horticulturae

IMPACT FACTOR 3.1

CITESCORE 3.5

Article

The First Protocol for In Vitro Propagation of *Kalanchoe beharensis* Through Adventitious Shoots, a Preliminary Study

Ignacio Moreno-García, Victoria Rodríguez, Begoña García-Sogo, Cristhian Ventura, Vicente Moreno and Benito Pineda

Special Issue
Tissue Culture and Micropropagation Techniques of Horticultural Crops

Edited by
Dr. Tolga Izgü, Dr. Özhan Şimşek, Prof. Dr. Maria Angeles Pedreño and Dr. Haiying Liang

MDPI

https://doi.org/10.3390/horticulturae10121379

horticulturae

MDPI

Article

The First Protocol for In Vitro Propagation of *Kalanchoe beharensis* Through Adventitious Shoots, a Preliminary Study

Ignacio Moreno-García †, Victoria Rodríguez †, Begoña García-Sogo, Cristhian Ventura, Vicente Moreno and Benito Pineda *

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de València, Carrer de l'Enginyer Fausto Elio, s/n, 46011 Valencia, Spain; nachomoreno21@gmail.com (I.M.-G.); victoria260299@hotmail.com (V.R.); btgsogo@gmail.com (B.G.-S.); cristhian.ventura.2014@gmail.com (C.V.); vmoreno@ibmcp.upv.es (V.M.)

* Correspondence: bpineda@btc.upv.es

† These authors contributed equally to this work.

Abstract: *Kalanchoe beharensis*, a vulnerable species according to the International Union for Conservation of Nature, is highly prized for its ornamental value and medicinal properties. Therefore, an efficient methodology to propagate this ecologically significant species would be of particular interest. The propagation of *K. beharensis* has traditionally been achieved by seed or cuttings, but these methods are limited in efficiency. Micropropagation provides a more efficient and controlled alternative by enabling the in vitro production of numerous plants in a small space and in a short period of time. Despite its advantages, no micropropagation protocol for *K. beharensis* has been reported in the literature. In this study, we report an efficient in vitro regeneration protocol for *K. beharensis*. In order to implement this, we evaluated the morphogenetic response of leaf and root explants in media supplemented with auxins, cytokinins, or a combination of both growth regulators. Surprisingly, the best results were observed in indole-3-acetic acid-supplemented media. Adventitious shoots were rooted in either hormone-free or auxin-supplemented media, with indole-3-acetic acid yielding the best results. Rooted plants were acclimatized in the greenhouse, achieving over 80% survival during acclimatization. This protocol improves multiplication rate, space utilization, and uniformity, providing a viable alternative to conventional propagation methods.

Keywords: indole-3-acetic acid (IAA); leaf explants; micropropagation; morphogenesis; root explants

Citation: Moreno-García, I.; Rodríguez, V.; García-Sogo, B.; Ventura, C.; Moreno, V.; Pineda, B. The First Protocol for In Vitro Propagation of *Kalanchoe beharensis* Through Adventitious Shoots, a Preliminary Study. *Horticulturae* **2024**, *10*, 1379. https://doi.org/10.3390/horticulturae10121379

Academic Editors: Tolga Izgü, Özhan Şimşek, Maria Angeles Pedreño and Haiying Liang

Received: 7 November 2024
Revised: 3 December 2024
Accepted: 20 December 2024
Published: 23 December 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The genus *Kalanchoe*, belonging to the Crassulaceae family, is widely recognized for its ornamental appeal and is used in traditional medicine in several countries. These plants, characterized by fleshy leaves and clustered flowers, are popular in gardening and floristry due to their easy cultivation, showy flowers, adaptability to drought, and prolonged flowering period [1]. *Kalanchoe beharensis* is one of the biggest genus members and one of the few kalanchoes that can genuinely be considered arboreal [2]. This species exhibits a remarkable ability to thrive in rugged areas, adapting readily to various soil types in temperate climates [2]. This ability to thrive in arid regions is due to its CAM metabolism. Some species of the genus *Kalanchoe*, such as *K. daigremontiana*, *K. fedtschenkoi*, and *K. tubiflora*, have served as model taxa in the study of the ecological relevance and mechanism of CAM metabolism [3]. However, the first comprehensive field study of CAM involved the use of *K. beharensis* [3,4]. Indeed, this species is able to maintain a full CAM yield throughout a dry season [3]. This ability is based on the maintenance of an adequate water status in the photosynthetically active leaves, even during prolonged drought periods [3].

As mentioned above, plants of the genus *Kalanchoe* are appreciated practically worldwide for their ornamental value. From an ornamental point of view, the cultivation of

K. beharensis has spread throughout various regions of the world in gardens, parks, and pots, especially in temperate zones, since it does not tolerate prolonged frosts [5]. *K. beharensis* is not only used in many areas of the world due to its drought tolerance, but is also distinguished by several characteristics that make it highly valued in the ornamental field [3]. Its large, triangular-shaped, woolly leaves add a visually attractive element to any environment. A thin layer of trichomes gives the leaves a woolly, velvety texture, further increasing their aesthetic interest [3]. The sculptural morphology of *K. beharensis* is also of great ornamental appeal. The plant has a robust habit with thick stems and leaves arranged at steep angles, creating a distinctive silhouette [3]. This sculptural structure allows the plant to stand out in gardens and interior spaces [6], providing a unique and aesthetically pleasing form that enhances the ornamental design.

Plants of the genus *Kalanchoe* are widely used in traditional medicine in several countries, such as India, China, South Africa, and Brazil. These plants are known for their therapeutic properties in the treatment of various conditions, and in the ethnomedicinal field, they have broad therapeutic properties in the treatment of inflammatory conditions, wounds, gastric ulcers, genitourinary disorders, and other diseases [7]. Studies in animal models support the pharmacological properties of some Kalanchoe species, thus validating traditional knowledge. The importance of this genus is reflected in the increasing number of pharmacological studies conducted in the last 30 years [7]. Regarding *K. beharensis*, it has been reported that its essential oils could be helpful in cancer treatments, mainly of the leukemia type, by targeting critical cellular resistance mechanisms [8]. *K. beharensis* essential oils may be an excellent alternative to traditional drugs to treat many different types of cancer, especially in drug resistant cancer [8].

Traditionally, plants of the genus *Kalanchoe* have been propagated for commercial exploitation mainly through seeds, a process that can be slow and can generate genetic variability [9]. Although Kalanchoe species can also be propagated by leaf and stem cuttings, these methods are not sufficiently efficient to satisfy market demand [10]. Traditional propagation methods for *K. beharensis* include seeds, stem segments or cuttings, and cut leaves with intact petiole [3]. Although propagation of this species is uncomplicated, its yield may be insufficient for commercial needs. In this context, other methods, such as micropropagation, would allow for more rapid, economical, and consistent production of plants on a large scale. In the genus *Kalanchoe*, several protocols for in vitro culture of various species based on both axillary and adventitious regeneration have been published (Table 1). In these protocols, different auxins (i.e., NAA, IAA, 2,4D, or IBA) have been used to induce cell division in adventitious regeneration experiments or shoot rooting in axillary regeneration experiments. Regarding cytokinins, only two cytokinins (i.e., 6 BA and TDZ) have been reported to induce adventitious morphogenesis (Table 1).

Table 1. Summary of different micropropagated species of the genus *Kalanchoe* via in vitro culture of vegetative explants.

