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Germination and Clonal Propagation of the Endemic Shrub *Corema album*, a Vulnerable Species with Conservation Needs and Commercial Interest

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In this study, we aimed to explore regeneration possibilities of *Corema album* (L.) D. Don by determining germination mechanisms and testing vegetative propagation methods. We analyzed seed viability under natural conditions, carried out germination treatments and a greenhouse experiment to study clonal propagation. We confirmed that *C. album* seeds present physiological dormancy, broken by ingestion by natural dispersers (rabbits and foxes), and that seed viability under natural conditions is lost after one year. *In vitro* germination was better achieved with a 200 ppm gibberellic acid treatment. Clonal propagation proved to be a successful technique for the production of *C. album*. Treating cuttings with IBA 0.2, w/v, at 20% resulted in the highest rooting percentage, while planting rooted cuttings in a substrate of perlite with vermiculite 1:1 was essential for plant survival. Our results show that both germination pretreatments and cutting propagation are powerful tools for the production of this valuable species. Both methods could be incorporated for population regeneration in natural habitats, and for the potential establishment of the species as a new crop for consumption and pharmacological purposes.

Keywords: *Corema album*, Clonal propagation, Endangered species, *Ex vitro* rooting, Germination treatment, Gibberellic acid, Seed dormancy, Seed viability.

Corema album (L.) D. Don (*Ericaceae* subfam. *Ericoideae* tribu *Empetrae*) is a dioecious woody shrub endemic to the Atlantic coast of the Iberian Peninsula, commonly known as Camarina or Camarinha. *C. album* is an important species in sand dune ecosystems, being the dominant species in the areas where it grows. *C. album*'s branches and fruits have traditionally played a useful ecological role not only in landscape conservation but also economically for local communities both in Spain and Portugal, due to its use for fuel or for commercialization of its edible fruits [1]. *C. album* berries were traditionally used in popular medicine as an antipyretic [1, 2] and recent studies have discovered important pharmacological properties of its fruits and leaves [3-5]. According to León-González *et al.* [3], extracts from *C. album* berries and leaves are rich in hydroxycinnamic acids and contain different amounts of flavonoids and stilbenes; they also found that human colon cells pre-treated with *C. album* fruit and leaf extracts showed an outstanding protection against challenge-induced damage. These findings support the traditional use of *C. album* as a medicinal plant. Recently, the species has been proposed as a suitable crop for berry production for the food industry [6].

Several factors make the natural regeneration of *C. album* difficult. Seeds show low germination under natural conditions and are endozoocorous, presenting physiological dormancy, which is broken after consumption by vertebrates like seagulls [7, 8], rabbits, and foxes [7, 9-11]. *C. album* seedling mortality rate in natural conditions is high, reaching 99% during the summer season [10]. Moreover, the habitat of *C. album* is dwindling with its populations being fragmented [9, 10]. Sand dune ecosystems along the distribution area of the species are largely affected by the expansion of tourist resorts and other anthropogenic-origin disturbance, such as large-scale plantations of pine trees and *Retama monosperma* [12], and the invasive shrub *Acacia longiforme* [13]. *C. album* has consequently been classified as a vulnerable species because of habitat loss and is included in the regional Red List of threatened vascular plants in Andalusia, Spain [14].

Facing the ongoing habitat loss and disturbance in *C. album* communities, regeneration under natural conditions is really low, both in the northern [7, 10] and southern limits of its biogeographical distribution area [9, 10, 15]. Under this scenario it is necessary to gain knowledge on the mechanisms underlying *C. album* propagation, not only for maintaining and regenerating degraded *C. album* populations, but also for future feasible agricultural and pharmacological use [3-6]. So far there is very limited information on the germination responses of *C. album* [16] and, to our knowledge, no clonal propagation techniques have been described for this endemic and vulnerable species.

