



In vitro propagation of *Limonium algarvense* Erben: towards the conservation of a rare and endemic halophyte

Luísa Custódio¹ · Leonardo Lescano¹ · Carolina Martins¹ · Maria João Rodrigues¹

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Abstract

Limonium algarvense, an endemic halophyte from the Southwest Iberian Peninsula, is listed as “Near Threatened” due to habitat degradation caused by climate change and human activities. Its medicinal properties and limited natural propagation make conservation efforts essential. In vitro propagation offers a sustainable *ex situ* conservation strategy for this species. Shoot tips from greenhouse-germinated seedlings were sterilized and cultured on Murashige and Skoog (MS) medium supplemented with various growth regulators for 2 cycles of multiplication, such as kinetin (KIN) and naphthalene acetic acid (NAA). Rooting was induced using various MS-based media, including supplements like calcium chloride (CaCl₂) and activated charcoal (AC), followed by a gradual acclimatization to *ex vitro* conditions. Aseptic cultures were successfully established in 75.6% of the explants using 0.1% HgCl₂. The optimal combination for shoot multiplication was 2.5 mg/L KIN+0.1 mg/L NAA, producing 15.7 shoots/explant after two cycles. The highest rooting percentage (67.6%) was obtained with MS medium supplemented with 10 mM CaCl₂, which also led to superior root length, shoot height, and rosette diameter compared to other treatments. However, survival during the acclimatization remained low (13%). This highlights the need for further optimization of the acclimatization phase to improve the *ex vitro* survival of propagated plants. Overall, the in vitro protocol provides a promising tool for conserving *L. algarvense*, but improvements are necessary for large-scale conservation efforts.

Key message

An efficient in vitro propagation protocol for *Limonium algarvense* was developed, supporting its conservation and sustainable use, despite challenges in ex vitro acclimatization that require further optimization.

Keywords Sea lavender · Micropropagation · Conservation · Plant tissue culture · Salt-tolerant plant

Introduction

The worldwide rapid degradation of ecosystems is leading to a massive loss of plant biodiversity, with high impacts on human livelihoods by negatively affecting natural ecosystems (Gulzar et al. 2018). Coastal habitats are major contributors to regional and global biodiversity, including many rare and endemic halophyte species, highly susceptible

to climate change and consequent habitat deterioration (Caperta et al. 2014).

Plant tissue culture (PTC) techniques are important *ex situ* conservation alternative approaches, particularly useful for the multiplication and preservation of threatened, rare and endemic species with limited self-propagation, mainly due to the reduced number of explants needed from the mother plant that produces a limited impact on wild habitats and their biodiversity (Twaij et al. 2020). PTC technologies allow the enhancement of shoot multiplication, hardening and rooting, and the obtaining of disease-free plants (Hutter and Schneider 2019). These systems can serve as a source of biological materials for in-situ conservation programs of threatened, rare, and endemic plant species aiming at their reintroduction into their natural habitats. These techniques have proved to be useful in the conservation of a wide range

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✉ Maria João Rodrigues
mjrodrigues@ualg.pt

¹ Centre of Marine Sciences (CCMAR/CIMAR LA), Faculty of Sciences and Technology, University of Algarve, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal

of endangered species, as the Hill's thistle (*Cirsium hillii*) in Bruce Peninsula National Park (Canada) (Bhatia and Sharma 2015), or the critically endangered species *Rubus humulifolius* that were successfully regenerated after a long-term storage at ultra-low temperatures to in vivo conditions in Botanical Gardens of University of Oulu (Finland) (WHO 2021). However, the application of PTC techniques for the conservation of coastal plant species is scarce.

The Algarve coast holds unique biodiversity, including several rare and endemic halophyte species, such as *Limonium algarvense* Erben, an endemic to the Southwest area of the Iberian Peninsula found in different salinity conditions, such as coastal dunes and salt marshes (Costa et al. 2014). The latest survey of the Red List of Vascular Plants for Mainland Portugal indicates this species as “Near Threatened”, as is close to meeting the criteria for “Vulnerable”, as it was identified a continued decline, with reduced extent of occurrence and area of occupation. The loss of habitat is mainly occurring due to increasing pressure from the expansion of exotic species, but other threats include the installation of fish farming tanks and works that affect the normal water flow in estuarine systems (Carapeto et al. 2020). Given the increasing threats to its natural habitat, innovative *ex situ* strategies are urgently required. Moreover, this is a medicinal species endowing highly bioactive flavonoids with biological properties relevant to human health, namely antioxidant, anti-inflammatory, antidiabetic, neuroprotective, anti-melanogenic, anti-wrinkle and photoprotective (Rodrigues et al. 2015, 2016, 2019, 2020; Lescano et al. 2024). PTC can thus also be used to create a nursery of plants for medicinal use with low impact on the native populations. Therefore, this work aimed at establishing and developing an in vitro conservation and multiplication protocol for *L. algarvense*. This included culturing seedling shoot tips as explants, and induction of new shoots by supplementation of MS medium with different combinations of growth regulators, followed by the rooting and acclimatization of obtained plantlets.