Species	Explant for Plant Regeneration	Propagation	PGRs [mg/L]	Regeneration Stage	Reference
K. rhombopilosa	Leaf derived from axenic plant	Axillary regeneration	NAA [0.1]	Rooting and shoot multiplication	[10]
K. blossfeldiana	Leaf derived from axenic plant	Adventitious regeneration	NAA [1.0] and BA [2.0]	Organogenesis induction and proliferation	[11]
K. blossfeldiana	Node derived from axenic plant	Axillary regeneration	IAA [0.1]	Rooting	[11]
K. laxiflora	Leaf derived from axenic plant	Adventitious regeneration	IAA [0.4] and TDZ [0.5]	Organogenesis induction	[12]
K. marmorata	Leaf derived from axenic plant	Adventitious regeneration	IAA [0.4] and TDZ [0.5]	Organogenesis induction	[12]

Table 1. *Cont.*

Species	Explant for Plant Regeneration	Propagation	PGRs [mg/L]	Regeneration Stage	Reference
K. peltata	Leaf derived from axenic plant	Adventitious regeneration	IAA [0.4] and BA [0.5]	Organogenesis induction	[12]
K. pinnata	Leaf derived from field grown plants	Adventitious regeneration	2,4 D [1.0] and 6 BA [2.0]	Organogenesis induction	[13]
K. tomentosa	Leaf derived from axenic plant	Adventitious regeneration	2,4 D [0.01]	Direct organogenesis induction	[14]
K. tomentosa	Leaf derived from axenic plant	Adventitious regeneration	2,4 D [0.2–0.5]	Indirect organogenesis induction	[14]
K. tubiflora	Leaf segments derived from axenic plant	Adventitious regeneration	IBA [1.0] and 2,4 D [2.0]	Callus induction	[15]
K. tubiflora	Leaf segments derived from axenic plant	Adventitious regeneration	BA [2.0], Kin [1.0], and IBA [1.0]	Organogenesis induction	[15]
K. tubiflora	Leaf segments derived from axenic plant	Adventitious regeneration	IAA [1.5], BA [1.0], and NAA [0.5]	Rooting	[15]

PGR = plant growth regulator; NAA = naphtalene acetic acid; BA = 6-benzylaminopurine; 2,4 D = 2,4-dichlorophenoxyacetic acid; IAA = indole acetic acid; TDZ = Thidiazuron; IBA = indole butyric acid.

As far as we know, no in vitro regeneration protocol for *K. beharensis* has been reported. The existence of an in vitro regeneration protocol for this species could have significant benefits, mainly due to its medicinal properties and ornamental use. In the same way, in vitro regeneration would ensure a constant and controlled production of plants. In addition, we found that the IUCN (International Union for Conservation of Nature) classifies *K. beharensis* as a vulnerable species (see https://www.iucnredlist.org/species/128093556/128098546 (accessed on 11 June 2024)) in the Red List of Threatened Species inventory. Specifically, this species is threatened by habitat loss due to the expansion of agricultural land and illegal collection in the wild [16]. For all these reasons, an efficient methodology to propagate this ecologically significant species would be of particular interest. This work describes a protocol for the adventitious regeneration of *K. beharensis* through direct organogenesis. This protocol improves multiplication rate, space utilization, and uniformity and may be a viable alternative to conventional propagation methods.

2. Materials and Methods

2.1. Plant Material and Introduction to In Vitro Conditions

Axenic plants of *K. beharensis* were used to perform the experiments. They were implanted in vitro from shoots of a *K. beharensis* plant located in the Universitat Politècnica de València ornamental plants greenhouse. For transfer under in vitro culture conditions, small shoots from the base of a three-year-old *K. beharensis* plant (Supplementary Figure S1) were collected. These shoots were divided into approximately 2–3 cm nodal segments, including at least one axillary bud. The nodal cuttings were first washed thoroughly with water and a 0.1% solution of Tween 20 detergent (Sigma-Aldrich, Burlington, MA, USA). They were then surface sterilized by immersion using a 30% volume-to-volume solution of domestic bleach (5.4% sodium hypochlorite) supplemented with 0.1% Tween 20 detergent (Sigma-Aldrich) for 20 min and rinsed three times with sterile distilled water. Once disinfected, the nodal segments were cultured on basal medium (BM) (Table 2). Axenic plants obtained from nodal segments were propagated every two months in 580 mL culture vessels with 40 mL of BM and maintained as in vitro stock plants. The cultures

were incubated in a climatic chamber at 25 °C under a 16 h/8 h (day/night) photoperiod with fluorescent light (photosynthetic photon flux of 100 μmol m^{-2} s^{-1}).

Table 2. Composition of culture media used for induction, development, and elongation of adventitious shoots from leaf and root explants.

Compounds	BM	IAA	NAA	BA	IAA/BA	NAA/BA
MS *,[1] solutions	100%	100%	100%	100%	100%	100%
Myo-inositol * (mg/L)	100	100	100	100	100	100
Sucrose * (g/L)	20	20	20	20	20	20
SH [2] vitamins	100%	100%	100%	100%	100%	100%
IAA * (mg/L)	-	2	-	-	2	-
NAA * (mg/L)	-	-	2	-	-	2
BA * (mg/L)	-	-	-	2	2	2
European bacteriological agar ** (g/L)	7	7	7	7	7	7

* Duchefa Biochemie, Haarlem, The Netherlands. ** Condalab, Torrejón de Ardoz, Madrid, Spain (batch number: AO1886, CAT. 1800.05). [1] [17]. [2] [18].

2.2. Plant Regeneration from Leaf and Root Explants

(a) Induction of the morphogenetic response

An experiment to evaluate morphogenesis in leaf or root explants was conducted on the following media: BM, IAA, BA, NAA, IAA/BA, and NAA/BA (Table 2). In each medium, 30 explants were used as the starting material. Leaves were excised from 2-month-old axenic plants, cut into 1–2 cm^2 pieces (1 explant per leaf), and cultured in a 90 mm Petri dish (5 explants per dish) containing 20 mL of medium with the abaxial surface in contact with the medium. Roots were also extracted from 2-month-old axenic plants by separating them from the axenic plant using a scalpel and culturing them in 90 mm Petri dishes as clusters of 7–15 roots. In this work, we used a concentration of 2 mg/L of each PGR to induce adventitious regeneration on both leaf and root explants. This is because in a preliminary experiment, we found that this concentration was adequate to induce adventitious regeneration on both leaves and roots based on previous studies in another species of the genus *Kalanchoe* [9]. All cultures were incubated in a climatic chamber, as described above.

In leaf explants, the percentage of explants that developed roots, the length of the longest roots, the number of primary roots, and the number of secondary roots were recorded after 30 days. The percentage of explants that developed shoots was also recorded. For root explants, the percentage of explants that developed roots was recorded after 30 days. Because the few developed roots were small and not very branched, other variables relating to the development of new roots were not evaluated. As in the case of leaf explants, the percentage of explants that developed shoots was recorded.

(b) Shoot elongation

After 30 days of the culture of leaf and root explants in morphogenesis induction media, the explants that had developed adventitious shoots were transferred to BM medium (Table 2) to promote their elongation. The explants were cultured in 380 mL glass containers (5 explants per container) with 40 mL of culture medium. All cultures were incubated in a climatic chamber, as described above. After 30 days, the number of shoots per explant was recorded. For the analysis of the mean number of shoots per explant, the percentage of explants that had developed shoots was considered. Therefore, the mean number of shoots was calculated relative to the total number of explants used in the experiment.