Clonal propagation by hardening off cuttings has been described for species related to *C. album* such as the American endemism *Ceratiola ericoides* [17], but studies involving *Ericaceae* propagation in the Iberian Peninsula or Mediterranean species are scarce [18]. In cuttings propagation, several phytohormones and growth factors such as indolebutyric acid (IBA) are described as effective rooting triggers [19]. The success of clonal propagation also depends on appropriate temperature, humidity, and substrate selection, which can be specific for each species, cutting type, season, and propagation system [20]. Various methods can be used for cuttings propagation of shrub species, but the one needed for *C. album* has not yet been described.

In this study we aimed to assess germination and clonal propagation requirements for *C. album* and to determine which regeneration constraints underlie its poor recruitment under natural conditions. We analyzed dormancy breaking and seed germination by means of different *in vitro* treatments (physical and physiological). We further evaluated the effectiveness of clonal propagation by cuttings hardening procedures in *C. album*, testing different over the counter growth regulators treatments and substrates. Our specific objectives were to: 1) establish a method to promote germination; 2) determine seed viability under natural conditions; and 3) evaluate the effectiveness of auxin treatments and different substrates on *C. album* clonal propagation under greenhouse conditions.

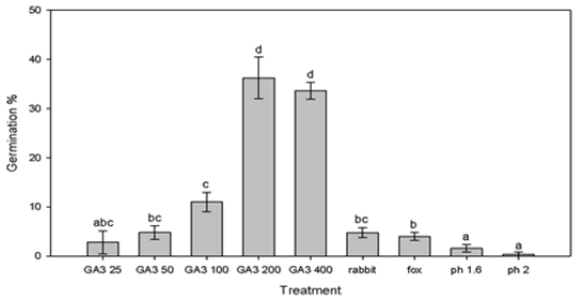

Figure 1: Total germination rates (%) for the different treatments. Treatments not included in the figure did not show any germinated seed (control, badger excrement, temperature shock, and scarification). Lower case indicates significant post-hoc Tukey test results.

We hypothesized that although this species can produce more than 40,000 seeds per plant every year [1,9,10], seed viability in the field is low and that clonal propagation is a feasible alternative procedure to produce *C. album* plants. Results can be relevant to establish a management protocol to foster the regeneration of *C. album* sand dune communities, as well as for its eventual commercial production for pharmaceutical and agronomic purposes.

Seed germination experiments: Gibberellic acid (GA_3) pretreatments broke dormancy successfully, reaching values near 40% of germinated seeds pretreated with 200 ppm and 400 ppm (Fig.1). Germination also occurred in seeds dispersed by rabbits (4.7%) and to a lower extent by foxes (4%), but not in seeds from badger excrements (ANOVA, F = 93.69; $P < 0.001$, Figure 1). pH treatments resulted in germination percentages below 2%, and the rest of the treatments (control, scarification, smoke, and temperature stratification) showed no germination (Figure 1).

Germination dynamics were similar for seeds from rabbit pellets and for all GA_3 treatments (29-30 days), with the exception of GA_3 25 ppm, which showed a longer time for t_0 (45 days). The time of first germination (t_0) was longer for seeds from fox excrements and for seeds under the pH treatments (63 and 129 days, respectively, Table 1).

Table 1: Germination parameters for the different treatments: t_0, t_{50}, and percentage of total germination. Treatments that are not shown in the table did not present any germination (control, pH 1.6, pH 2, smoke, scarification, and badger excrements).