Materials and methods

Explants disinfection and establishment

The disinfection and in vitro establishment of *L. algarvense* in vitro cultures followed the protocol previously described by Lescano et al. (2025), which was adapted from Khadka et al. (2019). Briefly, shoot tips excised from seedlings were washed, surface-sterilized with 0.1% (w/v) HgCl_2 for 5 min, and rinsed three-times with sterile distilled water (Khadka et al. 2019). The disinfected explants were placed in sterilized individual glass tubes containing Murashige and Skoog (MS) without plant growth regulators, supplemented with 2% sucrose and 0.8% agar. The key steps of explant preparation and in vitro establishment are illustrated in Fig. 1. Cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 16/8 h light/dark photoperiod with a light intensity of approximately $56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After one month, only the explants that remain free of contamination were used for subsequent multiplication experiments. If roots had developed during the establishment phase, they were carefully removed before subculturing and only shoot explants without roots were used in the multiplication experiments.

Multiplication

For shoot multiplication, explants obtained after disinfection were randomly assigned to five treatment groups, each corresponding to a different culture medium formulation:

- 1) MS basal medium (control, without plant growth regulators–PGRs),
- 2) MS + 2.5 mg/L 6-benzylaminopurine (BAP; $\approx 11.1 \mu\text{M}$) + 0.1 mg/L naphthalene acetic acid (NAA; $\approx 0.54 \mu\text{M}$),
- 3) MS + 2.5 mg/L kinetin (KIN; $\approx 11.6 \mu\text{M}$) + 0.1 mg/L NAA ($\approx 0.54 \mu\text{M}$),
- 4) MS + 2.5 mg/L BAP ($\approx 11.1 \mu\text{M}$) + 0.1 mg/L indole-3-butyric acid (IBA; $\approx 0.49 \mu\text{M}$),

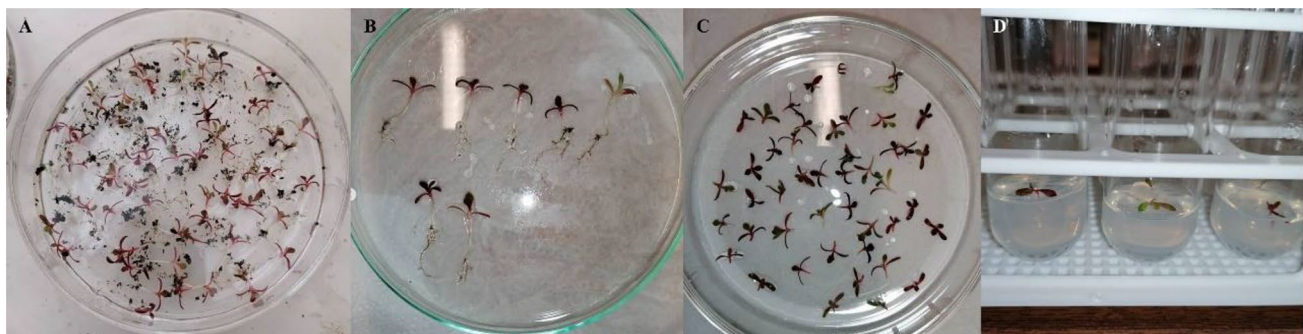


Fig. 1 In vitro disinfection and establishment of *L. algarvense*. (A) Seedlings removed from soil prior to in vitro introduction. (B) Seedlings washed thoroughly to remove soil residues, with intact root systems. (C) Seedlings with roots removed prior to culture initiation,

leaving only shoot tip explants for in vitro establishment. (D) Explants after disinfection, placed individually in glass tubes containing MS medium without plant growth regulators, during the establishment phase

- 5) MS + 2.5 mg/L KIN ($\approx 11.6 \mu\text{M}$) + 0.1 mg/L IBA ($\approx 0.49 \mu\text{M}$).

Explants were cultured in 250 mL borosilicate glass Erlenmeyer flasks, each containing 100 mL of medium. Each treatment consisted of three independent experiments, with five biological replicates per treatment and a total of fifteen explants per treatment ($n = 15$). Cultures were maintained under a 16/8 h light/dark photoperiod at $25 \pm 2^\circ\text{C}$ and light intensity approximately $56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The culture medium was supplemented with 2% (w/v) sucrose as the carbon source and solidified with 0.8% (w/v) agar. All media were adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min.

Shoot multiplication was performed in two consecutive 60-day cycles without medium renewal; explants remained in the same culture medium throughout. Only the best-performing treatment from the first cycle (2.5 mg/L KIN + 0.1 mg/L NAA) was selected for the second cycle. At the end of each cycle, the number of shoots per explant, shoot height, number of leaves, and rosette diameter were recorded.