(c) Rooting of adventitious shoots

Individual shoots were excised and transferred to different rooting media: BM, IAA^R, and NAA^R (Table 3). Each elongated shoot was transversally cut for further evaluation.

Shoots were cultured in 580 mL glass jars (4 shoots per glass jar) with 40 mL of culture medium. Approximately 60 shoots were cultured in each culture medium. All cultures were incubated in a climatic chamber, as described above.

Table 3. Composition of culture media used for rooting of shoots.

Compounds	BM	IAA^R	NAA^R
MS *,[1] solutions	100%	100%	100%
Myo-inositol * (mg/L)	100	100	100
Sucrose * (g/L)	20	20	20
SH [2] vitamins	100%	100%	100%
IAA * (mg/L)	-	1	-
NAA * (mg/L)	-	-	1
BA * (mg/L)	-	-	-
European bacteriological agar ** (g/L)	7	7	7

* Duchefa Biochemie, Haarlem, The Netherlands. ** Condalab, Torrejón de Ardoz, Madrid, Spain (batch number: AO1886, CAT. 1800.05). [1] [17]. [2] [18]. R: stands for rooting to differentiate this medium from the culture medium used to grow roots.

2.3. Acclimation of Axenic Plants

The axenic plants rooted in IAA^R medium were acclimatized to determine the survival rate. For this purpose, after removing the plants from the glass jars, they were placed on absorbent paper to eliminate most of the agar from their roots, taking care not to damage them. Subsequently, the roots were carefully rinsed with water and transferred to plastic pots containing coconut fiber. Taking into account the peculiarities of this species (i.e., CAM metabolism), micropropagated plants recently transferred to pots were covered with a plastic cup (24 plants), and the other half were not covered with the cup (24 plants). The plants were transferred to climatic chambers with the phytotron system programmed to maintain a constant temperature of 25 °C and a day/night cycle of 16/8 h. During the acclimatization period, irrigation with water and Hoagland's solution [19] were alternated to keep the substrate hydrated. The survival rate was recorded at 30 days of the acclimation period.

2.4. Statistical Analysis

We used the Statgraphics Centurion XVIII program (Statgraphics Technologies Inc., The Plains, VA, USA) to analyze our data sets. As the obtained data did not have a normal distribution, the Kruskal–Wallis method and Conover pairwise multiple comparison at $p < 0.05$ were used to evaluate the statistical significance of differences.

3. Results

3.1. Morphogenetic Response of Leaf Explants

The development of adventitious shoots had been observed on some roots of 60-day-old axenic plants (Figure 1). For this reason, and to promote root development from leaf explants, we decided to evaluate the morphogenetic response of leaf explants on auxin-supplemented media in our experiments. Likewise, we wanted to evaluate the morphogenetic response in a medium supplemented with cytokinin as well as in media combining auxins and cytokinin. Thus, regeneration of adventitious shoots was assessed on MS medium containing different plant growth regulators (PGRs) combinations (Table 2).

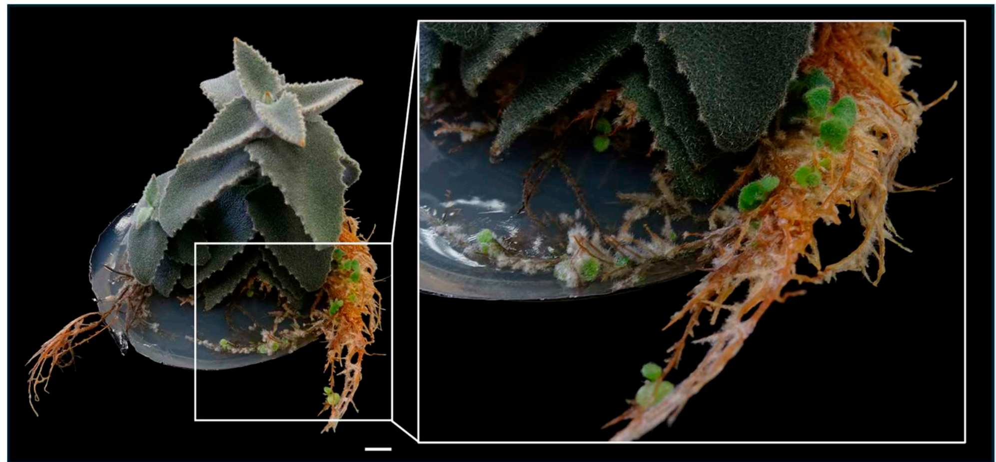

Figure 1. Axenic plant of *K. beharensis* cultured on hormone-free medium (i.e., BM) for 60 days (**left**). Notice the development of adventitious shoots emerging from the roots (**right**). Scale bars: 1 cm.

3.1.1. Root Development from Leaf Explants

Root development occurred in all treatments, although not all explants grown on cytokinin medium developed roots (Figure 2A). The longest roots developed on BM medium, and the shortest were those of explants grown on NAA medium (Figure 2B). The highest number of primary roots developed on NAA medium (Figure 2C), while the highest number of secondary roots developed on IAA medium (Figure 2D).

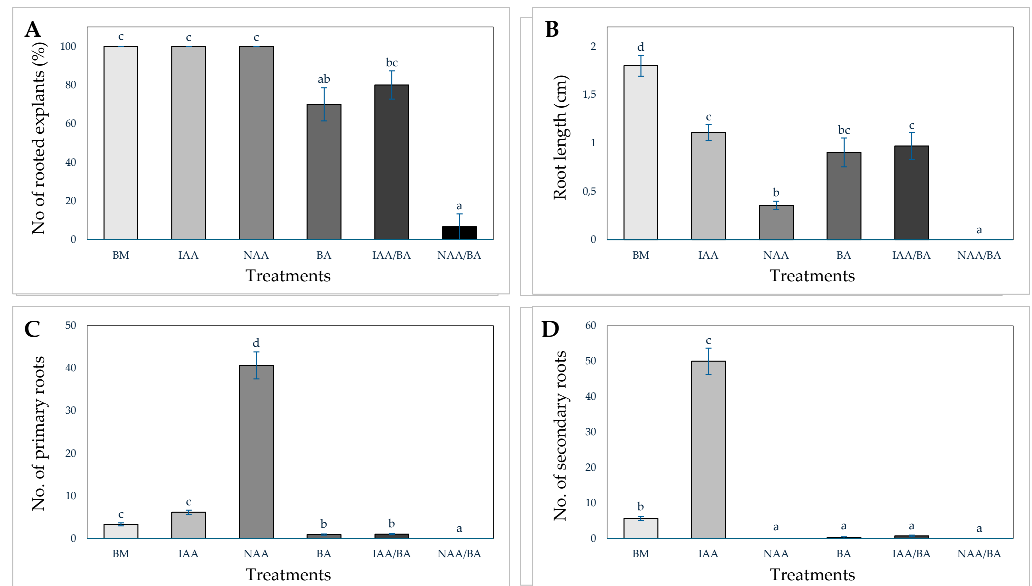

Figure 2. Root development from leaf explants. Results were recorded after 30 days of culture in the different treatments. (**A**) Percentage of explants developing roots. (**B**) Length of developed roots. (**C**) Mean number of primary roots per explant. (**D**) Mean number of secondary roots per explant. Data are means ± SE. Different letters indicate statistically significant differences ($p \leq 0.05$, Kruskal–Wallis test).