Treatment	t_0	t_{50}	t_{total}	t_{mean}
GA_3 25 ppm	45	57	67	50.5
GA_3 50 ppm	29	67	69	59.5
GA_3 100 ppm	29	54	71	54.6
GA_3 200 ppm	29	48	71	51.8
GA_3 400 ppm	29	48	71	49.4
Rabbit	30	35	59	47.9
Fox	63	67	71	66.5
pH 1.6	128	131	143	137.6
pH 2	131	131	144	136.3

Viability test and seed bank experiment: The tetrazolium viability test showed that seeds extracted from mature fruits presented an initial viability close to 52% (Fig. 2). Seeds from rabbit pellets reached 46.5% of viability. Seed viability decreased significantly along the study period, with seeds from bags unburied after 8 months presenting 26% of viability, thus a 50% viability loss during that period (Chi-square=10, df=1, $P<0.01$); after 16 months, almost all seeds from buried bags were non-viable (96%), and presented signs of desiccation (Chi-square=40.3, df=1, $P< 0.001$, Figure 2).

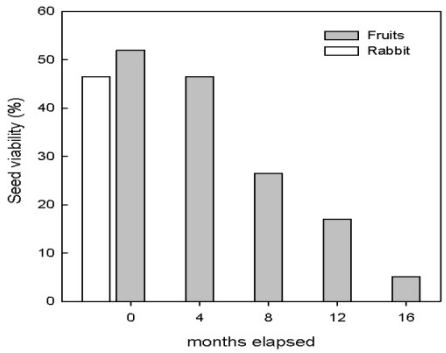

Figure 2: Seed viability from TZ test (%) for seeds extracted from fruits (initial), seeds from rabbit excrements (white bar), and seeds extracted from buried bags along the study period.

Clonal propagation experiment: Both the treatment and the substrate used for clonal propagation had a significant effect on the success of root production (Figure 3). The type of substrate strongly determined rooting success; PV substrate showed a higher percentage of rooted cuttings in relation to SP (Chi-square = 138.059, df = 1, $P < 0.001$). Hormonal treatments had a significantly different effect on root production (Chi-square = 15.847, df = 4, $P < 0.01$); IBA both at 0.2 and 0.4 %, w/v, was the treatment inducing higher root production, followed by Inabarplant, and control cuttings (Figure 4).

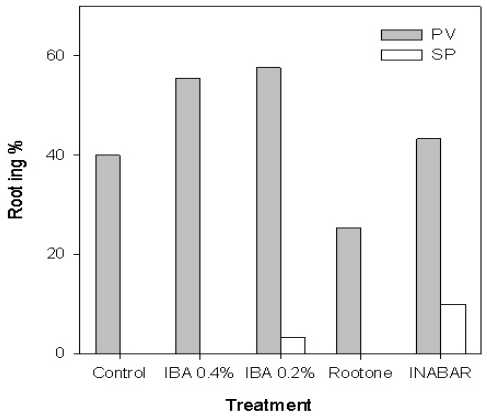

Figure 3: Total percentage of rooted cuttings for each treatment and substrate (PV perlite:vermiculite; SP sand:peat).

There were no significant differences in root production between cuttings from male and female plants (Chi-square = 0.218, P = 0.691). The most successful treatment was the combination of PV substrate with IBA treatment 0.2 and IBA 0.4%, w/v, with percentages of cuttings survival rates close to 60% (Chi-square = 17.161, df = 4, $P < 0.01$, Figure 3).

Within the surviving rooted cuttings, root number and length also differed between substrates, with cuttings planted in PV showing significantly higher number of roots than in the SP substrate (Mann Whitney U, Z = 11.78, df = 1, $P < 0.001$, Figure 4). Root number also varied among treatments (Chi-square = 15.16, df = 4, $P <$ 0.0044), with both IBA treatments showing the highest number of roots. Treatment also affected the total root length produced per cutting (ANOVA, F = 3.7, P < 0.01); roots were longer in control cuttings and in cuttings treated with Rootone (Figure 4).