Rooting

Individual shoots obtained from the second cycle of the multiplication phase (from the treatment with 2.5 mg/L KIN + 0.1 mg/L NAA) were transferred to rooting media and maintained for 60 days under the same culture conditions ($25 \pm 2^\circ\text{C}$, 16/8 h light/dark photoperiod, $56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The culture medium was not renewed during this period; explants remained in the same medium throughout the 60-day rooting phase. A total of six treatments were tested using Murashige and Skoog (MS) medium with different modifications:

- 1) Full-strength MS basal medium (control, no PGRs),
- 2) Full-strength MS supplemented with $5 \mu\text{M}$ indole-3-butyric acid (IBA),
- 3) Full-strength MS with 2 g/L activated charcoal (AC),
- 4) Half-strength ($\frac{1}{2}$) MS with 2 g/L AC,
- 5) Quarter-strength ($\frac{1}{4}$) MS with 2 g/L AC,
- 6) Full-strength MS supplemented with 10 mM calcium chloride (CaCl_2).

In this context, “half-strength” ($\frac{1}{2}$) and “quarter-strength” ($\frac{1}{4}$) MS media refer to formulations in which all components of the original MS medium (macronutrients, micronutrients, and vitamins) were reduced proportionally by 50% and 75%, respectively. All media were supplemented with 2% (w/v) sucrose as the carbon source and solidified with 0.8% (w/v) agar. The pH was adjusted to 5.7–5.8 prior to autoclaving at 121°C for 20 min. Explants were cultured

in 250 mL borosilicate glass Erlenmeyer flasks, each containing 100 mL of medium. The experimental design comprised three independent experiments, with five biological replicates, using a total of fifteen explants per treatment ($n = 15$). After 60 days, the number of rooted shoots was recorded, and root length, shoot height, and rosette diameter were measured. Visual assessments of plantlet health were also noted in order to determine the most effective medium for promoting vigorous and morphologically normal root development.

Acclimatization

Well-rooted plantlets, 60 days old, obtained from the rooting phase, specifically from the most effective treatment (MS + 10 mM CaCl_2), were selected for acclimatization. These plantlets exhibited well-developed root systems, with an average root length of 2.1 cm and shoot height of 2.3 cm. Plants were transferred to plastic cuvettes ($4 \times 4 \times 5.5$ cm per cell) each filled with a peat and perlite mixture (3:1, v/v), with one plant in each cell. Two acclimatization treatments were tested: (1) control (no treatment) and (2) spraying of plantlets with a 17.5% (v/v) paraffin solution to reduce water loss. A total of 15 plants were used per treatment ($n = 15$). Plants within the cuvettes were placed inside lidded transparent plastic boxes to maintain high humidity (90–100%) and kept indoors at $25 \pm 2^\circ\text{C}$ under natural light ($46\text{--}56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of photosynthetically active radiation–PAR). After 7 days under high-humidity conditions, lid opening was initiated and carried out daily in the morning (approximately 9:00 AM), when ambient temperature remained moderate, reducing thermal and desiccation stress during the initial stages of acclimatization. Plants were then progressively adapted to ex vitro conditions ($55\text{--}60\%$ relative humidity; $25 \pm 2^\circ\text{C}$) by gradually increasing ventilation time, starting at 1 h per day and extending daily, until the lids were fully open after 30 days. A liquid nutrient solution containing RetWin® Plus (N–P–K: 4–6–6) was applied once per week at a dilution of $150 \mu\text{L/L}$, with each plant receiving approximately 20 mL per cuvette cell to support nutritional needs during the acclimatization phase. Survival rates were subsequently assessed for both treatments.

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM), and experiments were led at least in triplicate. Significant differences were evaluated by ANOVA and Tukey’s honestly significant difference (HSD) test ($p < 0.05$). Statistical analyses were made using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation).

Results and discussion

Establishment

The obtaining of aseptic cultures is a crucial step to the in vitro culture, and since former reports on other *Limonium* species have highlighted the possibility of endophytic bacterial infections that may cause decreased growth and lead-tip necrosis (Khadka et al. 2019). To prevent that, in this work we followed the approach and the best disinfection method described by Khadka et al. (2019) for *L. sinuatum* L. Mill, as previously adopted by Lescano et al. (2024). At the end of this period, 75.6% of shoot tip explants remained free of contamination. Amongst these aseptic explants, 5.88% developed roots spontaneously.

Multiplication

The multiplication phase led to the production of new shoots from shoot tip explants over two consecutive 60-day cycles. Among the treatments tested, significant differences were observed in both survival and shoot formation rates (Fig. 2). In the first cycle, the shoots grown with the combination of 2.5 mg/L KIN + 0.1 mg/L NAA showed 100% survival, whilst in the second cycle only 75% of explants subjected to this treatment survived (Fig. 2), which was superior to the survival of explants cultivated in MS basal medium (control; 71.4%). Despite this, all living explants could produce new shoots (100%; Fig. 2).