3.1.2. Shoot Development from Leaf Explants

After 30 days of culture, we observed that adventitious shoots had developed in all culture media. (Figure 3A). It should be noted that the adventitious shoots were produced through direct organogenesis, which means that there was no callus formation. In the auxin-supplemented media, these adventitious shoots apparently arose from the roots. In contrast, adventitious shoots appeared only in one of the wound zones of the explant

in cytokinin-supplemented media. As can be seen in Figure 3B, the highest number of adventitious shoots per explant developed on the auxin-supplemented media, although there were no significant differences between the medium without PGRs and the medium supplemented with NAA.

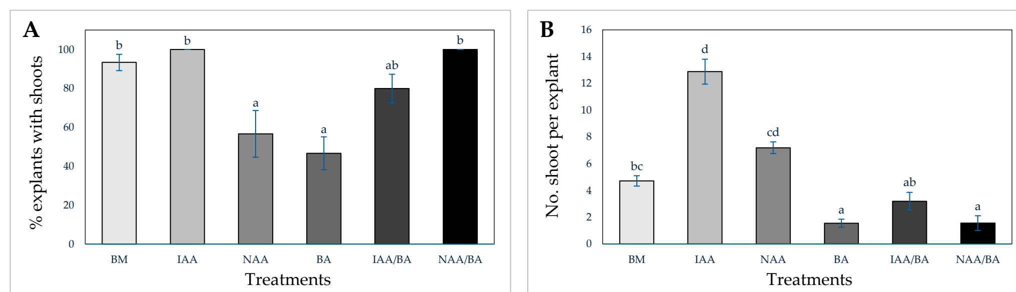

Figure 3. Shoot development from leaf explants. Results were recorded after 30 days of culture in the different treatments. (**A**) Percentage of explants that develop shoots. (**B**) Number of shoots per explant. Data are means ± SE. Different letters indicate statistically significant differences ($p \leq 0.05$, Kruskal–Wallis test).

To promote the elongation of adventitious shoots, leaf explants grown on the different culture media were transferred to hormone-free medium (i.e., BM medium). After 30 days of culture on the hormone-free medium, the number of shoots developed per explant was evaluated. As can be seen in Figures 3B and 4, explants originating from IAA and NAA media showed the best performance in terms of shoot production, significantly outperforming the shoot development on the other culture media. The results indicate that the media supplemented with auxins, where more roots are formed, produced a higher number of shoots. In contrast, media supplemented with cytokinin did not generate sufficient root development to give rise to quality shoots. In this sense, the adventitious buds that developed in the proximal region of the explant (i.e., the one closest to the petiole) did not give rise to shoots after transferring the explants to hormone-free medium (Figure 4, Supplementary Figure S2).

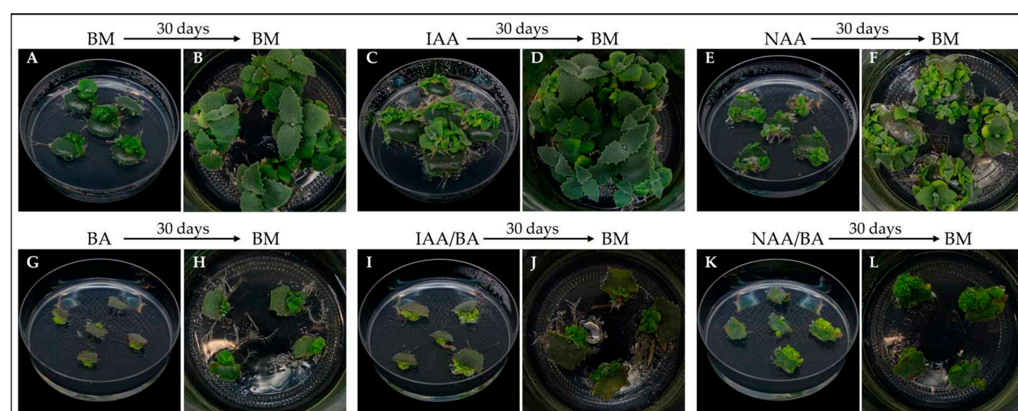

Figure 4. Root and bud induction from leaves after 30 days of culture on BM, IAA, NAA, BA, IAA/BA, and NAA/BA media (**A**,**C**,**E**,**G**,**I**,**K**), and shoot development after 30 days of culture on hormone-free medium (i.e., BM medium) (**B**,**D**,**F**,**H**,**J**,**L**).

3.2. Morphogenetic Response of Root Explants

We used the same culture media tested in the experiment with leaf explants to regenerate adventitious shoots from root explants. As in the experiment with leaf explants, we evaluated the development of new roots and the formation of morphogenetic structures in each tested media. The experiment was performed by culturing the explants in 7–15 root clusters.

3.2.1. Development of New Roots from Root Explants

New root development was not as profuse as in the experiment with leaf explants. In particular, in the media where a response was observed, these were small roots with limited branching (Figure 5).

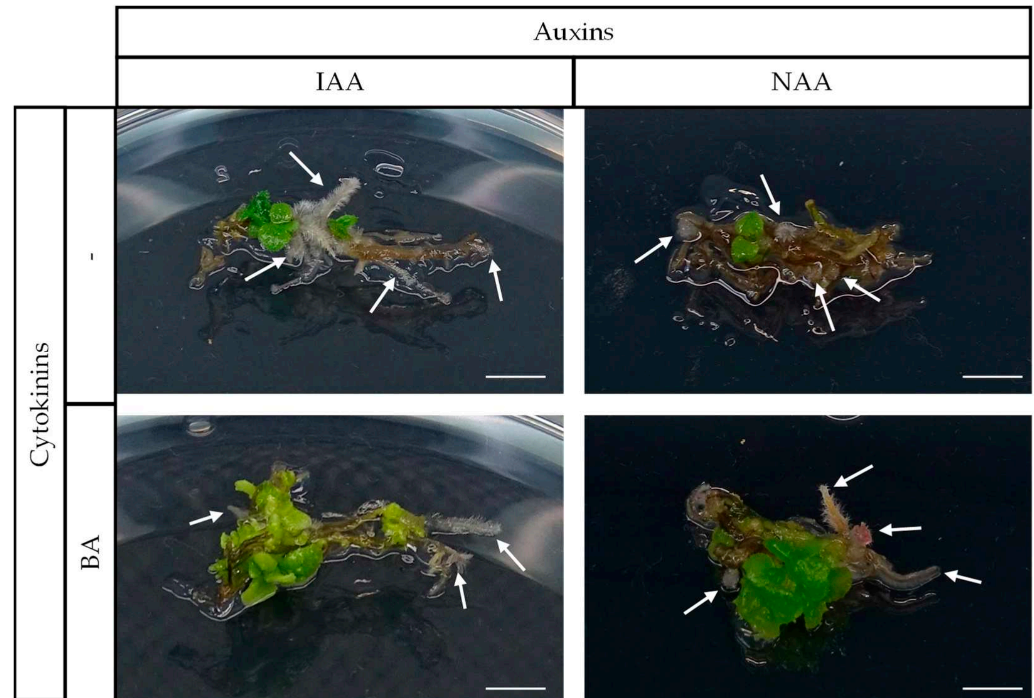

Figure 5. New root development from root clusters after 30 days of culture on IAA, NAA, IAA/BA, and NAA/BA media. Notice that there was very little new root development. Arrows indicate new root development. Scale bars: 0.5 cm.

