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First Ex Situ In Vitro Propagation Protocol of *Coronilla viminalis* Salisb., An Endangered Fodder Species Adapted to Drought and Salinity

Santiago Sierra [1], Carles Cortés-Olmos [1], Claudia Pallotti [1], Adrián Rodríguez-Burruezo [1], Benito Pineda [2,*] and Ana Fita [1,*]

[1] Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain; carcorol@upv.edu.es (C.C.-O.); adrodbur@doctor.upv.es (A.R.-B.)

[2] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de València, Carrer de l'Enginyer Fausto Elio, s/n, 46011 Valencia, Spain

* Correspondence: bpineda@btc.upv.es (B.P.); anfifer@btc.upv.es (A.F.)

Abstract: *Coronilla viminalis* Salisb. is a fodder leguminous plant from the Canary Islands and Northwestern Africa with adaptation to drought. Its conservation status is critical. Its low germination capacity and intense loss of viability of the seeds over time complicate its conservation, limiting its long-term maintenance in germplasm banks and hampering its potential use as a resilient fodder crop. Therefore, in this work, two alternative propagation methods have been addressed. The first was based on facilitating seed germination under aseptic conditions, supplementing the media with gibberellic acid (GA_3). The latter consisted of creating a micropropagation method from nodal segments and testing different media and pretreatments with indole-3-acetic acid (IAA). The quantity and quality of the roots were assessed, and the plant acclimation rate was measured. The results showed that the optimum concentration of GA_3 for germination, up to 98%, was 250 PPM. The experiments showed that using IAA in the micropropagation media is critical for in vitro rooting in this species. A hormonal pretreatment with IAA significantly improved the rooting efficiency compared to supplementing it into the culture media. One hundred percent of acclimated plants survived the process. These new protocols will help conserve the species and explore its possibilities as fodder crops.

Keywords: in vitro culture; micropropagation; conservation; germination; rooting; IAA induction; resilient plants; red list

Citation: Sierra, S.; Cortés-Olmos, C.; Pallotti, C.; Rodríguez-Burruezo, A.; Pineda, B.; Fita, A. First Ex Situ In Vitro Propagation Protocol of *Coronilla viminalis* Salisb., An Endangered Fodder Species Adapted to Drought and Salinity. *Horticulturae* **2024**, *10*, 201. https://doi.org/10.3390/horticulturae10030201

Academic Editors: Vasiliy A. Chokheli and Rupesh Kumar Singh

Received: 31 December 2023
Revised: 9 February 2024
Accepted: 16 February 2024
Published: 21 February 2024

1. Introduction

Coronilla viminalis Salisb. is a forage legume native to the Canary Islands and Morocco. It grows in cracks and ledges on cliffs, in poor or underdeveloped soil, forming part of rocky communities; it is generally observed in halophilic environments [1]. This legume is very appreciated by wild animals, and it is actively sought as food by rabbits and Moorish squirrels during the dry season, probably because of its high protein content [1–3]. Moreover, in *C. viminalis*, the contents of the essential elements, such as phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), and nitrogen (N), are within suitable ranges for its use as fodder for ruminant feeding [1,4]. Therefore, it shows great potential as a forage plant due to its ability to thrive in adverse climatic conditions and its good nutritional values, making it a suitable candidate for cultivation in arid and saline areas.

Nevertheless, the development of this species as a fodder crop is hampered by the lack of commercial material and, especially, by its status as a critically endangered species, CR B2ab (iii, iv, v) according to the IUCN codes [1,5]. *C. viminalis* is listed in the Spanish Red List of endangered species, as it faces an extremely high risk of extinction in the wild, with a

severely fragmented and limited geographic distribution and low population numbers [1]. Although the species can also be found in Morocco (no available data on its conservation state), the Canary Island populations are scarce, with only one population on the island of Lanzarote and eight populations in Fuerteventura, ranging from 90 to 21 individuals. This small population size is attributed to low germination rates, harsh climatic conditions, loss of habitat, and animal browsing [1].

Conservation efforts on *C. viminalis* have focused on its inclusion in protected areas on the island of Fuerteventura. However, to our knowledge, there are no known efforts to multiply the populations. In previous works, it was challenging to germinate the seeds, limiting the potential of seed storage as a way of conservation. In vitro culture has demonstrated high value for plant germplasm conservation and micropropagation [6]. Propagation protocols for *C. viminalis* could be used to reintroduce the species in protected areas and develop it as a potential fodder plant adapted to arid and harsh conditions. Unfortunately, no such propagation protocols have been described to date. In the literature, it is possible only to find some tissue-culture approaches for other family species (i.e., *Coronilla varia* L.) for which a protocol for somatic embryogenesis was developed [7,8]. In those trials, the auxin IAA was present in all protocols. It showed a significantly better response than its absence for initiating embryonic development and inhibiting malformations of embryos derived from calluses. Our group tested the propagation in previous experiments and found particular difficulties in maintaining the explants and rooting for *C. varia* and *C. viminalis* [9]. Similarly, there are no suitable protocols for the efficient germination of seeds showing a certain dormancy level.

Considering the potential of *C. viminalis* as fodder species, the lack of large stocks of plant material, and the necessity of its conservation due to its endangered status, the objectives of this research were: (i) find a suitable protocol for efficient seed germination and (ii) establish a protocol for micropropagation and acclimation of the species.

