem, grafting has been used empirically by many tree breeders as a strategy to confer juvenility properties to mature materials by establishing them onto young rootstocks in nurseries (reviewed by Huang et al. 1992) or in vitro (Read and Bavougian 2013). Mature scion grafting onto young rootstocks has been reported to increase the multiplication capacity of mature shoots compared to nongrafted mature material in *Eucalyptus* spp. (Titon et al. 2006) and other Meliaceae family members as *Khaya ivorensis*, *K. anthotheca*, *K. grandifoliola* and *K. senegalensis* (Opoku et al. 2018). Our group focuses on the establishment of appropriate strategies to select adult individuals showing phenotypic traits linked to high yield and to simultaneously improve a micropropagation protocol based on previous work to achieve the propagation of mature material (da Costa Nunes et al. 2002; Peña-Ramírez et al. 2010). We previously reported the grafting of mature *Cedrela odorata* L. scions onto young rootstocks as a tool to enhance adventitious shoot production in vitro. Some traits observed in grafted shoots, such as in vitro establishment, multiplication rate, and plantlet vigor, were significantly higher than those in nongrafted adult materials (Peña-Ramírez et al. 2010). Other researchers have evaluated the effects of successive rounds of in vitro grafting of adult material onto juvenile rootstocks, resulting in the generation of multiplication-competent adult material known as reinvigorated or rejuvenated explants. These materials exhibit a clear phase reversion (Huang et al. 1992) in which several traits, such as the fresh weight of shoots, leaf and stem form, rooting, chlorophyll content, photosynthetic efficiency and respiratory efficiency, resemble those of juvenile materials (Chen et al. 2013). To gain a better understanding of and to facilitate improvement in rejuvenation methodology, we explored serial grafting and micrografting of superior *C. odorata* materials onto juvenile plants with a focus on the preconditioning treatments of field material, sanitization, explant characterization, micropropagation competence traits, and early field performance. As *C. odorata* is considered by the International Union for Conservation of Nature (IUCN) as vulnerable species, the development of efficient propagation protocols of elite material could contribute to the sustainable use of this tropical resource.

Materials and methods

Mature superior tree selection and twig collection

By following the selection method described by Solís-Guillén et al. (2017), eight superior trees were chosen according to their phenotypic quality from mature 2d-type “Jobillo” forest (Martínez and Galindo Leal 2002) in 3 locations within the buffer zone of the Calakmul Biosphere Reserve (18°43'N, 89°24'W; 18°23'N, 89°54'W; and

18°37'N, 89°27'W) in the Álvaro Obregón and Conhuás ejidos, Calakmul municipality, Campeche, México. The selection of elite trees was carried out according to a ranking process involving nine different morphologic parameters. These parameters are related to yield (bole height, diameter at breast height), timber quality (bole straightness, branch thickness, branch insertion angle, grain straightness, phytosanitary status), and overall tree architecture (apical control, crown diameter) (Solís-Guillén et al. 2017). As a first step, thirty-two mature *C. odorata* trees corresponding to class 1 individuals according to Mexican legislation for tree germplasm management (Secretaría de Economía 2016) from the collection zones (Fig. 1a) were phenotypically evaluated according to Ideotype CT V 1.0 (Solís-Guillén et al. 2017). Eight of the trees received a score ≥ 85 (out of 100) (Figure S1). The selected superior trees were at least 30.4 cm in diameter at breast height, presented boles more than 10.5 m tall and were at least 30 years old according to their diametric class (Fig. 1b). These materials were employed as explant sources for further experiments requiring the use of vegetative material (twigs) and seeds.

From each selected superior tree, 100 dormant twigs 30 to 40 cm long and approximately 1 cm in diameter were obtained from the middle part of the crown. Once collected, the twigs were kept at 4 °C and wrapped in wet towels for transport. Additionally, 20 mature fruits containing approximately 40 seeds were collected from each selected superior tree. The fruits were kept at room temperature in plastic mesh bags for transport. Plant material collection took place for 2 days during the dry season in February 2014.