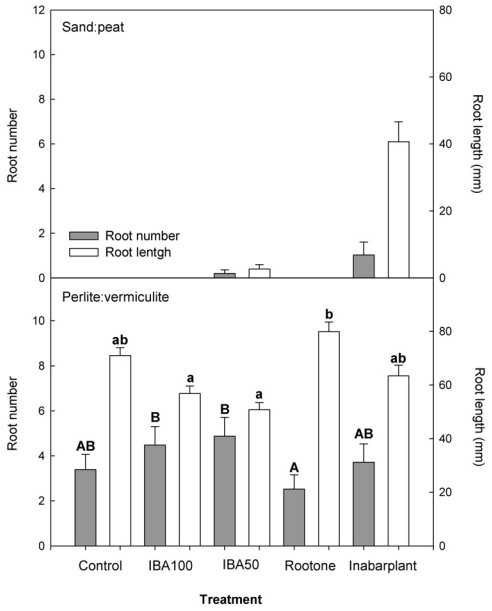

Figure 4: Number of roots per cutting and root length (mean ± standard error) per substrate (Perlite:vermiculite and sand:peat) and treatment. Gray bars represent root number and white bars root length. Significant post-hoc Tukey test results are shown for root length (lower case) and root number (upper case).

Plant survival three months after the transplant reached the highest value with the IBA treatment, with 52% survival, followed by control cuttings with 41%; while the treatment with Rootone had the lowest values (26%) (Chi-square = 16.55, $P < 0.01$). After three months, vegetative growth was similar for all treatments, reaching an average length of 23.5 ± 1.8 mm of new shoots produced per plant (Chi-square = 6.785, $P = 0.148$, Figure 5).

Germination constraints under natural conditions: We confirmed that *C. album* seeds present physiological dormancy [13, 16], which under natural conditions is broken by ingestion by natural dispersers such as rabbits and foxes (Figure 1). Fedriani and Delibes [13] described foxes and rabbits to be the most common dispersers of *C. album* seeds in the southern area of *C. album* distribution, the same as our study site, while Calviño-Cancela [10] described both rabbits and seagulls to be important but unspecialized dispersers. Our study corroborate these results, showing that rabbits and foxes are also much more efficient in breaking *C. album* seeds dormancy in relation to badgers, which did not increase germination percentages. We observed that the pattern of fruit digestion of the three dispersers differed significantly; seeds recovered from badger's droppings were surrounded by fruit pulp after digestion, which may reduce the potential digestion effects on seed dormancy release.

Viability tests in the field demonstrated that after 16 months *C. album* does not present a viable seed bank in the study site. These results would partly explain the lower population density in the southern area of the *C. album* distribution range, which has been attributed to lower precipitation and more severe drought during summers [15]. We found a strong decrease in seed viability within a year, with values under 5% of viable seeds after 16 months, which also presented clear signs of desiccation (Figure 2).

Our results reinforce the notion that *C. album* is an obliged endozoocorous species and unspecialized vertebrate dispersers such as rabbits and foxes play a key role in *C. album* regeneration in its

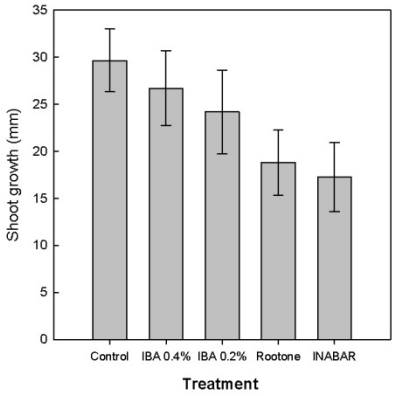

Figure 5: Shoot growth (mm) for the surviving plants from each treatment in the Perlite:Sand substrate (3 months after cutting planting).

southernmost distribution area, increasing germination by over 5%. This is in accordance with the study of Fedriani and Delibes [13], where rabbits were found to produce 6.7% of emerged seedlings in relation to no germination in control seeds.