2. Materials and Methods

2.1. Plant Material

The original plant material was a progeny of accessions stored at the germplasm bank 'Cesar Gomez Campos' and multiplied by Prof. Enrique Sanchis, who kindly provided them for the experiments. The set of plant material for the experiments consisted of (a) 12-year-old seeds derived from the original plant material collected in an expedition to Morocco and propagated by Prof. Enrique Sanchis, (b) two three-year-old plants that were grown in greenhouse conditions, and (c) 4 cm axenic plants containing two axillary buds grown under in vitro conditions that were already incorporated in tissue-culture conditions in previous experiments [9].

2.2. Germination Experiments

Two germination experiments were designed with dual objectives in mind. First, the aim was to preserve the species' genetic diversity, mainly focusing on populations with few individuals and addressing the challenge of poor germination. Second, given *C. viminalis*' potential as a fodder plant, it is imperative to establish an effective germination protocol for its cultivation. In the germination experiments, seeds underwent surface disinfection for six minutes using a 30% volume-to-volume solution of domestic bleach (5.4% sodium hypochlorite) supplemented with the surfactant Tween-20 (Sigma-Aldrich, Burlington, MA, USA). Subsequently, the seeds were rinsed three times for five minutes each with sterile distilled water.

In a first germination experiment (Exp #1), the seeds were germinated into 15 sterile plastic disposable Petri dishes with a sterile filter paper soaked with 5 mL of different filter-sterilized solutions consisting of (a) distilled water, (b) 250 PPM of gibberellic acid (GA_3), (c) 500 PPM of GA_3 (Duchefa Biochemie, Haarlem, The Netherlands) [10–12]. Fifty seeds were used per treatment, distributed in five replicates, and kept in the dark for

42 days at 25 °C. Germination was measured on days 1, 3, 5, 11, 18, 25, and 42 as the average number of germinated seeds obtained per condition.

A second experiment (Exp #2) was performed the same way as germination experiment 1, including two new concentrations of GA_3 to get a more precise gradient. The concentrations used were 125 PPM, 250 PPM, 375 PPM, and 500 PPM. Fifty seeds were used per treatment and distributed in five replicates for 250 seeds. Germination rates were measured on days 1, 4, 5, 11, 18, 25, and 42. The germination times were chosen explicitly in both experiments to meticulously track the initial 18 days of the experiment, corresponding to the expected peak germination capacity of the seeds within this timeframe. Concurrently, the other two time frames were selected to appraise any residual germination.

2.3. Propagation Media Development

After previous micropropagation attempts, it was found that a basal media without hormones was suitable for maintaining the plants' growth, but they were unable to root [9]. To find a suitable culture media for rooting, a first experiment was set using established, but not rooted, explants derived from mature greenhouse plants stored in basal media (from now on called CV basal) consisting of Murashige and Skoog basal medium (Duchefa Biochemie, Haarlem, The Netherlands) 2.2 g/L, sucrose 10 g/L (Sigma-Aldrich, St. Louis, MO, USA) and European bacteriological agar (Duchefa Biochemie, Haarlem, The Netherlands), pH 5.7. CV basal media was subjected to 5 modifications for this experiment, as shown in Table 1. The explants, characterized by cuttings approximately 4 cm in length and each containing two axillary buds, were sourced from the younger branches of the greenhouse plants. All explants underwent a six-minute surface disinfection, employing a 30% volume-to-volume solution of household bleach (containing 5.4% sodium hypochlorite) supplemented with Tween-20 surfactant (Sigma-Aldrich, St. Louis, MO, USA). The explants were subjected to three consecutive five-minute rinses using sterile distilled water. All explants were cultivated under in vitro conditions and kept in a growth chamber at 26 ± 2 °C, 16 h day length, and photosynthetic photon flux of 50 $molm^{-2}$ s^{-1} for eight months.

Table 1. Rooting media development experimental design.

Treatment	Concentration	Repetitions	Explants × Treatment
Control	n/a	4	16
Indole-3-butyric acid *	1 mg/L	4	16
Indole-3-acetic acid *	1 mg/L	4	16
Half sucrose	5 g/L	4	16
Activated charcoal	6 g/L	4	16
Activated charcoal + IBA	6 g/L + 1 mg/L	4	16

* This variation of basal media will be called CV rooting media. IBA = indole-3-butiric acid.

The rationale behind selecting these treatments was as follows: IAA and IBA, being naturally occurring auxins, have been widely reported to induce rooting in various other plant species [13,14]. The utilization of activated charcoal was motivated by its reported capacity to enhance rooting in different plant species, attributed to its role as an antioxidation agent [15]. The combination of both IBA and activated charcoal was employed to investigate whether a distinct response could be observed compared to the individual treatments. Lastly, incorporating a half-sucrose medium was implemented to reduce the osmotic stress experienced by the tissue [16].

2.4. Indole-3-Acetic Acid Induction to Improve Rooting

In the literature, auxin pretreatments have enhanced rooting efficiency in explants containing axillary buds. However, their duration and intensity (measured by the hormone concentration in the pretreatment) vary depending on the study, from shorter but more intense shock to mild induction through a longer timespan [17]. This experiment