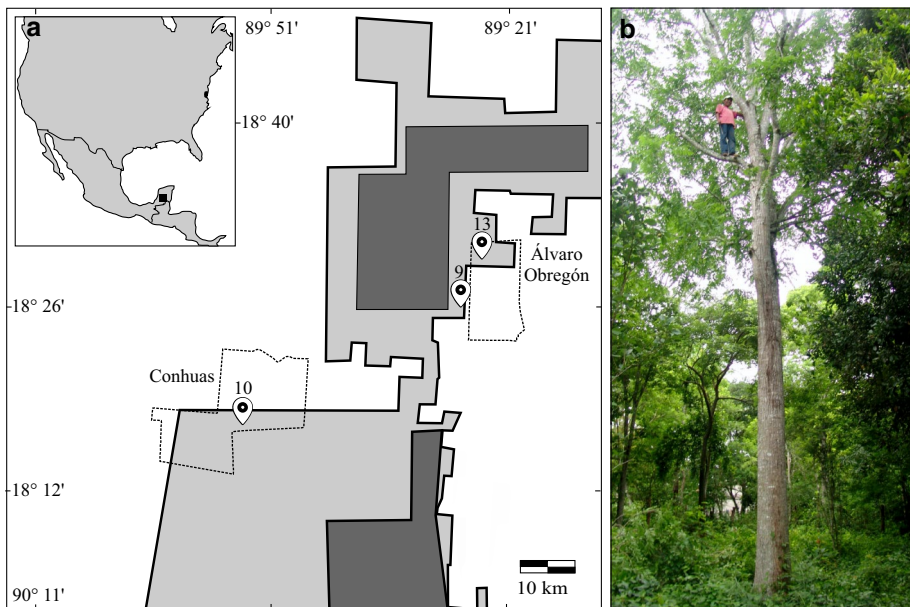


Fig. 1 Localization of superior trees and typical phenotype. **a** Collection zone in the Yucatán Peninsula. Light and dark gray correspond to the buffer and core zones of the biosphere reserve, respectively. Dotted perimeters delimit the Conhuás and Álvaro Obregón ejidos. Position marks indicate the locations of selected trees. The number above each position mark indicates the number of trees used as the selection base at each location. **b** Typical phenotype of superior *C. odorata* individuals selected as twig donors

Twig establishment and grafting

Twigs were immersed for 30 min in a 1 mL L⁻¹ solution of commercial Kasugamycin[®] (3-O-[2-amino-4-[(carboxyiminomethyl)amino]-2,3,4,6-tetra-deoxy- α -D-arabino-hexopyranosyl]-D-chiro-inositol) (Hokko Chemical Industry Co. Ltd., Tokyo, Japan). After this treatment, the twigs were washed with sterile water and segmented into 15 cm-long fragments for immediate processing to prevent the desiccation of the conductive tissues. The twigs from each superior tree were divided into two groups (Figure S2a). The first group of 50 twigs was employed as the scion over juvenile *C. odorata* trees as the rootstock (6-month-old, 70-cm-tall potted plants). To prepare materials for a single round of grafting, we proceeded as follows: once cleaned, 15 cm-long twig segments were cleft cut (Fig. 2a). Compatible cuts were excised into the rootstocks 15 cm from the soil, leaving at least 5 cm of surface contact between the two materials. The area of contact was secured with rubber bands and covered with Saran wrap to avoid dehydration (Fig. 2b). The second group consisting of 50 twigs from the selected trees served as a control and were grown directly under hydroponic conditions (zero rounds of grafting) (Fig. 2c) in Magenta[®] containers with 75 mL of 1 \times Woody Plant Medium (WPM) salt (Lloyd and McCown 1980) solution with the addition of 12 μ M indole-3-butyric acid. The grafted plants and twigs growing under hydroponic conditions were kept under greenhouse conditions (27 °C on average) with 50% shading. The twigs were sprayed twice per week with a 1 mL L⁻¹ solution of commercial Kasugamycin[®] to prevent fungal growth. One month after establishment, twig survival was measured by counting twigs exhibiting the clear proliferation of axillary buds, and twigs showing no axillary bud development were considered dead even in the absence of material decay or rot. Shoots that emerged from the proliferative lateral or apical buds of hydroponic twigs were excised, surface sterilized, and dissected into 9 mm-long stem segments (Figure S2b). Fifty of these segments were evaluated for their ability to produce adventitious shoots by direct establishment in semisolid medium (see “[In vitro procedures](#)” section). This material was considered the non-grafted group. In addition, shoots from the grafted twigs were excised, surface sterilized, and dissected also into 9 mm-long stem segments. These explants were divided into two groups. The first group was dissected and fifty 9 mm-long stem segments were established in semisolid medium (one round of grafting), and the second was micrografted (two rounds of grafting). The culture of stem fragments is described in the next section.