Afterwards, the multiplication of sea lavender explants was evaluated by determining their shooting capacity, by counting the number of new shoots and their leaves, and

by measuring their height and rosette diameter. Results are presented in Table 1; Figs. 3 and 4. Amongst all treatments, besides the plants grown in basal MS medium (control), only the explants cultivated with 2.5 mg/L KIN + 0.1 mg/L NAA also presented a normal and healthy appearance. In all the other conditions, the shoots had signs of hyperhydricity and the ones containing the auxin IBA were highly compact making their separation difficult. Furthermore, the 2.5 mg/L KIN + 0.1 mg/L NAA treatment induced an 8-fold increase in new shoot formation (8.11 shoots/explant) compared to the control (1 shoot/explant). In turn, this condition led to a reduction in the shoot height (1.17 cm), leaves number (4.90) and rosette diameter (1.53 cm) to control explants (Table 1; Fig. 3). For this condition, a second multiplication cycle was applied, and it induced an almost 2-fold increase in new shoot formation (15.7 shoots/explant), as well as an increase varying from 1.5 to 1.8 times in shoot height (1.43 cm), leaves number (7.19) and rosette diameter (2.07 cm).

It has already been noted for other plant species that the number of leaves and the height of shoots decrease as the number of shoots per explant increases, as the shoot spends more energy to multiply leaving less to invest in growth, while when the number of explants is smaller, the sprouts have more energy available to invest in their growth (Papafotiou et al. 2023). Moreover, consecutive multiplication cycles caused an increase in new shoots formation and their size, similar to what was observed for *L. giberitii*, other endemic species of the Iberian Peninsula (Martín and Pérez 1995), as well as for other halophyte species such as the sea knotgrass (*Polygonum maritimum*) (Custódio et al. 2022). Despite this, other Iberian endemic *Limonium* species (*L. dufourei*, *L. calaminare*, *L. dichotomum*, and