As expected, media supplemented only with auxins exhibited the best response (Figure 6). New root development also occurred on media supplemented with BA and NAA, or IAA, with the NAA/BA medium exhibiting the best response (Figure 6). However, in the medium supplemented only with cytokinins, no new root development occurred (Figure 6). The results suggest that auxin supplementation of the growing medium is necessary for new roots to develop from root explants.

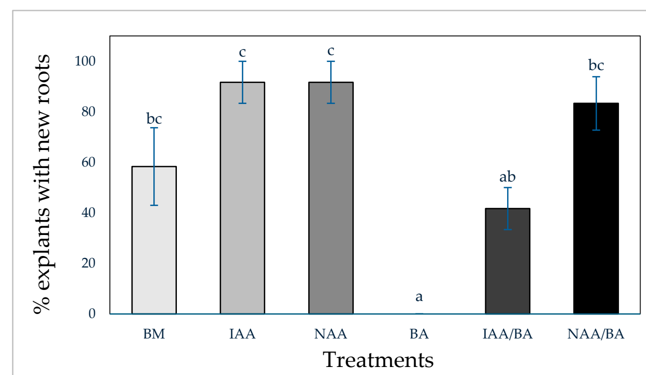

Figure 6. Root development from root explants. Results were recorded after 30 days of culture in the different treatments. Data are means ± SE. Different letters indicate statistically significant differences ($p \leq 0.05$, Kruskal–Wallis test).

3.2.2. Adventitious Shoot Development from Root Explants

After 30 days of culture, we calculated the percentage of explants that had developed adventitious shoots. We found that the three media supplemented with cytokinin (BA, IAA/BA, and NAA/BA) exhibited the most significant response (Figure 7A). The IAA medium exhibited the highest percentage response of the two auxin-supplemented media (IAA and NAA), with response percentages similar to those observed in the cytokinin media (Figure 7A). It should be noted that the adventitious shoots were produced through direct organogenesis, which means that there was no callus formation.

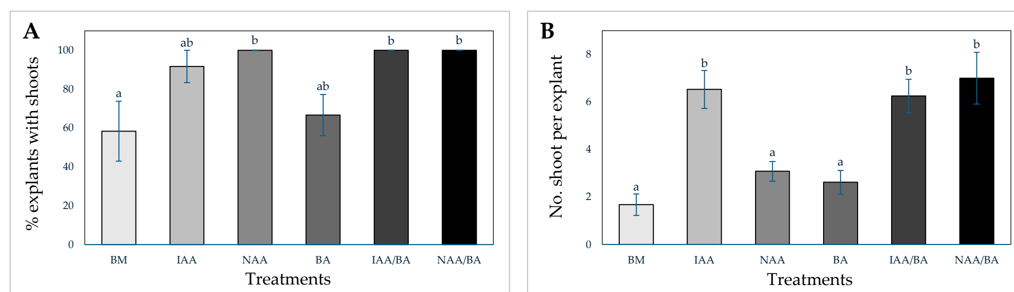

Figure 7. Shoot development from root explants. Results were recorded after 30 days of culture in the different treatments. (**A**) Percentage of explants that develop shoots. (**B**) Number of shoots per explant Data are means ± SE. Different letters indicate statistically significant differences ($p \leq 0.05$, Kruskal–Wallis test).

As in the experiment with leaf explants, root explants cultured in the different culture media were transferred to hormone-free medium to promote the elongation of adventitious shoots. After 30 days of culture on this medium, the number of shoots developed per root cluster was evaluated. In contrast to what was observed with leaf explants, the root explants grown on cytokinin-containing media (i.e., BA, IAA/BA or NAA/BA) showed the best performance in shoot production, significantly outperforming shoot development on the other culture media (Figures 7B and 8). However, it should be noted that the shoots obtained were much smaller than those obtained from leaf explants (Figure 4 vs. Figure 8).

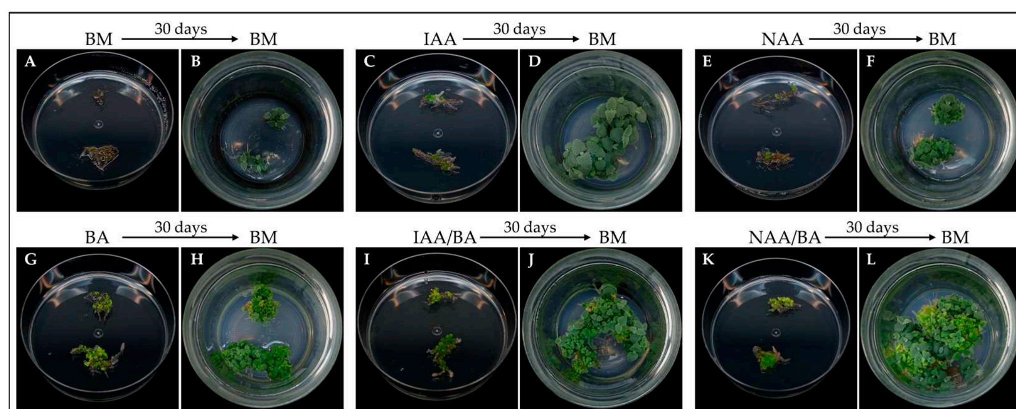

Figure 8. Root and bud induction from roots after 30 days of culture on BM, IAA, NAA, BA, IAA/BA, and NAA/BA media (**A**,**C**,**E**,**G**,**I**,**K**), and shoot development after 30 days of culture on hormone-free medium (i.e., BM medium) (**B**,**D**,**F**,**H**,**J**,**L**).

3.3. *Rooting of Adventitious Shoots*

Single adventitious shoots generated from leaf and root explants in the different culture media were separated from leaf and root cultures and transferred to hormone-free (i.e., BM) or auxin-containing (i.e., IAA and NAA) media. Shoots grown on all media developed adventitious roots, although differences in the number and length of roots were observed

depending on the culture medium. Shoots grown in NAA medium developed significantly more primary adventitious roots (Table 4, Figure 9). By contrast, shoots grown on IAA medium developed significantly more secondary adventitious roots (Table 4, Figure 9). In addition, shoots grown on hormone-free medium (i.e., BM) and medium supplemented with IAA developed longer roots (Table 4, Figure 9). In general, the formation of longer roots, with a more significant number of secondary roots, allowed a greater development of the aerial part (Figure 9). Therefore, the best medium for rooting and adventitious shoot development was IAA, followed by BM.

Table 4. Root development in adventitious shoots. Percentage of shoots developing adventitious roots (response rate), mean number of primary and secondary roots per shoot, and average maximum root length per shoot on BM, IAA, and NAA media.

Item	BM	IAA^R	NAA^R
Response rate (%)	100 ± 0.0 (a)	100 ± 0.0 (a)	100 ± 0.0 (a)
Number of primary roots	7.2 ± 0.6 (a)	14.8 ± 1.2 (b)	27.6 ± 2.7 (c)
Number of secondary roots	40.8 ± 7.1 (b)	75.2 ± 2.9 (c)	12.0 ± 0.6 (a)
Maximum root length (cm)	2.7 ± 0.2 (b)	2.4 ± 0.4 (b)	0.7 ± 0.1 (a)

The values are given as the mean ± SE. Means within each row followed by different letters are significantly different according to the Kruskal–Wallis test ($p \leq 0.05$). R: stands for rooting to differentiate this medium from the culture medium used to grow roots.