In vitro procedures

Micrografting was performed as described by Murashige et al. (1972), employing 2 to 4 cm-long shoots from grafted mature twigs (Figure S2c). Once separated from a twig, the leaves were removed from the shoots, and the shoots were then immersed in a solution of 1 mL L⁻¹ Tween[®] 80 for 10 min. Then, the shoots were washed with sterile distilled water, immersed again in 10% (w/v) calcium hypochlorite for 10 min, and rinsed again with sterile distilled water. Using a sterile scalpel, two 3 mm-long oblique cuts were immediately made at the base of the shoot; the shoot was then inserted as a cleft graft on the stem of a 3-week-old *C. odorata* plantlet approximately 6 cm from the root. The rootstock plantlets had been previously produced by germinating seeds on sterile 1 \times Murashige and Skoog medium (MS; Murashige and Skoog 1962) with vitamins, 30% (w/v) sucrose and 2.5 g L⁻¹ Gelrite[®] (Duchefa Biochemie, Haarlem, Netherlands) following previously reported

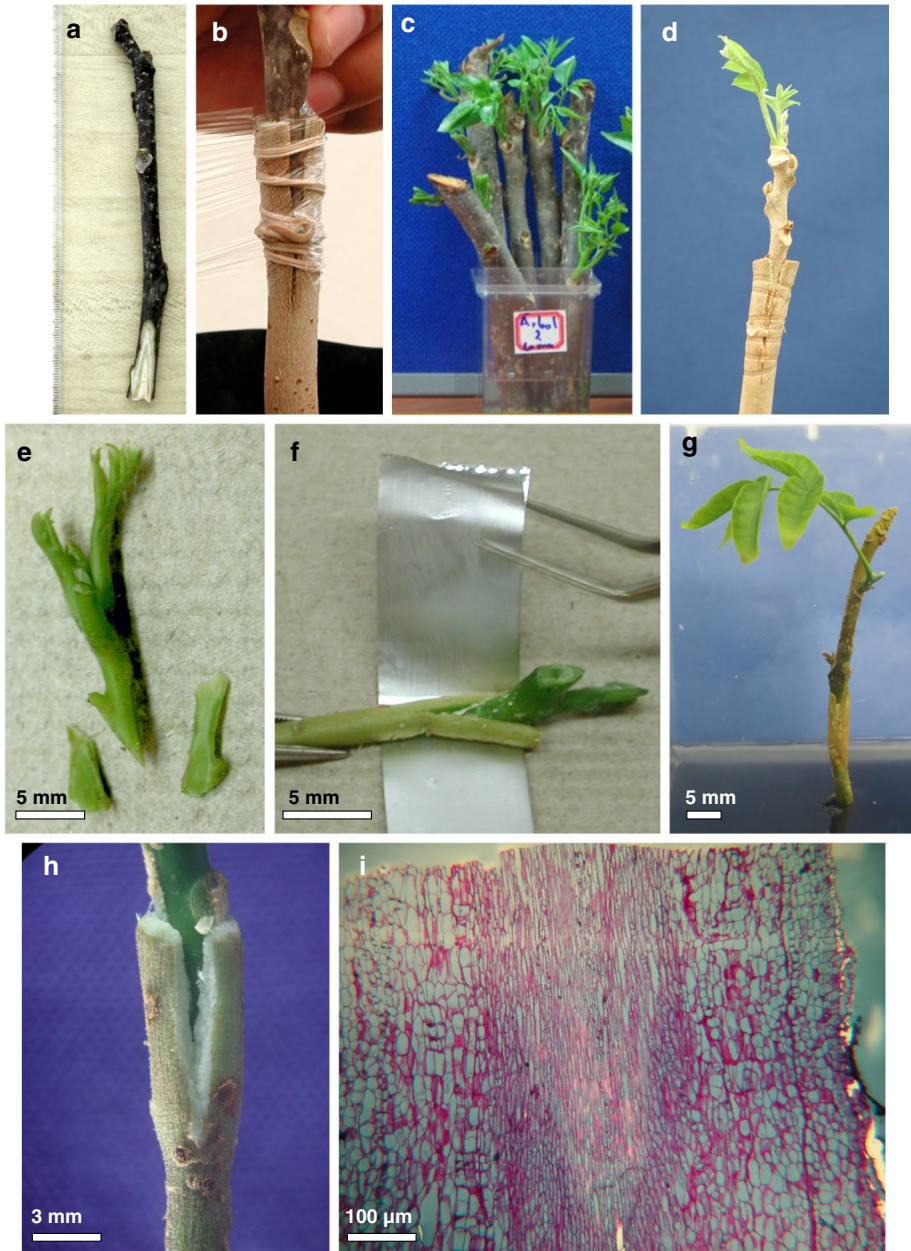


Fig. 2 Mature twigs from superior tree establishment, shooting and micrografting. **a** Twig employed as scion, **b** cleft junction graft, **c** hydroponic twig with shoots, **d** successful graft, **e** shoot prepared as microscion, **f** micrograft joint, **g** successful micrograft, **h** junction area in micrograft with calli surrounding the wound, and **i** micrograph of a longitudinal slide of the graft at the junction area, showing annealing and restored fusion of conductive tissues

sodium hypochlorite-based surface-sterilization methods (Peña-Ramírez et al. 2010). The union was secured using sterile pieces of aluminum foil (5 cm long and 1.5 cm wide). Micrografted plants were kept *in vitro* in an incubation room for 3 months at 28 °C with a photoperiod of 16 h light (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; Sylvania T12 Gro-Lux[®]; Waltham, MA, USA). Six weeks after micrografting, scion stems were dissected to obtain fifty 9 mm-long stem segments.

Histological analysis

Representative micrografts were dehydrated and embedded in hydroxyethylmethacrylate and stained with toluidine blue for microscopic observation as previously described (Peña-Ramírez et al. 2011).