raises an exploration of concentrations and exposure times using indole-3-acetic for this species. With the rooting media decided (hereafter referred to as CV rooting media), efforts were made to optimize root induction in *C. viminalis* explants by assessing the effects of four treatments with indoleacetic acid (IAA, Duchefa Biochemie, Haarlem, The Netherlands). This experiment aimed to fine-tune the conditions for root induction, building upon the outcome of the previous experiment and enhancing the impact of IAA on *C. viminalis* explants. Two different explant sources were used for this experiment to test the viability of the protocol: (a) axenic plants grown under in vitro conditions and (b) mature plants grown in the greenhouse. Two concentrations of IAA and four exposition times were used as inductors to promote rooting, in contrast with explants cultivated directly in CV rooting media (positive control) or CV basal media (negative control) (Table 2). The induction was made as follows: the first approach consisted of a shock with a high IAA concentration of 1000 mg/L for 30 or 120 s. In the second approach, a lower concentration of IAA (6 mg/L) was applied for 6 or 24 h using a medium containing IAA at that concentration. The stock solution was prepared at 5000 mg/L and filter-sterilized. The shocks of 30 and 120 s duration were performed by putting individual explants in IAA solution droplets of 100 µL, ensuring that the explant's base was in contact with the IAA solution the whole time. The inductions of 6 h and 24 h were done in a culture media with a composition of distilled water and European Bacteriological Agar (6 g/L, Intron Biotechnology, Seongnam, Republic of Korea) supplemented after autoclaving with sterile IAA stock solution until reaching a concentration of 6 mg/L, again, contacting the base of the explant. After the induction, all induced explants were cultivated in CV rooting media except the negative control.

Table 2. Indole-3-Acetic acid induction experimental design.

Explant Type	IAA Concentration	Exposition Time	Repetitions	Explants per Repetition	Explants per Treatment
In vitro axillary nodes	1000 mg/L	30 s	4	5	20
		2 min	4	5	20
	6 mg/L	6 h	4	5	20
		24 h	4	5	20
	Positive control (1 mg/L IAA)		4	5	20
	Negative control		4	5	20
Mature plant axillary nodes	1000 mg/L	30 s	4	5	20
		2 min	4	5	20
	6 mg/L	6 h	4	5	20
		24 h	4	5	20
	Positive control (1 mg/L IAA)		4	5	20
	Negative control		4	5	20

Rooting data were recorded for 42 days. The number of roots per petri dish was counted on day 42, and the general appearance of the explant was evaluated. Five rooted plants were selected for each treatment, explant source, and induction time to acquire quantitative data from the experiment. Scans of these plantlets were performed (both in grayscale and color) with the program WinRhizo, an image analysis software specifically designed for root measurement (Regent Instruments Inc., Quebec, QC, Canada) to obtain the maximum information about their root length and surface area.

2.5. Acclimation

To determine the survival of the explants for standard cultivation, rooted plantlets from the in vitro culture were rinsed with deionized water to remove any excess medium and then transferred to 80-cell trays containing soil mix composed of 50% peat and 50% vermiculite. Trays were placed in minigreenhouses for 30 days, and the lid was removed gradually for five days before the complete exposition of the plant to the growth-chamber

conditions. The watering schedule was twice a week. The growth chamber was programmed to have a constant temperature of 25 °C and a day–night cycle of 16/8 h. The survival rate was recorded at 15 and 30 days of the acclimation period.

2.6. Statistical Analysis

For analyzing our data sets, we used the Statgraphics Centurion XVIII program (Statgraphics Technologies Inc., The Plains, VA, USA). ANOVA test was performed to check the effect of the different treatments at $p < 0.05$. The significance of the germination rate was tested by a Student's *t*-test, using the critical values for significance at $p < 0.05$. The means differing significantly were compared using the LSD test at the 5% probability level. Transformation with natural logarithm (Ln) was necessary for both length and root area to normalize the dataset.

3. Results

3.1. Germination Experiments

The effects of gibberellic acid in the germination of *C. viminalis* were evaluated by testing its germination with water and GA_3 concentrations of 250 PPM or 500 PPM. Significant differences were found between the treatments for the number of seeds germinated from the fifth day after sowing (DAS) (Figure 1). In particular, the treatment with GA_3 250 PPM showed a higher number of germinated seeds from day 5 to 42. However, no significant differences were found between the control and the treatment with a gibberellic acid at a concentration of 500 PPM during the whole experiment (Supplementary Table S1). (Figure 1).

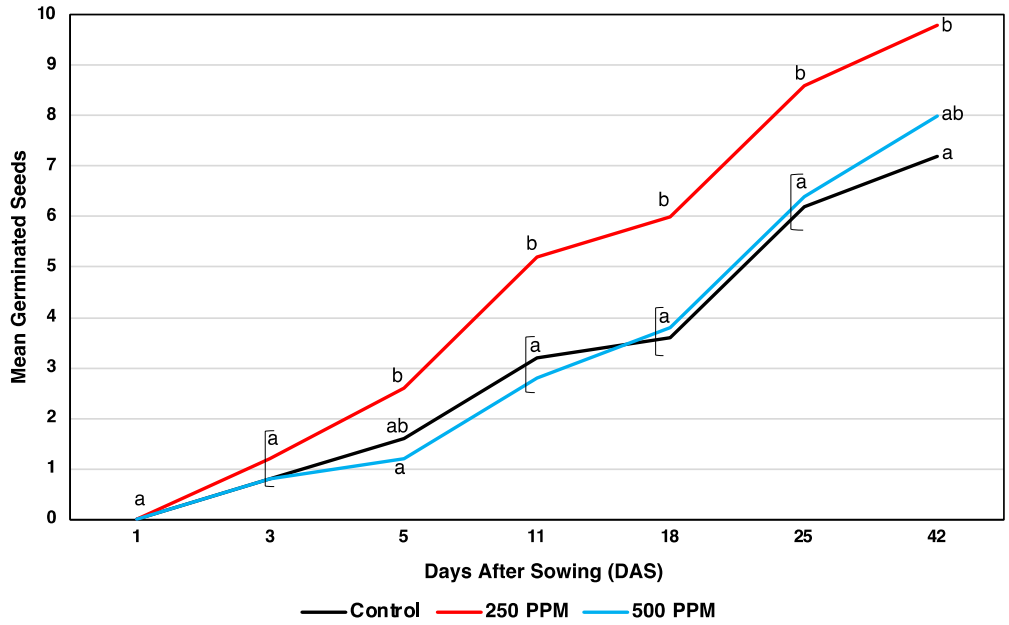

Figure 1. Graphic representation of the mean germinated seeds per plate and per treatment over time in the germination experiment. The same letter between treatments in the same DAS indicates no significant differences according to the LSD post hoc test. Error bars were omitted to obtain a more explicit graph. All data are provided in Supplementary Table S1.