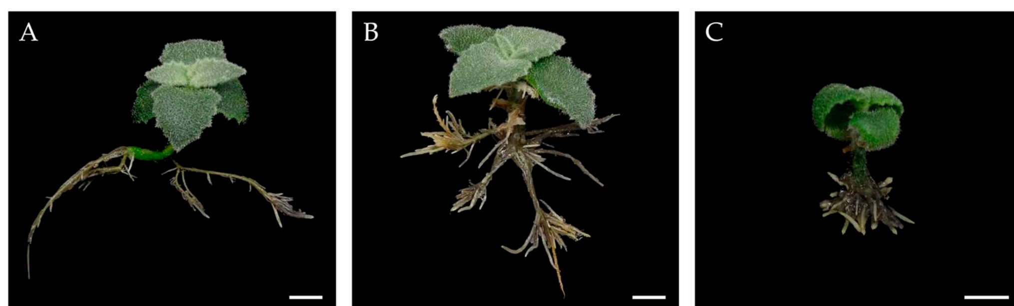

Figure 9. Root development in adventitious shoots grown in hormone-free medium (**A**), medium supplemented with IAA (**B**) and medium supplemented with NAA (**C**). Scale bars: 0.5 cm.

3.4. Transfer to Ex Vitro Conditions: Acclimation of Axenic Plants

The success of a micropropagation process lies in the ex vitro acclimation phase of the regenerated plants, which is usually measured based on the survival percentage [20]. In order to determine the survival rate of axenic plants, 48 plants were transferred to ex vitro conditions, 24 of which were covered with plastic cups and the remaining 24 without cups. The acclimation results were successful in both conditions. After 30 days, we found that the survival rate was approximately 80% in both the cup-covered and uncovered plants. Currently, three months after acclimatization, the plants show robust and sustained growth (Figure S3).

4. Discussion

4.1. Auxins Induce the Formation of Shoots in High Numbers

We had previously observed shoot development on the roots of adult axenic plants grown on a basic rooting medium. For this reason, for the establishment of a micropropagation protocol for *K. beharensis*, in addition to evaluating culture media supplemented with cytokinins that promote organogenesis [21], we assessed culture media supplemented with auxins that are characterized by promoting root development [21,22].

The analysis of root development from leaf explants revealed remarkable differences between the different culture media used in the experiment. All explants grown on auxin-supplemented media (i.e., IAA or NAA) or hormone-free medium developed roots. In the medium supplemented with IAA, the explants did not develop many primary roots but did

develop a significant number of secondary roots. This finding is important, since secondary roots increase the surface area for nutrient uptake, enhancing the survivability and growth of regenerated plants. The opposite occurred in the medium supplemented only with NAA, as explants developed more short primary roots but did not develop secondary roots. Both IAA and NAA induced adventitious root formation in *Kalanchoe marnierianum*, although IAA promoted the formation of longer roots, while naphthalene acetic acid promoted the development of shorter adventitious roots [22]. These results indicate that auxin type and concentration not only affect the number of roots able to develop, but also influence the kind of roots formed.

K. beharensis seems to have a high capacity to develop plants from its roots. Therefore, the best results were obtained in those media where the explants developed more roots. Thus, the average number of roots per explant, considering both primary and secondary roots, was low in BM medium. For this reason, when transferred to basic elongation medium, explants grown on BM only gave rise to about 4–5 plants per explant. A lower number of plants were obtained in media supplemented with cytokinins. Although cytokinins are antagonists of root development [21], explants grown on cytokinin-supplemented media developed some roots. However, the percentage of explants that developed roots and the mean number of roots per explant were low. Our results suggest that BA, alone or with auxins, inhibits root proliferation. The results also indicate the existence of a strong interaction between BA and auxins, especially between BA and NAA.

Therefore, and in terms of efficiency, our results indicate that in this species, the most suitable media to promote plant development from leaf explants are those that induce more significant root development, that is, auxin-supplemented media. Although our results suggest that the PGRs used in this study delay root elongation, since longer roots developed in the medium without PGRs, the auxins NAA and IAA stimulate root proliferation and root branching, respectively. In fact, an average of 40 primary roots per explant developed in the NAA medium. In the case of the IAA medium, although the number of primary roots was low, almost 50 secondary roots developed per explant. Consequently, when transferred to an elongation medium, explants cultivated on auxin-supplemented media, especially in the medium containing IAA, yielded the highest number of plants per explant. All in all, our results indicate that the higher root proliferation and branching promoted by auxins, or the higher root length in the medium without PGRs, results in a higher number of shoots per explant. In contrast, the inhibition of root proliferation promoted by BA, both alone and in its interaction with the auxin NAA, resulted in fewer adventitious shoots per explant.

4.2. Root Explants Grown in Cytokinin-Supplemented Media Develop a More Significant Number of Adventitious Shoots

Given the high capacity of *K. beharensis* for developing adventitious shoots from its roots (i.e., shoots), we decided to use this type of explant as starting material. The analysis of the development of new roots from root explants revealed that the best culture media were, as expected, those supplemented with auxins. However, evaluation of the development of adventitious shoots indicated that the best responses were being obtained on cytokinin-supplemented media. In root explants of *Broussonetia papyrifera*, it was observed that three hormones, BA, IBA, and NAA, were necessary for new roots and shoots to develop from root explants [23]. They suggested that BA was involved in callus formation, IBA in adventitious shoot development, and NAA in root formation and growth [23]. In a study with root explants of *Scaevola sericea*, it was found that the presence of cytokinins in the culture medium was necessary to generate adventitious shoots, since media supplemented only with auxins did not induce adventitious shoot development [24]. Our results indicate that root explants grown on media supplemented only with auxins or without growth regulators can develop adventitious shoots. However, root explants grown on media supplemented with cytokinins develop significantly more adventitious shoots, regarding the number of root explants with adventitious shoots and the number of adventitious shoots developed in each explant. Similar results were observed in root explants of the

semiparasitic herb *Monochasma savatieri* Franch [25]. Indeed, although the best results were obtained on cytokinin-supplemented media, the authors verified that auxin-supplemented media, and even those without hormones, could induce some adventitious bud formation when the culture period was extended above 40 days.

4.3. Culture Medium Supplemented with IAA Enhances Root Development in the Shoots

Shoots regenerated from leaf or root explants can be transferred to hormone-free or auxin-supplemented media to promote rooting and growth in the axenic plant. In this work, we have found that *K. beharensis* was able to develop primary and secondary roots on a basic culture medium without supplementation of growth regulators (i.e., BM). However, the shoots culture on medium supplemented with 1 mg/L IAA (i.e., IAA^R) resulted in a higher number of primary and secondary roots. Consequently, axenic plant growth was higher in the IAA^R medium than in the BM medium. When shoots were grown on medium supplemented with 1 mg/L NAA (i.e., NAA^R), significantly more primary roots were formed, but the number of secondary roots was significantly less than those developing on shoots grown on IAA^R medium and even on BM medium. Also, the roots were significantly shorter than those of shoots grown on IAA^R or BM media. As a result, the growth of axenic plants was weaker than that of plants grown on IAA^R or BM media. Therefore, NAA^R medium does not seem suitable for promoting the rooting of adventitious shoots. These results suggest that lateral roots play an essential role in nutrient uptake from the culture medium and, thus, in plant growth. In line with our results, it has been observed in another species of the genus *Kalanchoe* (*K. marnierianum*) that auxin IAA promotes the growth of long-length roots, whereas NAA tends to form shorter roots [22].