Organogenic induction

The organogenic capacity of the stem segments from the tree sources (zero, one and two rounds of grafting (Figure S2d) was measured by inducing adventitious shoot formation by establishing them in WPM (Lloyd and McCown 1980) macronutrients, MS micronutrients and vitamins supplemented with 30 g L⁻¹ sucrose, 2.5 g L⁻¹ Gelrite[®] and the proper amount of 6-benzylaminopurine (BAP) (0, 4.4, or 13.2 μM) combined in a matrix design with dicamba (0, 4.5, or 13.5 μM) following a previously reported protocol (Peña-Ramírez et al. 2010). The number of emerging adventitious shoots per explant and the length of new shoots (distance in mm from the explant to the tip of the adventitious shoot) were determined one month later. Each combination of plant growth regulators was assayed three times using at least 10 shoots of each type of material in each case. Adventitious shoots were individualized and rooted by culture them for six weeks in the same medium containing 4.92 μM Indole Butyric Acid.

Plant acclimatization and field evaluation

Twenty randomly chosen rooted shoots from the micrografted material were established in Sunshine Mix #3[®] substrate (Sun Gro Horticulture, MA, USA) by covering the base of the shoots in commercial rooting powder formula (Radix 1500[®] Intercontinental Import Export, S.A. de C.V. Salamanca, Mexico). The use of rooting is a commercial practice followed by commercial growers who germinate seeds before transfer them to trays. We consider to follow commercial procedures to avoid differences with seedlings used as growing reference. Plants once established in 130 cm³ capacity trays were maintained under greenhouse conditions (27 °C on average, 50% shading, 80 to 90% relative air humidity), with irrigation every 2 days. Plant survival and morphology were evaluated after 3 months of development. Individuals from the three treatments (zero, one and two grafting rounds) were compared to identically aged juvenile plants derived from *in vitro*-germinated seeds. Plant height was determined from the soil level to the apical meristem, internode distance was measured from the fifth internode to the apical meristem, and leaf length was determined by measuring the longest leaf on each plant.