To further adjust the concentration necessary to obtain optimal germination under GA_3, the effects of the gibberellic acid in the germination of *C. viminalis* were evaluated by testing its germination within a calibration-curve design, using concentrations of 125 PPM, 250 PPM, 375 PPM, and 500 PPM. Significant differences were found between the treatments (*p*-Value = 0.004, at a 95% confidence level). A further analysis using an LSD test showed three different homogeneous groups. Treatment with GA_3 250 PPM differed significantly and was the best compared to the others at the end of the experiment (day 42).

Significant differences were also found between the control and the treatment with 125 PPM of GA_3. In comparison, no significant differences were found between the treatments with a GA_3 concentration of 375 PPM and 500 PPM compared with the control (Supplementary Table S2) (Figure 2).

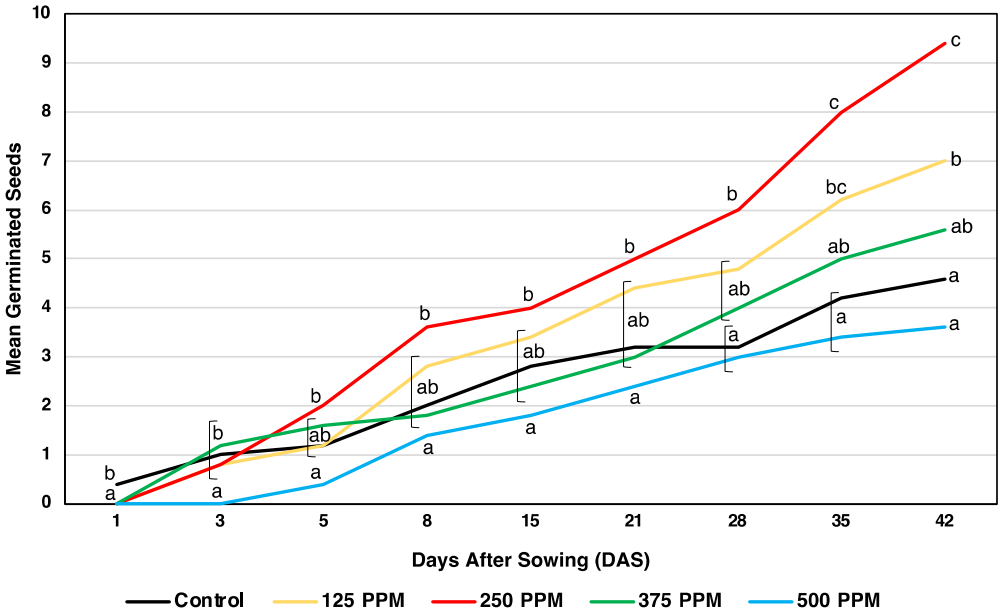

Figure 2. Graphic representation of the mean germinated seeds by plate and per treatments over time in germination experiment 2. The same letter between treatments in the same DAS indicates no significant differences according to the LSD post hoc test. Error bars were omitted to obtain a more explicit graph. All data are provided in Supplementary Table S2.

3.2. Propagation Media Development

In previous experiments [9] the foundation of the protocol was established with a soft disinfection of the plant materials followed by in vitro maintenance in basal media without hormones (see Section 2). However, those in vitro axenic plants were unable to root, showing in some cases a high oxidation rate and/or some unproductive callus. Thus, the effects of the different treatments in the rooting of nodal explants were evaluated by different supplements, such as (1) activated charcoal (6 g/L), (2) ½ sucrose (5 g/L), (3) indole-3-butyric acid (IBA) (1 mg/L) + activated charcoal (6 g/L), (4) IBA (1 mg/L), and (5) IAA (1 mg/L), that were incorporated into the media. For this experiment, the propagation process started from mature plants. Significant differences (p-Value 0.0286, at a 95% confidence level) regarding the rooting percentage were found between the treatments (Figure 3). The control, one-half sucrose, activated charcoal, and combined IBA + activated charcoal treatments showed no rooting response (Figure 3). Treatment with IAA 1 mg/L, with a rooting percentage of 25%, was the best treatment and significantly different from the others. The IBA treatment showed a moderate response of in 10% of rooted explants.

3.3. Indole-3-Acetic Acid Treatments to Optimize Rooting

To optimize *C. viminalis* rooting, four induction treatments plus two controls (positive and negative) were used. No rooting events were detected for negative control conditions, so these data were not included in the subsequent analyses to avoid bias in the results. In this case, two different explant sources were used: (a) axenic plants grown under in vitro conditions from the previous experiments and (b) mature plants grown in the greenhouse.