Our results indicate that, in *K. beharensis*, IAA is a potent inducer of adventitious root development, especially promoting lateral root development. These results are congruent with previous research in *K. bossfeldiana*, where the use of IAA also resulted in a high rooting rate and robust root development [11]. However, other authors have evaluated the effect of three auxins (i.e., IAA, IBA, and NAA) and the combination of two of them (i.e., IBA and NAA) on adventitious rooting of *Scaevola sericea* [24]. They concluded that the highest rooting percentage was obtained in the culture medium supplemented with 2.5 mg/L NAA. Nevertheless, it has been observed that axenic *Monochasma savatieri* plants developed more roots on the medium supplemented with NAA, although these were easily browned (oxidized) [25]. In contrast, axenic *Monochasma savatieri* plants grown on medium supplemented with IAA or IBA formed fewer roots but developed better. These results show that no universal auxin is suitable for adventitious root development in all species.

4.4. K. beharensis Does Not Require Protection from Water Loss During the Acclimatization Process

K. beharensis has a CAM metabolism, which allows it to minimize water loss by opening its stomata mainly during the night and keeping them closed during the day, a period that concentrates the greater number of hours with high temperatures. This strategy reduces transpiration and improves water use efficiency. This metabolism could facilitate a gentler transition from controlled in vitro growth conditions to the less predictable and more variable ex vitro growth conditions. Different species are acclimatized by transferring the in vitro plants to pots with substrate and using a transparent plastic pot as a cover [20]. The pot creates a microclimate of high humidity that prevents dehydration of the plants during the first days of acclimatization. In most species, this technique allows the removal of the pot after 7–15 days. In the present study, taking into account the metabolism of this species, half of the plants were acclimatized by covering them with the plastic pot, while the other half were acclimatized without the pot. This simple experiment allowed us to verify that *K. beharensis* does not require the protection system provided by the plastic pot necessary for the acclimation of other species. In fact, the acclimatization process without the plastic pot had already been performed in another species with CAM metabolism, on which we have worked in the laboratory. Indeed, the acclimatization of axenic plants of *Sedum sediforme* and *S. album* without using any cover to protect them from water loss allowed us to verify

that these two species did not require the use of the plastic pot during the acclimatization process [26]. Overall, these results indicate that CAM species do not require special care for successful transfer to ex vitro conditions. In short, the CAM metabolism of *K. beharensis* allows its acclimation without a cover that protects it from water loss, facilitating and cheapening its acclimation process from in vitro to ex vitro conditions.

4.5. Implementation of a Micropropagation Protocol of K. beharensis for Conservation and Multiplication

The micropropagation program of *K. beharensis* that we have developed involves the following stages: (1) introduction into tissue culture and development of axenic plants on medium with 1 mg/L of IAA, (2) culture of leaf explants on medium with 2 mg/L of IAA, (3) shoot elongation on hormone-free medium, (4) rooting on medium with 1 mg/L of IAA, and (5) acclimation of axenic plants.

The introduction of shoots (see Supplementary Figure S1) to the in vitro culture conditions, and the development of an axenic plant with leaves to initiate the micropropagation process, would require about 60 days (see Supplementary Figure S2). We could obtain five leaf explants from an axenic plant, and from each leaf, twelve plants would be obtained after a 90-day culture period (stage 2 = 30 days, stage 3 = 30 days and stage 4 = 30 days, see Figures 2B, 3 and 7). That is, 60 axenic plants would be obtained from one axenic plant in 5 months. From this point on, each of these 60 plants could give rise to another 60 plants in 3 months, which would mean 3600 plants in 8 months from a single axenic plant. Given the capacity of the leaves to generate adventitious shoots in the culture medium supplemented with IAA, more than 200,000 axenic plants could be obtained in 1 year.

Adventitious regeneration of plants under in vitro culture conditions can cause genetic and epigenetic changes in crops, called somaclonal variation [27,28]. Among the most important factors in the frequency of somaclonal variation are the type of organogenesis, the culture period, and the number of subcultures. Regarding the type of organogenesis, in adventitious regeneration of plants by direct organogenesis, no variation is to be expected, and if there is any, it is usually minimal. Conversely, adventitious regeneration of plants by indirect organogenesis, with a process of callus formation, is of fundamental importance in inducing somaclonal variation [27]. Here, we describe a protocol for adventitious regeneration of *K. beharensis* through direct organogenesis without callus formation. Therefore, we recommend this protocol for the cloning of valuable cultivars.

As we have indicated in this work, the acclimation process would not involve great difficulties, and the plants could be acclimatized without needing protection to avoid dehydration. However, we have estimated that a period of 4 months would be necessary to obtain a plant of about 10 cm in height (Supplementary Figure S3).

5. Conclusions

K. beharensis has been classified as vulnerable in the Red List of Threatened Species and is of great interest both for its ornamental value and medicinal potential. Propagation of *K. beharensis* has traditionally been carried out by conventional methods (i.e., seeds and cuttings), although these may have limitations regarding efficiency and genetic conservation. There is no standardized micropropagation protocol for this species in the scientific literature, highlighting the need for detailed research such as the one presented in this paper. In this work, we present a micropropagation protocol that allows us to obtain thousands of plants in less than 1 year from an axenic plant. The best results are obtained by culturing leaf explants in a medium supplemented with 2 mg/L of IAA, subsequent elongation of adventitious shoots in hormone-free medium, and rooting in a medium with 1 mg/L of IAA. The last stage consists of acclimatization of the axenic plants, which is performed directly without the protection used to reduce plant dehydration, thus reducing the cost of the micropropagation process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121379/s1, Figure S1: Three-year-old *K. beharensis* plant cultivated in the greenhouse; Figure S2: Morphogenetic response from leaf explants cultured in cytokinin supplemented media; Figure S3: *K. beharensis* three months after acclimation.

Author Contributions: Conceptualization V.M. and B.P.; methodology, I.M.-G., B.G.-S. and B.P.; investigation, I.M.-G. and V.R.; resources, B.P.; data curation, C.V.; writing—original draft preparation, I.M.-G. and B.P.; writing—review and editing, V.M. and B.P.; visualization, I.M.-G.; funding acquisition, V.M. 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 their use in other unpublished articles.

Acknowledgments: B.P. and V.M. are thankful for the granting of the R+D+i projects PID2019-110833RB-C32 and PID2023-151867OB-C32, funded by MICIU/AEI/ 10.13039/501100011033. The authors thank David Harry Rhead for reviewing the manuscript in the English language.