Statistical analysis

Data were collected from three independent replicates and analyzed using standard ANOVA procedures unless stated otherwise. Differences between means were identified by Fisher's least significant difference (LSD) test with the assistance of the Statistica® software package (StatSoft, Tulsa, OK) at a confidence level of $P \leq 0.05$.

Results

Grafted and micrografted twig establishment

This work focused on the use of grafting and micrografting tools as strategies for the rejuvenation and successful establishment and reproduction of selected mature *C. odorata* trees selected from two different provenances. Twigs from eight superior trees were used to evaluate the organogenic ability of shoots derived from twigs under hydroponic conditions (nongrafted), one round of grafting, or grafting followed by micrografting (two consecutive rounds of grafting) (Figure S2). The twigs from different provenances had different survival rates (Table 1), with the grafted twigs showing a survival rate ranging from 71 to 98%. (Fig. 2d) and a 31 to 43% survival rate in the hydroponic treatment.

Both hydroponic, and grafted materials produced a similar number of shoots from apical and axillary buds, yielding an average of 1.5 shoots per twig. Materials from different provenances did not significantly differ in terms of shooting frequencies or survival (Table 1); thus, the data are presented for both provenances together. Healthy shoots from established twigs (248) (Fig. 2c, d), were employed for micrografting (Fig. 2e–g), and monitored weekly to identify the formation of calli in the area surrounding the wound (Fig. 2h). Some typical surviving micrografts were fixed, and histological observation showed the regeneration of connective tissues between the scion and the rootstock (Fig. 2i).

Table 1 Twig establishment and shooting frequency per provenance

Donor tree number	Provenance	Survival (percentage)	
		Hydroponic	Grafted
1	Álvaro Obregón	35	82
7	Álvaro Obregón	33	92
12	Álvaro Obregón	43	98
16	Álvaro Obregón	30	80
18	Álvaro Obregón	37	73
22	Álvaro Obregón	40	94
24	Conhuás	41	77
29	Conhuás	31	71

Kruskal–Wallis non-parametric variance analysis show no significant differences at $P \leq 0.05$ among donor trees (provenances)

Organogenic capacity in nongrafted, grafted and micrografted explants

To evaluate the organogenic response of the different materials, shoots were collected from 87 hydroponic twigs, 100 grafts, and 100 micrografts were used as explant sources. Stem segments, 9 mm-long, were evaluated for their ability to produce new adventitious shoots when cultured in medium containing different dicamba and BAP concentrations. BAP at 4.4 and 13.2 μM has a significant positive effect on the number of new adventitious shoots formed per explant (shooting frequency), compared to treatments without BAP; dicamba also has this effect, but stronger differences were noticeable in the presence of 13.2 μM BAP. Shooting frequency under this conditions was significantly higher in grafted materials in relation to that observed among the non-grafted materials. Non-significant but slight differences were observed when comparing materials that underwent one or two rounds of grafting (Fig. 3a, b). Shoot length was also measured in these cultures and exhibited a positive trend respect to the number of grafting rounds. Strong statistically significant differences ($\alpha=0.05$) occurred between non-grafted and micrografted explants evaluated at the doses of dicamba $\geq 4.5 \mu\text{M}$.

Comparison of traits among juvenile, hydroponic, and successively grafted plants

Phenotypic differences were observed in the adventitious shoots derived from micrografted and hydroponic twig materials, namely, longer primary roots and more abundant secondary roots were observed in the later. Additionally, a larger leaf size was observed in