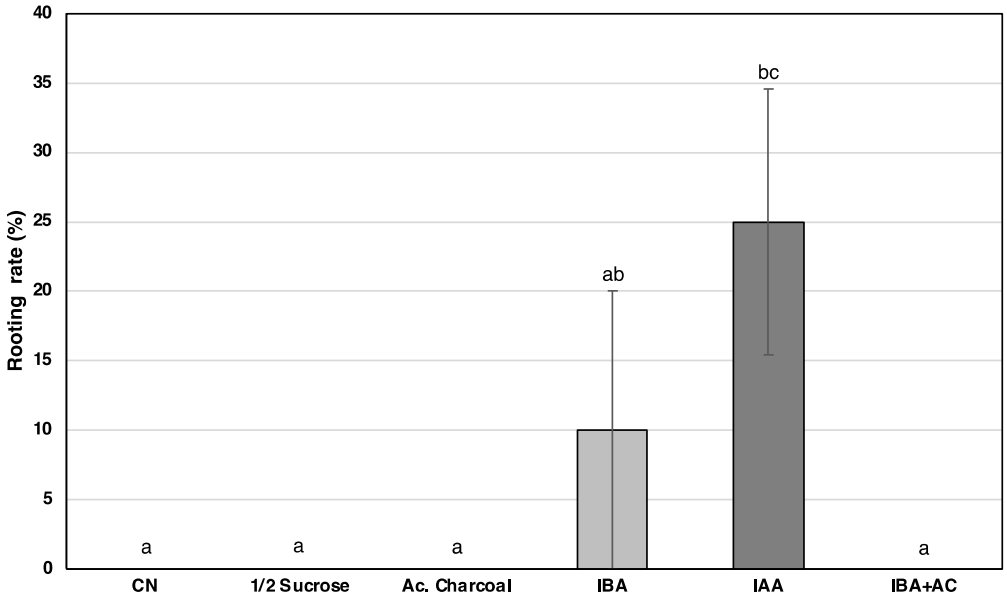

Figure 3. Graphic representation of the difference in rooting percentage between treatments in the exploratory rooting experiment. CN = control group, AC = activated charcoal. The same letter between treatments indicates no significant differences according to the LSD post hoc test.

The statistical analysis of the results showed the effect of the treatments on the rooting response (Figure 4). Treatments of 30 s shock, 2 min shock, and 24 h induction significantly differed from the positive control in the case of in vitro explants. In contrast, for the greenhouse explants, the treatments of 30 s of shock and 6 h of induction were different from the positive control group (Figure 3, LSD test). The optimum treatment was the 30 s shock with IAA at 1 mg/mL for the greenhouse explants with a rooting of 85%, while the 2 min shock was better for the in vitro plants with a rooting of 85%.

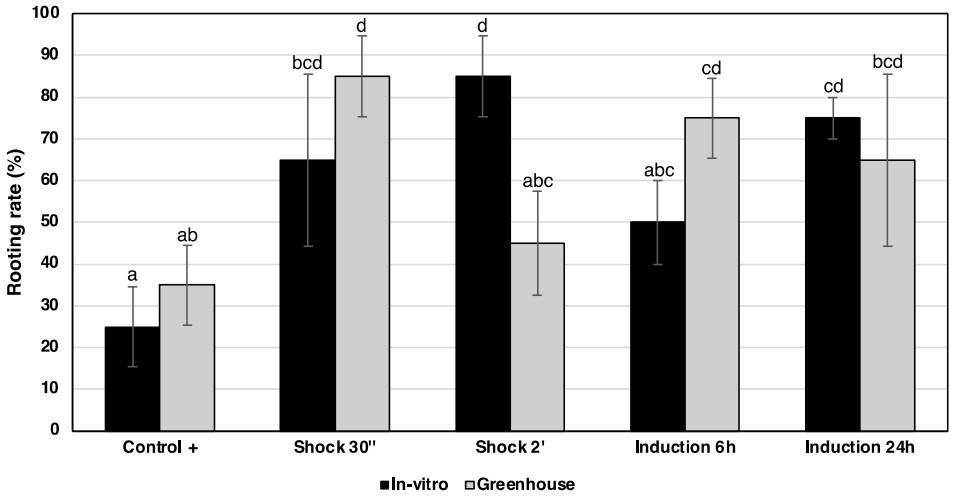

Figure 4. Graphic representation of the difference in mean rooting percentage between treatments and explant types in the IAA induction rooting experiment. Bars with different letters were significantly different according to the LSD test at the 5% probability level.

3.4. Root Measurements Analyses

The effects of the different treatments on the root length and area of *C. viminalis* nodal explants (also considering their origin) were evaluated in the same experiment by scanning each plant's root system (Figure 5A).