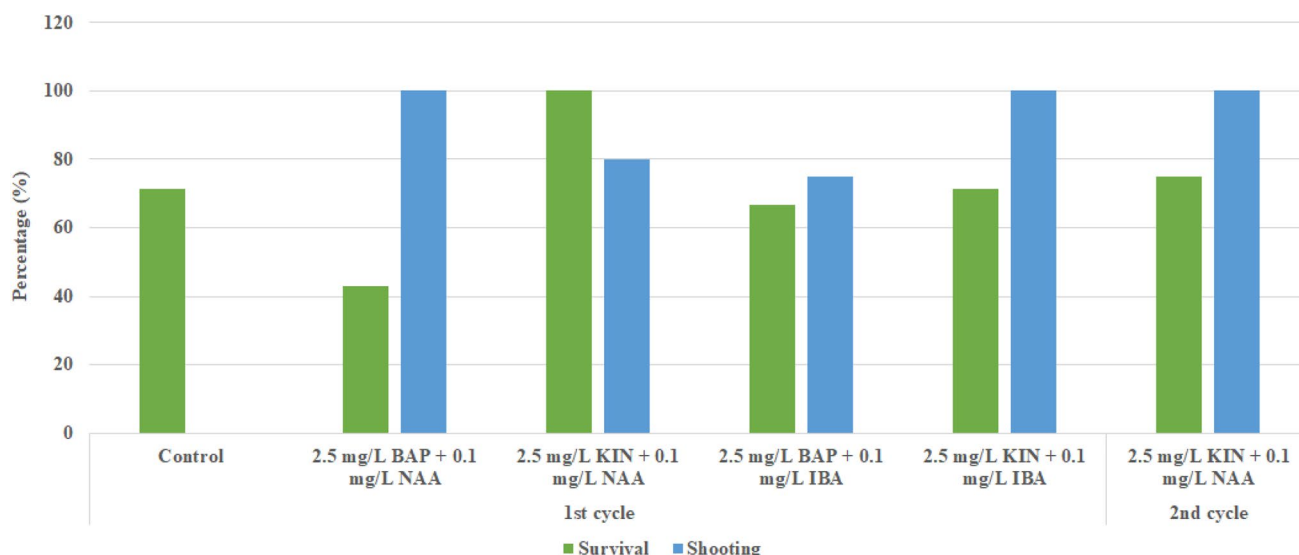


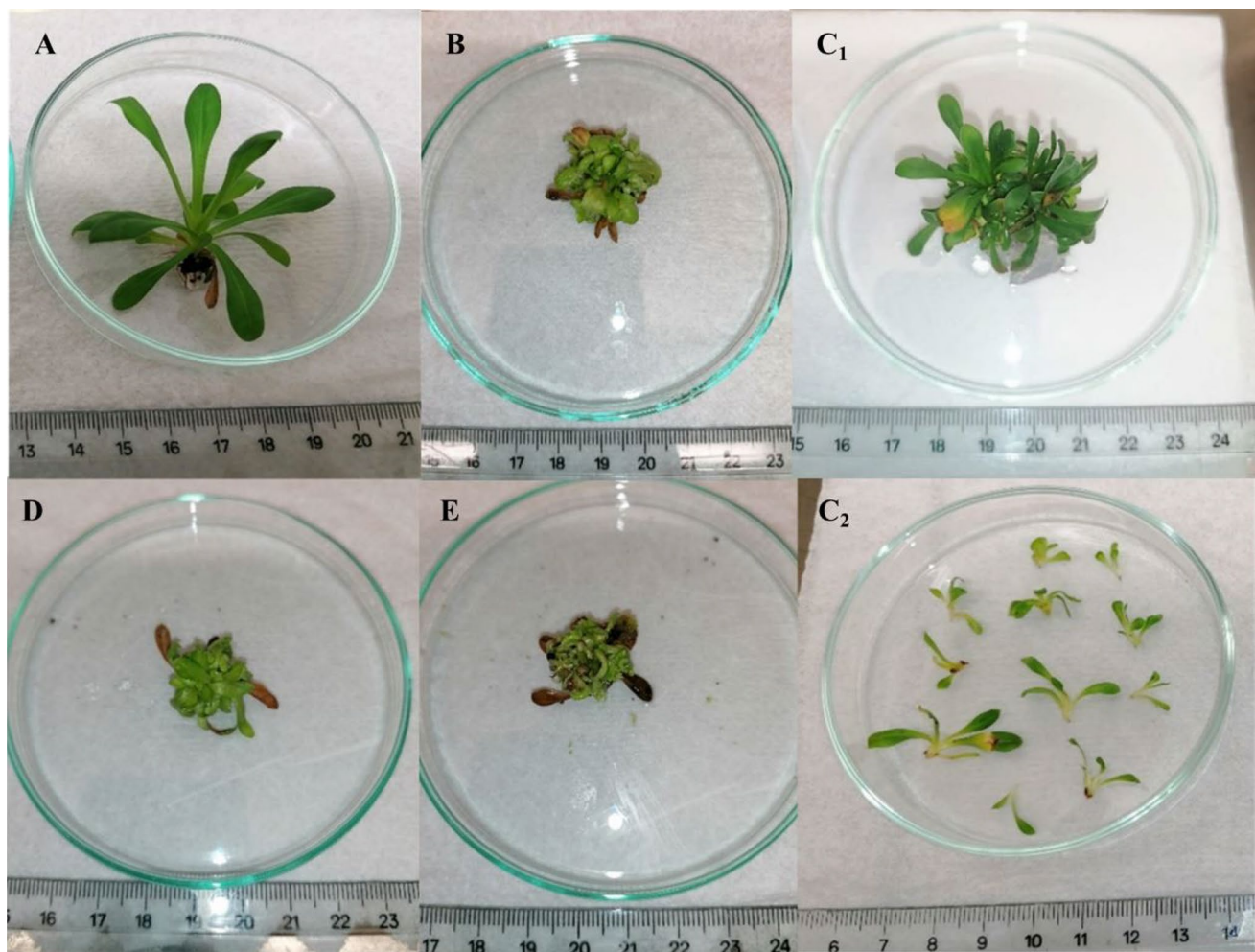
Fig. 2 Percentage of survival and shoot formation of *L. algarvense* explants after 60 days in MS medium without plant growth regulators (PGRs, control) and supplemented with different combinations of

cytokinins and auxins: 2.5 mg/L BAP + 0.1 mg/L NAA, 2.5 mg/L KIN + 0.1 mg/L NAA, 2.5 mg/L BAP + 0.1 mg/L IBA, and 2.5 mg/L KIN + 0.1 mg/L IBA. Bars represent the average values from 15 explants

Table 1 Shoot multiplication of *L. algarvense* explants grown for 60 days in MS basal medium free of PGRs (control), MS medium supplemented with 2.5 mg/l of BAP and 0.1 mg/l of NAA, 2.5 mg/l of KIN and 0.1 mg/l of NAA, 2.5 mg/l of BAP and 0.1 mg/l of IBA, and 2.5 mg/l of KIN and 0.1 mg/l of IBA

Treatment	Shoot number	Shoot height (cm)	Leaves number (cm)	Rosette diameter (cm)	Observations
<i>1st cycle</i>					
Control	1.00 ± 0.00 ^b	4.38 ± 0.74 ^a	12.7 ± 1.2 ^a	6.13 ± 0.43 ^a	Normal appearance
2.5 mg/L BAP + 0.1 mg/L NAA	10.0 ± 2.8 ^a	0.91 ± 0.06 ^b	4.61 ± 0.41 ^b	1.09 ± 0.09 ^b	Hyperhydric
2.5 mg/L KIN + 0.1 mg/L NAA	8.11 ± 1.21 ^a	1.17 ± 0.04 ^b	4.90 ± 0.22 ^b	1.53 ± 0.09 ^b	Normal appearance
2.5 mg/L BAP + 0.1 mg/L IBA	Nd	Nd	Nd	Nd	Hyperhydric; Difficult to separate
2.5 mg/L KIN + 0.1 mg/L IBA	Nd	Nd	Nd	Nd	Hyperhydric; Impossible to separate
<i>2nd cycle</i>					
2.5 mg/L KIN + 0.1 mg/L NAA	15.7 ± 2.4 [*]	1.43 ± 0.05 [*]	7.19 ± 0.29 [*]	2.07 ± 0.08 [*]	Normal appearance

Nd: Not determined. Values correspond to mean ± SEM of three independent experiments ($n = 15$). For the same column, values marked with different letters (a, b) are considered statistically different at $P < 0.05$ (Tukey's HSD), while values marked with * are significantly different from the same condition in the first cycle ($P < 0.05$)