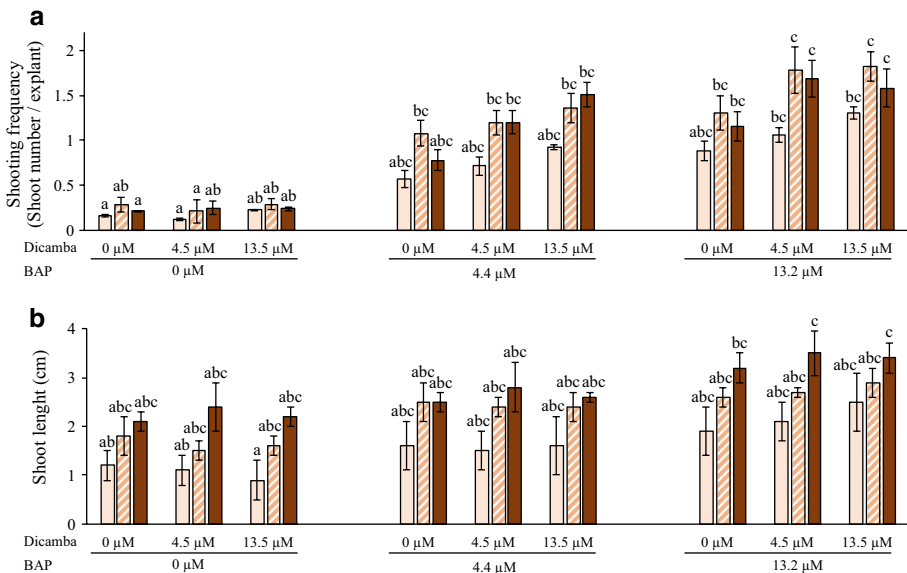


Fig. 3 Organogenic capacity of shoot segments. Adventitious shoot number (**a**) and length (**b**) of shoot stem segments originating from hydroponic twigs (zero rounds of grafting, represented by light brown bars), grafted twigs (one round of grafting, represented by cross-hatched bars) and micrografts (two consecutive rounds of grafting, represented by dark brown bars) cultured on MS medium containing different concentrations of dicamba and BAP were measured. Error bars correspond to standard deviations. Letters above bars indicate significant differences by LSD tests at $P \leq 0.05$; $n = 25$ per repetition

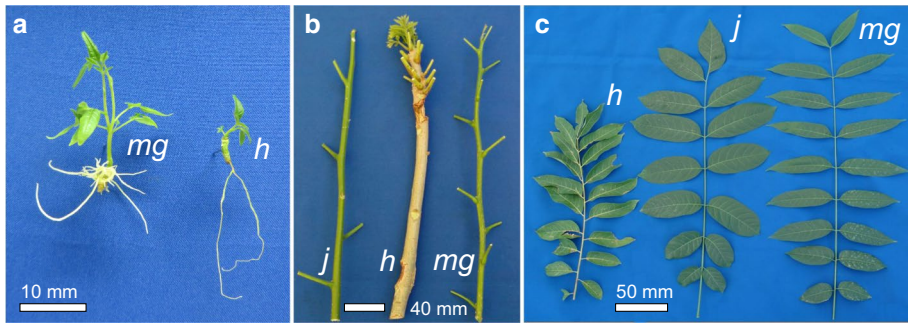


Fig. 4 Qualitative traits of plants derived from micrografts, hydroponic shoots and seeds. **a** In vitro organogenic response of micrografted (mg) and hydroponic-derived (h) stem fragments. **b** Apical shoot development of juvenile (j), hydroponic-derived (h), and micrograft-derived (mg) 3-month-old plants. Leaves were removed from the plants for better visualization of internode distance. **c** Leaves of 3-month-old plants collected from hydroponic-derived (h), juvenile (j) and micrograft-derived trees (mg). Bars in the pictures indicate size

shoots from micrografted stems (Fig. 4a). After micrografted-derived rooted shoots were transferred to the soil ($\bar{x} \geq 90\%$ survival rate), early development of these plants was followed under nursery conditions. As early as three months after being transferred to pots, visual differences began to be notorious when comparing to hydroponic-derived materials, showing a more vigorous and rapid development (Figure S3a). Six months after being transferred to soil, plants derived from micrografts and hydroponic twigs were compared to juvenile trees that developed from seeds. Plant height, internodal distance, leaf number and leaf length were determined (Fig. 5). Micrografted plants showed heights similar to those of juvenile plants (Figure S3b, S3c), in contrast to the slower-growing hydroponic-derived control materials (Fig. 5a). The internodal distance and stem phenotype of plants derived from the micrografted material were very similar to those of juvenile trees. In both cases, rapid vegetative growth was characterized by long internodes (approximately 5 cm) and thin green stems. In contrast to material from micrografts and juvenile trees, hydroponic twigs produced proliferating shoots with shorter internode distances, a more rapidly endured cortex, and thick stems with very limited vegetative elongation (Figs. 4b, 5b). The leaves of juvenile and micrograft-derived plants were also very similar in number, size, form and texture, with both being dark green and soft. Hydroponic twig-derived plants, in contrast, showed smaller leaves that were pale green and more lignified (Figs. 4c, 5c, d, S3b). Finally, micrograft-derived plants were transferred to soil in an experimental field next to seed-derived plants (Figure S3d).