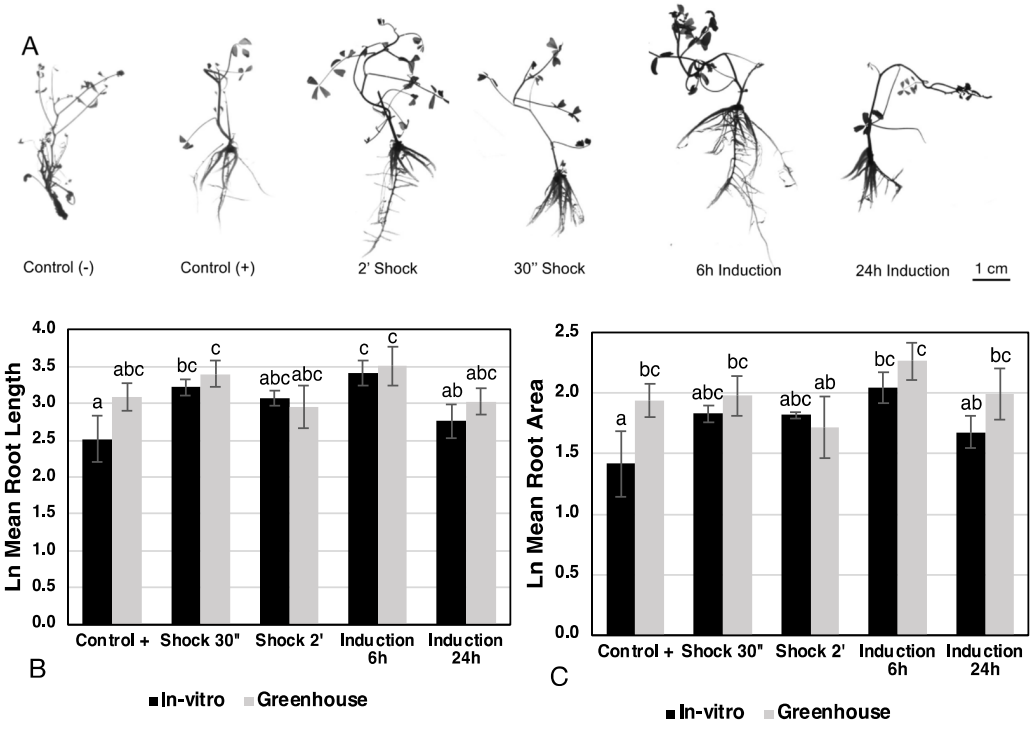

Figure 5. (**A**) Plants scanned from the indole-3-acetic acid treatments experiment. (**B**) Graphic representation of the difference in mean natural logarithm of the root length between treatments and explant types at the IAA induction rooting experiment, (**C**) Graphic representation of the difference in mean natural logarithm of the root area between treatments and explant types at the IAA induction rooting experiment. Bars with different letters were significantly different according to the LSD test at the 5% probability level. All measurements were done in the plants from the indole-3-acetic acid treatments experiment.

Regarding root length, the 6 h induction produced the longest roots for both in vitro and greenhouse-sourced explants (Figure 5B). Also, the 30 s shock in greenhouse explants grew longer than in the control group. Considering root area, the treatment with the best overall results was the 6 h of induction for both in vitro and greenhouse-sourced explants (Figure 5C).

Regarding the micropropagation rate originating from greenhouse-cultivated plants, it has been determined that each plant can yield 40 explants. With the 85% rooting rate obtained in the IAA 30 s shock, an in vitro micropropagation rate of 34 plants per greenhouse plant can be achieved, and those plants gain axenic status. The axenic in vitro plants have an aerial length of, on average, 12.4 $\pm$ 2.6 cm and bear an average of 8.3 $\pm$ 1.1 usable buds (including the terminal bud) within 90 days. In the next round of micropropagation, the two-minute shock treatment with IAA is recommended. This results in the generation of four explants on average from each axenic plant. Subsequent rooting, with an 85% success rate, leads to a micropropagation rate of three plants per round. This establishes a progression that follows the formula Y = I + 3n in subsequent rounds (Y = number of plants in that propagation round, I = initial number of axenic plants, and n = propagation round), potentially yielding three plants per initial axenic plant in the first round, nine plants in the second, 27 plants in the third, and so forth.

3.5. Acclimation

The results from the acclimation experiment were 100% successful after one month of growth in minigreenhouses. All the plants derived from the culture experiment survived the whole month regardless of the treatment and tissue source (Figure 6). However, the plants derived from greenhouse explants showed more vigorous growth (visual observation).

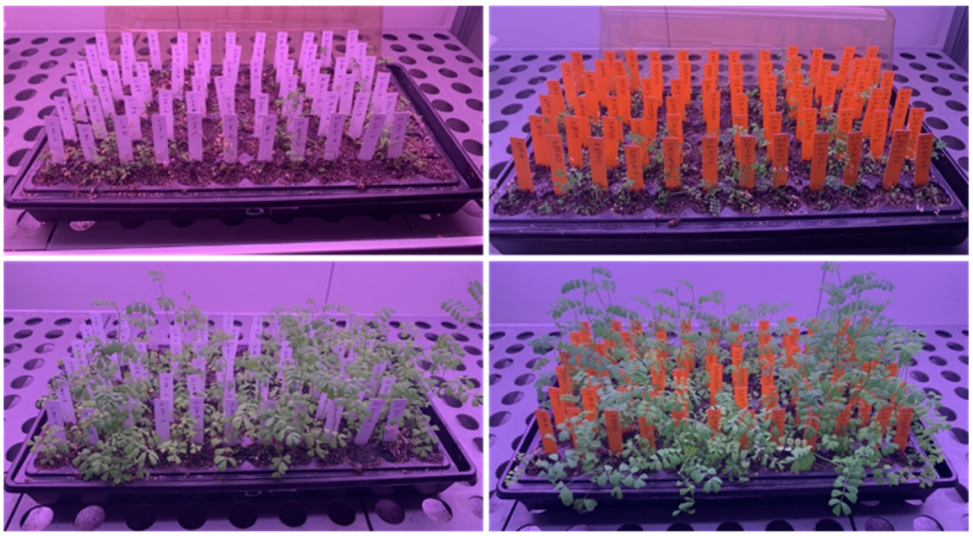

Figure 6. Acclimatation experiment. (**Left up**)—freshly transferred plants from tissue culture to mini greenhouses derived from sourced explants. (**Left Down**)—one-month acclimated plants derived from in vitro-sourced explants. (**Right up**)—freshly transferred plants from tissue culture to mini-greenhouses derived from greenhouse-sourced explants. (**Right down**)—one-month acclimated plants derived from greenhouse-sourced explants.