**Fig. 3** Morphological appearance of *L. algarvense* shoots after 60 days of culture under different treatments. (A) Control (MS without PGRs); (B) 2.5 mg/L BAP + 0.1 mg/L NAA; (C₁) 2.5 mg/L KIN + 0.1 mg/L

NAA– shoot cluster; (C₂) 2.5 mg/L KIN + 0.1 mg/L NAA– separated shoots; (D) 2.5 mg/L BAP + 0.1 mg/L IBA; (E) 2.5 mg/L KIN + 0.1 mg/L IBA. All images taken after the first cycle of multiplication

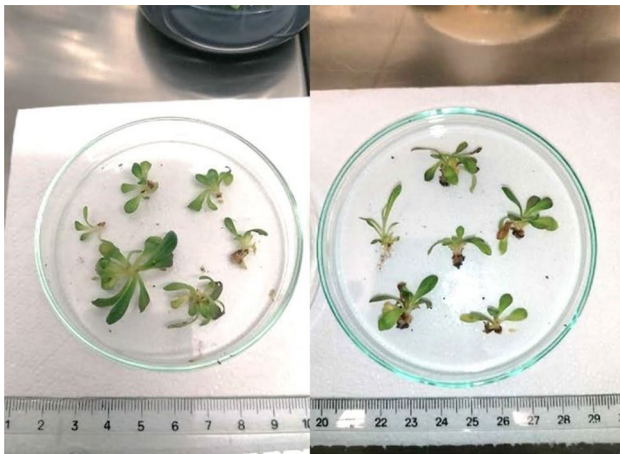


Fig. 4 Morphological appearance of *L. algarvense* of separated shoots after 60 days of culture in the second multiplication cycle on MS medium supplemented with 2.5 mg/L KIN + 0.1 mg/L NAA

L. catalaunicum) presented an opposite trend (Martín and Pérez 1995). Other work reporting the micropropagation of *L. sinuatum* also had good results on shoot multiplication using a combination of KIN and NAA, presenting 15.4–20 shoots per explant and 5.84–6.36 cm of average shoot height. Despite this, they did not observe signs of hyperhydricity when explants were cultured with IBA (Khadka et al. 2019). In turn, inflorescence explants from *L. cavanillesii* showed high multiplication rates when cultured with MS medium supplemented with 5 mg/L 2iP or KIN alone (9.4–10.0 shoots/explant, respectively) (Amo-Marco and Ibañez 1998), as well as nodal segments from seedlings of 5 *Limonium* species endemic to Iberian Peninsula that have successfully multiplied with a mixture of BAP and IBA (Martín and Pérez 1995). All these differences are probably related to this species' interspecific differences and ecological variations, despite belonging to the same family and genus.

The improved results observed after two multiplication cycles in this study could be attributed to the acclimatization of explants to in vitro conditions, a phenomenon well-documented in tissue culture research. During the first subculture, plantlets may undergo stress from the initial introduction to the artificial environment, which could hinder growth and multiplication. However, after one or more cycles, the explants often adjust to the microenvironment, leading to enhanced growth rates and shoot proliferation in subsequent cycles (George et al. 2008). This acclimatization process allows plants to better adapt to the hormonal and nutritional conditions of the culture medium, which may reduce physiological stress and promote more efficient resource allocation for shoot and root development (Sivanesan et al. 2021). Multiple studies have demonstrated that successive subcultures can improve shoot multiplication, particularly in recalcitrant species or those initially slow to

respond to tissue culture conditions (Mangal et al. 2019). Thus, the increased shoot formation observed in the second cycle aligns with broader evidence that successive subculturing helps overcome initial adaptation challenges and supports robust propagation in various plant species.

In this study, the highest shoot multiplication rate was achieved with MS medium supplemented with 2.5 mg/L kinetin (KIN) and 0.1 mg/L NAA, confirming the effectiveness of this cytokinin–auxin combination for *L. algarvense*. However, to further enhance multiplication efficiency, future efforts could focus on fine-tuning the hormonal balance, such as testing higher or lower concentrations of kinetin, or evaluating alternative cytokinins like zeatin, which has shown lower rates of physiological stress and improved shoot quality in other species (Masekesa et al. 2016). Additionally, supplementing the medium with organic additives such as coconut water or casein hydrolysate could also promote in vitro shoot multiplication (Miladinova-Georgieva et al. 2022). Refining environmental conditions, particularly light intensity or spectral composition, might also improve uniformity and shoot development (Cavallaro et al. 2022). In addition, elevated CO₂ concentrations during in vitro culture have been shown to stimulate shoot proliferation by enhancing photosynthetic activity and reducing photorespiration, ultimately promoting more vigorous and sustained shoot development (Pinheiro et al. 2021). Overall, improving the multiplication protocol based on the promising results from the KIN + NAA treatment can significantly increase the production of robust plantlets, reinforcing the potential of micropropagation as a tool for the conservation and restoration of this rare and endemic halophyte.