Gibberellic acid pretreatment was the most successful method to germinate seeds of *C. album.* We found that GA_3 both at 200 and 400 ppm significantly increased germination in *C. album* seeds, reaching 45% of the total in relation to the other experimental treatments, which presented very low germination results (lower than 5% for pH treatments), and to control seeds, with no germination (Figure 1). Taking into account that under natural conditions the viability of seeds from fresh fruits is close to 50% (Figure 2), we can conclude that GA_3 treatment had an effect of almost 100% germination success. This treatment allowed us to produce seedlings in a relatively short period (2 months), and these we transplanted and grew in the greenhouse, proving that it is a powerful method that could be used for production of *C. album* plants for conservation and commercialization purposes. This method would be particularly useful when a genetically varied pool of individuals is needed, for example for transplants in a regeneration program or conservation of natural areas where *C. album* is the dominant shrub species.

GA_3 has been previously used for conservation purposes in endangered species [19, 21], and has been proposed as a successful method for *in vitro* production. In our study, the combined use of growth regulators in agar proved to be a key factor in the germination success. The same method was proposed by Rossini *et al.* [22] for the endemic shrub *Erica andevalensis* (Ericaceae). Our results also corroborate those of Santos *et al.* [16], who recently described hormonal methods as a means of germinating seeds from *C. album* from populations across the coast of Portugal. Nevertheless, our results differ from those of Santos *et al*. [16], who treated seeds with a combination of GA_3 at 1000 ppm and low pH, obtaining lower germination rates (30.3%) in a much longer period (175 days). Thus, we can conclude that treating seeds with 200 ppm GA_3 in agar medium is a successful method to promote germination of *C. album*.

Clonal propagation by cuttings hardening was a successful method to produce *C. album* plants. Both the substrate and the growth regulator treatment used in the cuttings hardening process had a significant effect on the rooting success and later cuttings survival.

The substrate of combined perlite and vermiculite (PV), that we choose for having been successful in the related American species

Ceratiola ericoides, was essential in the success of the experiment. Cuttings in PV showed an overall survival of 44% in comparison with cuttings grown in sand and peat (SP), with only 2% of surviving cuttings at the end of the experiment.

We also found that the treatment with IBA growth regulator was essential for the success in *C. album* plant production. Cuttings treated with IBA increased survival up to 60% in relation to controls, which resulted in only 40% of surviving plants (Figure 3). The surviving cuttings showed different degrees of root length and number; control and IBA- produced cuttings were the ones showing higher root number in relation to Rootone treated cuttings (Figure 4). Thus, we can conclude that the best treatment to produce long-living plants of *C. album* is to plant cuttings without the bottom leaves, treated with IBA 0.2, w/v, at 20% in a PV substrate.

In spite of the interest of producing *C. album* as a cultivar and the species vulnerable status due to habitat fragmentation, there have been very few attempts to develop a protocol of *ex situ* regeneration. We provide results for successful methods for the production of *C. album,* including both germination and clonal reproduction, which we believe could be useful tools in the conservation of this endemic species. Our results also showed that *C. album* seed germination under natural conditions is limited by both the seeds physiological dormancy and the lack of a viable seed bank. These results corroborate that *C. album* natural regeneration strongly depends on natural dispersers.

Together with habitat loss due to human activities, the ongoing global change projections of higher drought intensity and frequency in *C. album* distribution area threaten to constrain the populations of this vulnerable species even further [15]. The results in this study could constitute the methodological basis for protocols for the production of this valuable species. The production of *C. album* could be essential both for the species regeneration in natural habitats and for the species great potential as a new crop, due to the interest in its fruits for consumption and pharmacological purposes.

Experimental

Seed germination experiment: A population of *C. album* located in Doñana Natural Park (SW Spain) was chosen to collect ripe fruits and vertebrate excrements containing predated fruits in August. *C. album* is the dominant species in the study sand dune shrub community, and the only one that produces large amounts of fleshy fruits during the summer.