Discussion

The efficient clonal propagation of superior *C. odorata* trees represents a promising but underutilized tool for capturing and rapidly propagating interesting germplasm. The low frequency of class 1 elite trees available for capturing germplasm indicates the need for efforts to develop clonal propagative methods suitable for application to adult individuals. In this work, we reported that only 8 of 32 trees received the highest rating when qualified according to the previously reported ideotype (Solís-Guillén et al. 2017); although

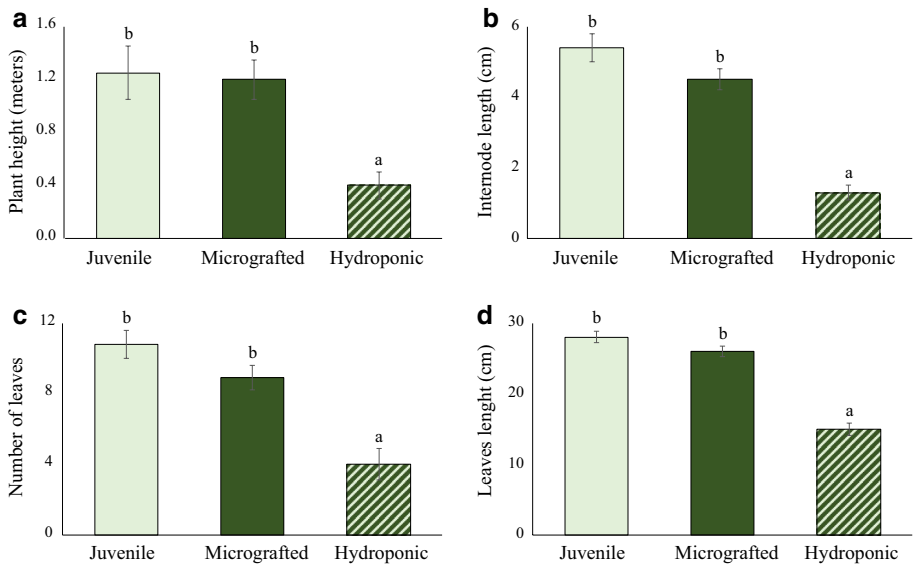


Fig. 5 Quantitative traits of plants derived from micrografts, hydroponic shoots and seeds. Plant height (a), internode length (b), number of leaves (c), and leaf length (d) of 3-month-old plants derived from micrografted shoots, hydroponic twigs and juvenile seed-derived plants were measured. Error bars represent standard deviations. Letters above bars indicate significant differences by LSD tests at $P \leq 0.05$; $n = 25$ per repetition

this number appears to be high, the 32 trees analyzed were preselected by land owners as class-1 trees (CONAFOR 2016), neglecting the much more greater number of class 2, 3 and 4 individuals (Solís-Guillén et al. 2017). To conserve this scarce and valuable type of individuals through clonal propagation, several authors recognize rejuvenation strategies, such as grafting, as a prerequisite for adult clonal propagation (Franclet et al. 1987; Pierik 1990). In this work, we evaluated the effects of successive grafting rounds combined with BAP and dicamba treatments on the organogenic capacity of explants and the presence of noticeable juvenility-associated traits under in vitro and nursery conditions. Our results show that grafting and micrografting in treatments with the highest BAP and dicamba concentrations were able to increase the rate of adventitious shoot formation. BAP positive effect was significant in the two concentrations evaluated showing statistical differences among treatments, dicamba effect however was very slight, appearing to reach a saturation level at 4.5 μM , contrasting to previous experiments performed by our group (Peña-Ramírez et al. 2010), where was demonstrated that high doses (13.5 μM) showed a stronger effect on adventitious shoot induction in the same kind of explant; however, those experiments were performed using an undetermined medium containing coconut water, thus, the plant growth regulators balance could be the source of variation. For shoot elongation, both BAP and dicamba showed a weaker effect respect explants in media without plant growth regulators, being in this case dicamba the growth regulator with the highest effect, this finding is consistent to classic auxin effect of cell elongation. Adventitious shoot frequency and particularly shoot elongation values in explants derived from micrografted shoots resulted significantly higher than hydroponic-derived ones and closer to values obtained from juvenile explants (Juarez-Gómez 2013). Moreover, ex vitro leaf size and internode distance in micrografted-derived plants show morphometric values closer to

juvenile plants. After 1 year in the field, plants did not show any sign of flower emergence in contrast to early flowering characteristic of this species when adult twigs are established in soil (Juarez-Gómez 2013), suggesting that at least, a partially rejuvenated stage might have been reached for these materials.