Rooting

Induction of a well-developed root system is a critical step of a successful micropropagation process, giving more chances for a successful establishment of plants into the soil and consequent survival to ex vitro acclimatization (Abdalla et al. 2022). The rooting performance of *L. algarvense* under different treatments is summarized in Table 2; Fig. 5.

In the control treatment (MS basal), as well as the MS medium supplemented with 5 µM IBA, no measurable root development was observed, and these treatments resulted in 100% browning of the plant tissue. This suggests that neither the MS basal medium nor the addition of IBA at this concentration was conducive to rooting, leading to complete tissue necrosis. In contrast, the addition of 2 g/L AC to the MS medium produced healthier outcomes. Plantlets grown in MS + 2 g/L AC showed a rooting percentage of 34.8%, with root lengths of 1.53 ± 0.17 cm and shoot heights of 1.66 ± 0.24 cm. The rosette diameter was also relatively large (1.73 ± 0.19 cm), and all plantlets were classified as

Table 2 Rooting response of *L. algarvense* shoots after 60 days of culture in different media: MS basal medium free of pgrs, MS supplemented with 5 μ M IBA, MS with 2 g/l AC, $\frac{1}{2}$ MS with 2 g/l AC, $\frac{1}{4}$ MS with 2 g/l AC, and MS supplemented with 10 mM CaCl_2

Treatment	Rooting (%)	Root length (cm)	Shoot height (cm)	Rosette diameter (cm)	Observations
MS basal	Nd	Nd	Nd	Nd	100% browning
MS + 5 μ M IBA	Nd	Nd	Nd	Nd	100% browning
MS + 2 g/L AC	34.8	1.53 ± 0.17^{ab}	1.66 ± 0.24^a	1.73 ± 0.19^b	Healthy, moderate vigor
$\frac{1}{2}$ MS + 2 g/L AC	15.2	1.76 ± 0.22^{ab}	1.54 ± 0.37^a	1.38 ± 0.11^b	Slightly pale, reduced vigor
$\frac{1}{4}$ MS + 2 g/L AC	13.5	1.26 ± 0.20^b	1.35 ± 0.06^a	1.04 ± 0.19^b	Small, weak rosettes
MS + 10 mM CaCl_2	67.6	2.12 ± 0.22^a	2.31 ± 0.22^a	3.47 ± 0.18^a	Vigorous, dark green, compact rosette

Nd: Not determined. Values correspond to mean $\pm$ SEM of three independent experiments ($n = 15$). Columns marked with different letters (a, b) are considered statistically different at $P < 0.05$ (Tukey's HSD)