Also, the growth of the plants derived from the in vitro experiments was monitored for the next two and a half years. Plants resulting from both experiments developed normally during the first six months (Figure 7). Vegetative growth was similar in both groups; however, the plants from the in vitro micropropagation experiment exhibited more growth than the seedlings (Figure 7A,B). After a 6-month growth period, plants derived from the IAA rooting induction experiment continued to show robust and sustained growth.

Figure 7. (**A**) 3 3-month-old plants resulting from the seed germination experiment using gibberellic acid. (**B**) Close-up images of two plants derived from the IAA rooting induction experiment and acclimated for three months. (**C**) Plants derived from the IAA rooting induction experiment acclimated for six months.

After two years, the in vitro-derived plants from the IAA induction experiment displayed blossoming, reaching maturity (Figure 8A,B). A closer examination of the inflorescence of these two-year-old plants showed normal flower development (Figure 8B). This contrasted with the absence of flowering in plants derived from the germination experiment with gibberellic acid (Figure 8C).

Figure 8. (**A**) Flowering two-year-old plants obtained from the IAA rooting induction experiment. (**B**) A close-up image of the inflorescence of these two-year-old plants. (**C**) Plant growth after two years of acclimation of in vitro-derived seedlings from the GA_3 treatment.

4. Discussion

4.1. Gibberellin Treatments for Seed Germination

The development of germination protocols for wild species is hard to achieve. Usually, wild species have low germination rates caused by survival mechanisms such as seed dormancy [18]. Previous efforts for germinating *Coronilla viminalis* have been unsuccessful in obtaining high germination rates. Fabaceae family seeds usually show either physical dormancy due to their impermeable seed coat, or combinational dormancy, combining physical and physiological dormancy [18]. Therefore, in previous experiments with the related species *C. varia*, GA_3, sulfuric acid, and HCl were tested. The best results were obtained using GA_3, contrary to other Fabaceae species, where mechanical abrasion or sulfuric acid improves germination [19,20]. With this information, an experiment to compare different concentrations of GA_3 was set.

GA_3 plays an essential role in seed dormancy, having an antagonistic effect to abscisic acid (ABA), leading the seeds to break dormancy and start germination processes, as it promotes embryo expansion by softening the endosperm and promoting the growth of embryogenic cells [21–23]. The effect of GA_3 regulator in moderate concentrations increases germination, possibly by activating the expression of the α-amylase gene, leading to starch

degradation in the seeds and making available monosaccharides for the embryo [21]. Also, GA_3 can induce the expression of many other genes necessary for enzyme expression, such as proteases and even lipases, which can be essential in embryo development and seed germination [24]. The results showed that soaking seeds in gibberellic acid solutions positively affected germination when used in moderate doses. The germination rate significantly increased when 250 PPM of gibberellic acid was added to the seeds in sterile controlled conditions, reaching more than 90% germination. However, a reduction in the germination rate has been observed when using higher concentrations of GA_3. This could be due to toxicity caused by the increased hormone concentrations leading to a de-regulation of the embryo metabolisms, which has been found in other species [11,25]. For example, in the case of garlic (*Allium sativum*), treatment of 500 ppm gave the lowest percentages of germination and presented a relatively higher rate of abnormality in garlic plantlets [26]. In our experiment, the possible beneficial effect of bleach sterilization, which can have acted as a mild scarification pretreatment, cannot be discarded.

4.2. Auxin Treatments for Shoot Rooting

As growth regulators, auxins play a vital role in a series of complex metabolic routes in plants, interacting with many other elements to control plant growth and development. Probably, the most critical auxin is indole-3-acetic acid (IAA), a phytohormone synthesized inside the tryptophan pathway or hydrolyzing aminoacidic conjugates containing IAA [27]. The second most crucial auxin is the indole-3-butyric acid (IBA), formed by reducing IAA. Both hormones are synthesized in the plant's apical meristem and transported through the whole plant to the root system, stimulating growth. These hormones are inactivated using mechanisms such as conjugation and direct oxidation [27,28]. The literature has widely reported the role of IAA and IBA in both root initiation and root number increase [29]; in the case of adventitious roots derived from an in vitro culture, root formation consists of two stages: primordia initiation after induced damage and root emergence and development. IAA acts in the first stage of the rooting process as a gene activator, promoting the early formation of root cells [27]. At the same time, IBA is supposed to mediate the interaction between endogenous IAA and amino acids, leading to the protein synthesis of root-promoting factors [27]. However, there is no complete understanding of the rooting process since the plant's interactions with the ambient and other growth regulators greatly influence root generation [28].