Juvenility trait induction by phase reversion through serial grafting has been previously described for several species (reviewed by Wendling et al. 2014). However, successive rounds of grafting are long-term efforts that require up to 1 year between grafting cycles (Moon et al. 2008). Micrografting techniques, can be an alternative to reduce this periods, and allowing controlled conditions to perform more detailed studies of the rejuvenation phenomena at physiological or molecular level. Juvenility induction in mature material has been attributed to a microlocalized mode of action for inducing juvenility via plant growth regulators (Liu et al. 2018). This suggests that phase change requires a particular balance and distribution of plant growth regulators among specific cell layers in meristematic tissues, a condition that might be achieved when juvenile cells from rootstocks act as “nurse tissue”, providing appropriate plant growth regulators to scion tissues or even at individual scion meristematic cells. This effect also involve the participation of epigenetic shifts mediated by siRNA (Chuck and Connor 2010; Molnar et al. 2010; Chen et al. 2013). Molecular evidence indicates that the migration of siRNA, proteins and other putative regulatory molecules from rootstocks to scions, are able to activate key targets, such as triglyceride lipase, which is involved in jasmonic acid biosynthesis and root growth retardation (Chen et al. 2013), evidence suggest that translocation can occur through symplastic routes established after graft functional establishment. Recent experiments show that the differential expression of miR156 and miR172 are correlated with juvenility traits in woody species such as *Eucalyptus* spp. and *Acacia* spp. However, changes in phase conversion from juvenile to adult are subtle; *ergo*, hard to detect even by following these particular siRNAs (Wang et al. 2011), and may be assumed that reversed-phase transition (rejuvenation) occurs in the same way, presenting subtle changes at molecular level. Here relies the relevance of the establishment of protocols for micrografting in vitro, where highly controlled experiments can be carried out.

Our data show that *C. odorata* phase reversion is a process in which juvenility-related traits are partially present in micro-grafted shoots. Partial rejuvenation is suggested by the shooting frequency, which was not as high in micrografted plants as that obtained when juvenile material was evaluated (Juarez-Gómez 2013). Nonetheless, successive rounds of grafting appeared to promote shoot length (Fig. 3b, S3a, S3b, S3c) and rooting ability (a higher number of roots and increased biomass) (Fig. 4a). Micrograft-derived plants maintained juvenility-resembling-traits 6 months after establishment in soil, suggesting that at least partial juvenility persisted during the ex vitro acclimatization phase, which may be indicative of an epigenetic shift maintained by somatic mechanisms. Shoot appearance, internode length, plant height, leaf length and leaf number showed values closer to those of juvenile control trees and were significantly different from those of nongrafted adult-derived plants. For other perennial species, such as Hazelnut (*Corylus avellana*) Chesnut (*Castanea sativa*) and Grape (*Vitis vinifera*), the loss of juvenility traits after in vitro culture, including early flowering, and the loss of tendril formation in the case of *Vitis vinifera*, has been reported (Nas et al. 2002). In our case, flowering was still absent on micrografted plants after 2 years of field culture, possibly due to the juvenile rootstock effect through siRNA or plant growth regulation control that may trigger epigenetic somatic memory (Smulders and de Klerk 2011). Further work addressing changes in micrografts in vitro at molecular level, *vg*, in DNA methylation; siRNA expression or translocation; plant growth regulator shifts and their respective responses in reciprocal grafting experiments (young

scion over adult rootstock), would provide additional evidence of the occurrence and duration of phase change in this valuable species, providing a reliable tool for the rescue of unique superior individuals.

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Compliance with ethical standards

Conflict of interest The authors state that they do not have any conflicts of interest.

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