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

References

1. Vargas, A.; Herrera, I.; Nualart, N.; Guézou, A.; Gómez-Bellver, C.; Freire, E.; Díaz, P.J.J.; López-Pujol, J. The genus *Kalanchoe* (Crassulaceae) in Ecuador: From gardens to the wild. *Plants* **2022**, *11*, 1746. [CrossRef]
2. Walker, C.C. Two shrubby Madagascan kalanchoes. *N. Z. Cactus Succul. J.* **2019**, *72*, 5–7.
3. Smith, G.F.; Figueiredo, E.; Walker, C.C.; Condy, G. *Kalanchoe beharensis*. Crassulaceae: Kalanchooideae. In *Flowering Plants of Africa*; Grobler, A., Condy, G.S., Eds.; South African National Biodiversity Institute (SANBI Publishing): Pretoria, South Africa, 2021; Volume 67, pp. 50–59.
4. Kluge, M.; Razanoelisoa, B.; Ravelomanana, D.; Brulfert, J. *In situ* studies of crassulacean acid metabolism in *Kalanchoe beharensis* Drake Del Castillo, a plant of the semi-arid southern region of Madagascar. *New Phytol.* **1992**, *120*, 323–334. [CrossRef]
5. *Kalanchoe beharensis*. Available online: https://jardinbotanico.uma.es/jb-cra-10/ (accessed on 15 June 2024).
6. Club Global Flowers: *Kalanchoe beharensis*. Available online: https://club.global.flowers/en/kalanchoe/19941-kalanchoe-beharensis (accessed on 15 June 2024).
7. Nascimento, L.B.D.S.; Casanova, L.M.; Costa, S.S. Bioactive compounds from *Kalanchoe* genus potentially useful for the development of new drugs. *Life* **2023**, *13*, 646. [CrossRef]
8. Poma, P.; Labbozzetta, M.; McCubrey, J.A.; Ramarosandratana, A.V.; Sajeva, M.; Zito, P.; Notarbartolo, M. Antitumor mechanism of the essential oils from two succulent plants in multidrug resistance leukemia cell. *Pharmaceuticals* **2019**, *12*, 124. [CrossRef] [PubMed]
9. Sanikhani, M.; Frello, S.; Serek, M. TDZ induces shoot regeneration in various *Kalanchoe blossfeldiana* Poelln. cultivars in the absence of auxin. *Plant Cell Tissue Organ Cult.* **2006**, *85*, 75–82. Available online: https://link.springer.com/article/10.1007/s11240-005-9050-6 (accessed on 13 June 2024). [CrossRef]
10. Kertrung, T.; Junkasiraporn, S. In vitro propagation of *Kalanchoe rhombopilosa* (Crassulaceae). *NU. Int. J. Sci.* **2018**, *15*, 37–48. Available online: https://www.thaiscience.info/Journals/Article/NUJS/10988681.pdf (accessed on 13 June 2024).
11. Castelblanque, L.; García-Sogo, B.; Pineda, B.; Moreno, V. Efficient plant regeneration from protoplasts of *Kalanchoe blossfeldiana* via organogenesis. *Plant Cell Tissue Organ Cult.* **2010**, *100*, 107–112. [CrossRef]
12. Frello, S.; Venerus, E.; Serek, M. Regeneration of various species of Crassulaceae, with special reference to Kalanchoe. *J. Hortic. Sci. Biotechnol.* **2002**, *77*, 204–208. [CrossRef]
13. Santos, M.R.A.; Ferreira, M.G.R.; Guimarães, M.C.M.; Lima, R.A.; Oliveira, C.L.L.G. Callogenesis in leaves of *Kalanchoe pinnata* Lam. by 2,4-D and BA action. *Rev. Bras. Plantas Med.* **2014**, *16*, 760–764. [CrossRef]
14. Pakum, W.; Inmano, O.; Kongbangkerd, A. TDZ and 2,4-D on in vitro propagation of panda plant from leaf explants. *Ornam. Hortic.* **2020**, *27*, 41–48. [CrossRef]
15. Sathya, C.; Abubacker, M.N. In vitro Organogenesis and Epiphyllous Secondary Buds Formation in *Kalanchoe tubiflora* (Harvey), Hamet, (Crassulaceae). *Biosci. Biotechnol. Res. Asia* **2023**, *20*, 1433–1442. [CrossRef]
16. Rabehevitra, A.D. *Kalanchoe beharensis*. *IUCN Red List. Threat. Species* **2019**, e.T128093556A128098546. Available online: https://doi.org/10.2305/IUCN.UK.2019-2.RLTS.T128093556A128098546.en (accessed on 11 June 2024).
17. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–479. [CrossRef]
18. Shahin, E.A. Totipotency of tomato protoplasts. *Theor. Appl. Genet.* **1985**, *69*, 235–240. [CrossRef]
19. Hoagland, D.R.; Arnon, D.I. The Water-Culture Method for Growing Plants without Soil. *Calif. Agric. Exp. Stn.* **1950**, *347*, 1–39.
20. Cristiano, G.; Murillo-Amador, B.; De Lucia, B. Propagation Techniques and Agronomic Requirements for the Cultivation of Barbados Aloe (*Aloe vera* (L.) Burm. F.)-A Review. *Front. Plant Sci.* **2016**, *7*, 1410. [CrossRef] [PubMed]

21. Kurepa, J.; Smalle, J.A. Auxin/Cytokinin Antagonistic Control of the Shoot/Root Growth Ratio and Its Relevance for Adaptation to Drought and Nutrient Deficiency Stresses. *Int. J. Mol. Sci.* **2022**, *23*, 1933. [CrossRef]
22. Kulka, R.G. Hormonal control of root development on epiphyllous plantlets of *Bryophyllum* (*Kalanchoe*) *marnierianum*: Role of auxin and ethylene. *J. Exp. Bot.* **2008**, *59*, 2361–2370. [CrossRef] [PubMed]
23. Lin, J.; Zou, J.; Zhang, B.; Que, Q.; Zhang, J.; Chen, X.; Zhou, W. An efficient in vitro propagation protocol for direct organogenesis from root explants of a multi-purpose plant, *Broussonetia papyrifera* (L.) L'Hér. ex Vent. *Ind. Crops Prod.* **2021**, *170*, 113686. [CrossRef]
24. Liang, H.; Xiong, Y.; Guo, B.; Yan, H.; Jian, S.; Ren, H.; Zhang, X.; Li, Y.; Zeng, S.; Wu, K.; et al. Shoot organogenesis and somatic embryogenesis from leaf and root explants of *Scaevola sericea*. *Sci. Rep.* **2020**, *10*, 11343. [CrossRef] [PubMed]
25. Zhang, Y.; Chen, Y.; Zhang, X. Adventitious Shoot Induction from Internode and Root Explants in a Semiparasitic Herb *Monochasma savatieri* Franch ex Maxim. *J. Plant Growth Regul.* **2017**, *36*, 799–804. [CrossRef]
26. Moreno-García, I. Micropropagación de *Sedum sediforme* y *S. album*, una Estrategia Rápida y Eficiente para la Conservación y Multiplicación a Gran Escala de Estas Especies. Bachelor's Thesis, Universitat Politècnica de València, Valencia, Spain, 2019. Available online: https://m.riunet.upv.es/handle/10251/128034 (accessed on 5 November 2024).
27. Ferreira, M.d.S.; Rocha, A.d.J.; Nascimento, F.d.S.; Oliveira, W.D.d.S.; Soares, J.M.d.S.; Rebouças, T.A.; Morais Lino, L.S.; Haddad, F.; Ferreira, C.F.; Santos-Serejo, J.A.d.; et al. The Role of Somaclonal Variation in Plant Genetic Improvement: A Systematic Review. *Agronomy* **2023**, *13*, 730. [CrossRef]
28. Larkin, P.; Scowcroft, W. Somaclonal variation—A novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet* **1981**, *60*, 197–214. Available online: https://link.springer.com/article/10.1007/BF02342540 (accessed on 15 November 2024). [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.