In the Fabaceae family, generating roots from plant cuttings is problematic since these plants are usually recalcitrant to tissue culture. However, it has been reported in multiple experiments that using IBA and IAA can help overcome this obstacle [29]. In the case of *C. viminalis*, previous efforts showed that results could be hit and miss due to the recalcitrancy of this species, as vegetative propagation cannot be fully achieved due to the lack of explant rooting. After months of growth, in those experiments, explants showed an enlargement at the base, with necrotic tissue surrounding them, which led to the top of the development of the aerial part and its decline. Therefore, the exploration for further improving the culture media was necessary. That is why we planned to promote rooting in *C. viminalis* using auxins in conjunction with activated charcoal to reduce necrosis caused by oxidation [30]. The best results were obtained using IAA at one mg/L with a rooting success of 32%, probably because this auxin is naturally occurring and is the most suitable for this species, deferring from the lacking results of IBA at around 12% rooting. We performed a shock experiment using different concentrations and induction times to improve the rooting rate further. In this sense, it has been reported that a short but intense exposure to auxins could produce improved rooting in the Fabaceae family [14], considering both the treatment and the explant origin. The percentage of rooted explants for *C. viminalis* increased by adding a further treatment with the auxin IAA independently from the explant source. However, slight differences were seen. The best-performing treatment for the explants derived from in vitro plants was the 2 min shock with a 1mg/mL solution of IAA, leading to an 85% rooting. This could be due to the previous exposure of

these explants to exogenous levels of IAA before the initiation of the experiment, leading to epigenetic changes that finally lead to significative desensitization of this tissue to the hormone [31–33]. This can be contrasted with the results obtained for greenhouse-derived explants, in which the optimum treatment was the 30 s shock with a solution of IAA at a concentration of 1mg/mL, accomplishing a rooting efficiency of 80%. This suggests that tissues from the greenhouse, with exposure to only endogenous IAA concentrations, show greater sensitivity to exogenous IAA treatment than explants from in vitro culture, with prior exposure to IAA in the culture media.

4.3. Acclimation

Before the acclimation process, a scan of the roots derived from in vitro culture was done to measure the length and superficial area. This evaluation aimed to assess if those factors affect the survivability of the plantlets after one month of acclimation and to see if significant differences can be seen between treatments and explant origin during the acclimation period. Minimal differences were found, giving the 6 h induction the best root quality for all the measurements and all the explants, but this does not correlate with the rooting performance results. After evaluating the acclimation survival after one month, 100% survivability was achieved for all the treatments and explants(no matter their origin) and the vegetative development through the following two years has gone as expected, implying that the root parameters are not relevant for plant survival for this species after one month of acclimation. However, the plants derived from in vitro micropropagation presented earlier flowering than those germinating from seeds, suggesting a priming effect on the plants induced by the stress in the in vitro culture. This followed similar cases reported in the literature [34–37]. Looking ahead, potential challenges or limitations in acclimating these plants to their arid and saline natural habitat need careful consideration by conservationists or cultivators. Factors such as soil composition, water availability, and salt tolerance may hinder the successful transition from in vitro culture to the challenging external environment.

5. Conclusions

With our protocol, seed germination was successful on seeds between 5 and 10 years old. Using gibberellic acid (GA_3) at a concentration of 250 PPM improved the germination rate of *C. viminalis* compared to the control and higher doses of GA_3, achieving up to 98% germination. Higher doses provoke lower germination efficiency, possibly by a toxicity effect. Our results contrast with previous experiments in which very low germination rates were obtained, from 0 to 13.9% in the best case (unpublished data). Indole-3-acetic acid (IAA) is critical for in vitro rooting in this species compared with other auxins or treatments aimed at reducing polyphenolic oxidation. In addition, hormonal treatment with IAA, shock, or induction significantly improved the rooting efficiency compared to just supplementing it into the culture media.

Once rooted and established under in vitro culture conditions, in a 90-day culture period in a rooting medium, the plants reach an average length of 12.4 cm and contain about six to nine buds, including the terminal bud. In successive cloning rounds (i.e., every 90 days), our protocol would allow us to obtain a micropropagation rate corresponding to three plants from each mother explant.

Historical challenges in propagating *C. viminalis* have hindered conservation efforts. The experimentally proven enhanced rooting efficiency provides a breakthrough, addressing the need for successful propagation. This opens avenues for larger-scale cultivation and reintroduction initiatives crucial for species conservation. Beyond conservation, *C. viminalis*' adaptability to arid and saline conditions holds the potential for sustainable agriculture, offering alternatives to solving problems such as water scarcity and salinity and, thus, could enhance agricultural resilience in arid environments.

The protocols and media presented here can be used to multiply the *C. viminalis* materials to reintroduce plants into the territory. But, it can also generate a large set of plant

materials suitable for cultivating this tolerant species to harsh conditions such as forage. In parallel, studies on the genetic diversity of the remaining populations would be very interesting to preserve and improve this species.

6. Future Perspectives

In mapping out the future perspectives of the research on Coronilla viminalis preservation, the forthcoming work can be focused on adopting a more comprehensive approach, incorporating elements of habitat restoration, in situ conservation, and genetic diversity assessment. In addition, recognizing the potential of alternative ex situ preservation protocols, it will be interesting to explore methodologies such as slow-growth storage and cryopreservation. These innovative techniques can address the challenges associated with the conservation of *Coronilla viminalis*, focusing on providing pragmatic solutions to the complexities inherent in effectively preserving endangered plant species.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10030201/s1, Table S1. Germination Experiment 1; Table S2. Germination Experiment 2.

Author Contributions: Conceptualization, C.C.-O., B.P., C.P., A.R.-B. and A.F.; Methodology, S.S., C.C.-O. and B.P.; investigation, S.S., C.C.-O. and C.P.; resources, A.R.-B., A.F. and C.P.; data curation, S.S. and C.C.-O.; writing-original draft S.S.; writing—review and editing, S.S., C.C.-O., B.P., A.R.-B. and A.F.; visualization, S.S. and C.C.-O.; funding acquisition, A.R.-B. and A.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data will be available upon request to the authors. The data are not publicly available due to the use in other unpublished articles.

Acknowledgments: The authors acknowledge the support of Enrique Sanchis by providing plant materials and a description of the species.

Conflicts of Interest: The authors declare no conflicts of interest.

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