Germination treatments were carried out under laboratory conditions to determine factors breaking seed dormancy. *C. album* seeds were extracted from the fruits and excrements and washed carefully from any pulp debris. A disinfection treatment was applied to avoid fungal proliferation in the seeds, consisting of an immersion in NaOCl 1% for 1 min followed by ethanol 70% for 30 sec. After disinfection, seeds were washed thoroughly with distilled water and planted in an 8% agar medium [22] in Petri dishes, applying the following treatments:

1-5) Gibberellic acid treatments. Agar medium with dissolved gibberellic acid (GA_3) to concentrations of 25, 50, 100, 200, and 400 ppm before solidification. GA_3 regulates germination in numerous ways and its effect on germination has been observed in many species [22, 23].
6-8) Natural dispersers. Seeds were extracted from recent excrements from rabbits (*Oryctolagus cuniculus*), foxes (*Vulpes vulpes*), and badger (*Meles meles*), collected from the study site at the same time as fresh fruits. Seeds that presented predation signs were discarded.
9-10) pH treatments. We treated seeds with different concentrations of hydrochloric acid at pH 1.6 (similar to fox stomach pH), and pH 2 (similar to rabbit gut pH, [25]) during 10 min. Once treated, seeds were planted in the agar medium.
11) Smoke treatments: seeds were stored in a closed plastic container (10 x 10 cm) attached to a smoker device (used for apiculture) containing leaves and wood from shrubs from the *C. album* plant community (*Halimium halimifolium, H. commutatum, Corema album, Cistus salvifolium*, and *Stauracanthus genistoides*). We kept seeds in the smoke for 1 h before planting.
12) Cold stratification: seeds were kept at 4ºC for one month prior to planting (following [26]).
13) Scarification with sandpaper. Seeds were carefully sandpapered until the seed's woody endocarp was perforated.
14) Control. Seeds were extracted from ripe fruits and washed with distilled water before planting in the agar medium.

We prepared 5 Petri dishes per treatment, each with 50 seeds (5 replicates per treatment, 50 seeds, 14 treatments). Seeds were placed in a germination chamber with temperature-controlled conditions with a cycle of 12 h of light at 24ºC and 12 h in dark at 15ºC. We checked seeds periodically for germination during 4 months, daily during the first month, and weekly during the rest of the experiment. We considered seeds with an emerging radicle as germinated and removed them from the Petri dishes.

Germination values for each individual Petri dish (N = 5) were calculated. We then calculated the time of initial germination (t_0), time when 50% germination was reached (t_{50}), and final percentage of germination for each treatment. The parameter t_0 is useful to study germination dynamics when the germination rate is low [22].

Seed viability analysis: We tested seed viability by means of the tetrazolium test (TZ). The test was carried out in control seeds from the same collected fruits used for germination experiments and in the seeds extracted from rabbit pellets. In addition, to assess the existence of an active seed bank of *C. album* in natural conditions, seeds were placed inside mesh bags in the field (100 seeds per bag). We prepared 12 plastic mesh bags where the seeds were placed, and we buried them at a 15 cm depth in the study site. Three bags were collected every 4 months (month 4, 8, 12, 16) and seeds tested for viability. Seeds were cut longitudinally with a scalpel, and treated with a 1% solution of 2, 3, 5 tripiphenyl tetrazolium chloride (TZ) for 24 h at room temperature [26]. We visually evaluated the embryos under a dissecting microscope; dried or un-colored embryos were considered as non-viable and red-colored embryos as viable.

Clonal propagation experiment: In order to assess the possibility of producing this long-lived species for regeneration of natural populations, forest restoration and commercial purposes, we carried out a clonal propagation experiment under greenhouse conditions. In *C. album* both sexes present significant reproductive allocation differences that may alter vegetative growth [24]; thus, we collected shoots cuttings in the field site from 10 male and 10 female individuals (approx. 40 shoots per plant). Cuttings were obtained from mature plants in January, when the reproductive season had not yet started. We carefully selected yearly shoots after the previous node in the stem to ensure shoot age homogeneity; this is possible as in *C. album* each year's flowering leaves a node in the stem. Cuttings were kept watered in plastic containers until arrival at the laboratory. We then recut cuttings to a length of 9 cm, washed them with distilled water, and removed the leaves from the bottom 4

cm of each cutting. One set of cuttings was used as controls and the other set was treated with 5 different growth regulator treatments from commercially distributed agricultural components:

1) and 2) Liquid solution of indolebutyric acid (IBA) at 0.2 and 0.4%, w/v (Exuberone ®, Bayer).
3) Rooting powder growth regulator mix of 0.056%, w/w, IBA, 0.032%, naphthaleneacetic acid (NAA), and 0.078%, w/w, alpha naphthaleneacetamide (Rootone, Compo ®).
4) Rooting dust growth regulator mix of IBA 0.1% and NAA 0.1, w/w (Inabarplant, INABAR®).
5) Control shoots without any treatment but with leaf removal.
For the liquid treatment IBA 0.2 and IBA 0.4%, the base of each cutting was submerged in the solution for 10 s, and then left to dry before planting. For the powder treatments (Rootone and Inabarplant) the cuttings were previously soaked in distilled water and then covered with the powder.

We planted all shoots into 2 substrates, perlite with vermiculite 1:1 (hereafter PV) and sand with peat 1:1 (hereafter SP); these substrates were chosen for being the most successful in related *Ericaceae* species such as *Ceratiola ericoides* [17]. We prepared 3 cuttings per individual, treatment, and substrate (N = 600, 3 shoots x 10 individual x 2 sexes x 5 treatments x 2 substrates).

Cuttings were planted in 5 cm wide and 12 cm high pots (3 cuttings per pot). Trays were kept in the shade in an air-conditioned controlled-temperature greenhouse (22^{o}C) with high RH (minimum 60%), and were watered daily. Additionally, pots were watered every 15 days with the systemic fungicide polioxine-B 2%, w/v, to avoid proliferation of pathogenic fungi in the roots. Pots were relocated every week to avoid site-specific effects. After 3 months we checked for signs of rooting in each cutting and counted and measured all main roots when present.

Cutting survival was calculated as the total cuttings that rooted and survived until the end of the experiment per treatment. Dead or non-rooted cuttings were discarded.

To assess the success of each treatment, we planted the rooted cuttings in 10 cm wide pots containing a mix of sand from the natural distribution area with commercial peat in a 1:1 proportion, following Thetford *et al*. [17]. We placed the hardened cuttings in a greenhouse under natural environmental conditions; pots were relocated every week. Three months after planting, we calculated plant survival and final vegetative growth as the accumulated length of vegetative shoots produced after the last node in the stem [24].

Statistical analyses: We analyzed germination treatment effects by an ANOVA for the effect of treatment on the final germination percentage; we applied a Tukey post-hoc test to analyze significant differences between the different treatments.

We tested the effect of treatment on cutting propagation success with a Chi-square to analyze the percentage of rooted cuttings per treatment and substrate; we also tested for sex effects to discard any differences in rooting and growth between cuttings from male and female plants; g-tests were used to assess differences among treatments when necessary. Root number per cutting was analyzed by a Kruskal-Wallis non-parametric test for independent groups. Total root length produced per cutting was analyzed by a nested ANOVA with treatment and substrate as fixed factors and individual plant origin of the cuttings as within subject factor; we used a post-hoc Tukey test to compare pairwise differences. Final growth of plants obtained from the hardened cuttings was analyzed by a nested ANOVA with treatment as fixed factor and plant origin of cuttings as within subjects factor. We log-transformed the variables root length and vegetative growth to fit normality assumptions. We calculated the proportion of surviving plants from hardened cuttings as the plants that survived the transplant after 3 months and analyzed survival of plants with a Chi-square test.

All statistical analyses were performed with SPPS 17 software package (Chicago, IL, USA) and statistica 6 StatSoft (Tulsa, OK, USA).

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