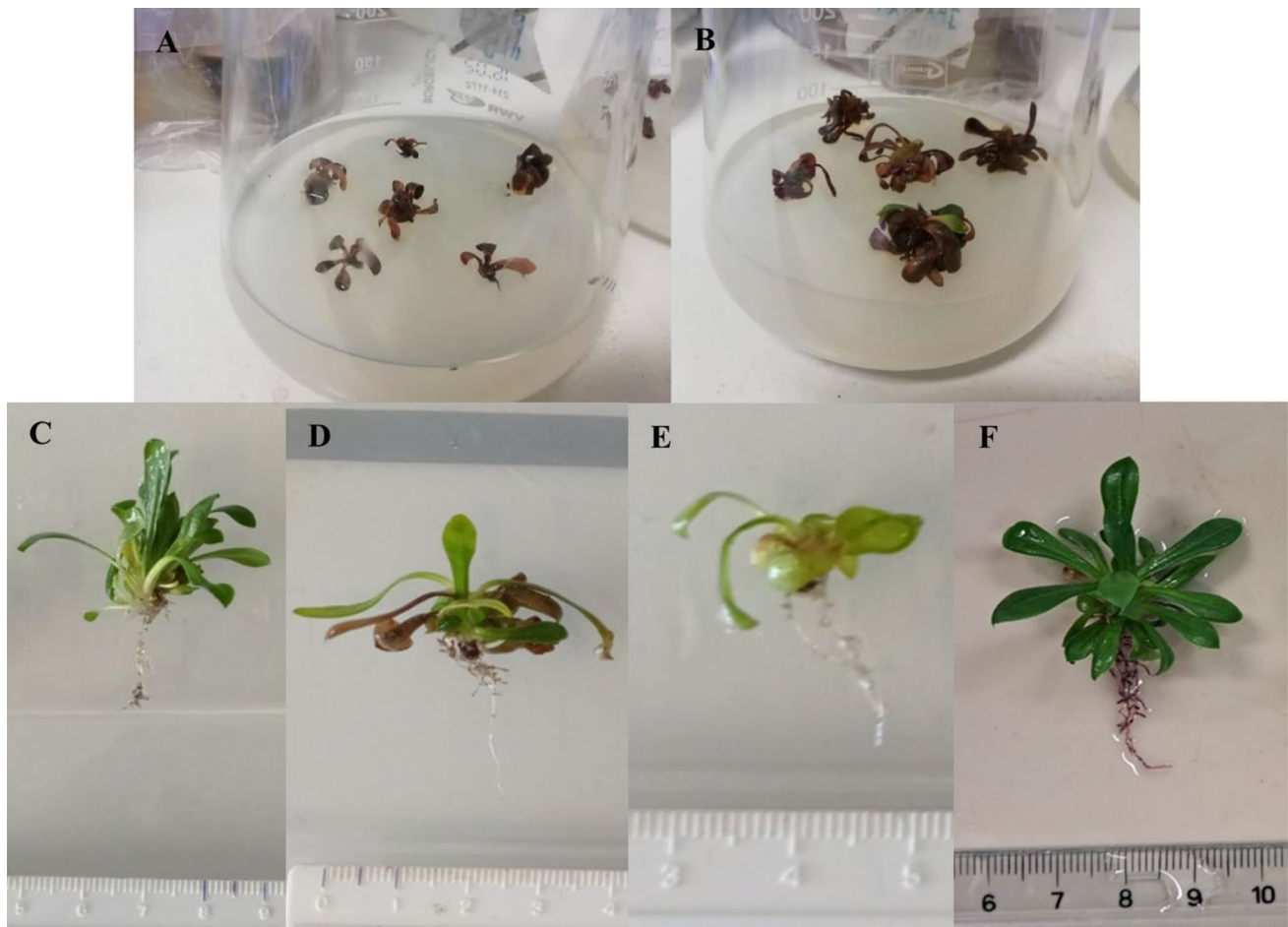


Fig. 5 Rooting response of *L. algarvense* shoots under different treatments after 60 days of culture. (A) MS basal medium without plant growth regulators (control), (B) MS supplemented with 5 μ M IBA,

(C) MS with 2 g/L AC, (D) $\frac{1}{2}$ MS with 2 g/L AC, (E) $\frac{1}{4}$ MS with 2 g/L AC, and (F) MS supplemented with 10 mM CaCl_2

healthy. Treatments with $\frac{1}{2}$ MS + 2 g/L AC and $\frac{1}{4}$ MS + 2 g/L AC led to root development, though with reduced rooting percentages (15.2% and 13.5%, respectively). While the root length and shoot height were comparable across these AC treatments, the rosette diameter was notably smaller in the $\frac{1}{4}$ MS treatment (1.04 ± 0.19 cm), suggesting a concentration-dependent effect on overall plantlet size. Moreover,

plantlets grown under these conditions appeared less vigorous, showing reduced turgor, paler leaf color, and overall weaker morphology. The most significant results were obtained with the treatment of MS + 10 mM CaCl_2 , where plantlets exhibited the highest rooting percentage (67.6%), along with superior root length (2.12 ± 0.22 cm), shoot height (2.31 ± 0.22 cm), and rosette diameter (3.47 ± 0.18

cm). These values were significantly higher than those recorded for the other treatments ($P < 0.05$), indicating that the addition of calcium chloride to the MS medium greatly enhanced plantlet growth and development. Plantlets from this treatment also exhibited a robust and healthy appearance, characterized by well-developed root systems, intense green leaf color, and compact, symmetrical rosettes, indicating strong physiological status. This further supports the efficacy of CaCl_2 in promoting successful in vitro plantlet propagation.

Overall, the results of this study demonstrate the significant role of calcium chloride in enhancing in vitro rooting, shoot growth, and overall plantlet development. The MS + 10 mM CaCl_2 treatment significantly improved plant growth parameters, such as root length, shoot height, and rosette diameter, compared to other treatments. This is consistent with recent studies showing the beneficial effects of calcium supplementation in tissue culture media. Calcium is a crucial signal transducer involved in regulating several physiological processes, including cell wall stabilization, membrane integrity, and enzyme activity, all of which are essential for cell growth and differentiation (Hepler 2005; Edel and Kudla 2016). In particular, calcium has been shown to play a key role in maintaining the structural integrity of plant cells and facilitating nutrient uptake, which may explain the increased rooting observed in the MS + 10 mM CaCl_2 treatment (Zhang et al. 2020). Research has confirmed that calcium ions stabilize cell membranes and cell walls by binding to pectin, a major component of the cell wall, which is necessary for maintaining cell structure and function during plant development (Hepler 2005; Baez and Bacete 2023). Additionally, calcium's role in modulating hormonal responses, such as auxin and cytokinin levels, may contribute to the improved shoot growth and overall plantlet health observed in this study (Gong et al. 2021; Liu et al. 2022).

The use of activated charcoal in the other treatments also contributed to healthy plantlet development, although its effects on growth were less pronounced than CaCl_2 . AC is known to absorb inhibitory phenolic compounds released during tissue culture, which can otherwise hinder plant development (Thomas 2008). This aligns with previous findings that AC improves medium conditions, allowing for better rooting and shoot growth by reducing toxicity in the culture environment (Pan and Van Staden 1998; Zhao et al. 2013). However, AC's effect may be limited in the absence of additional essential nutrients, such as calcium, that directly support cellular functions. On the contrary, treatments with MS basal medium and MS + 5 μM IBA resulted in 100% browning, indicating poor outcomes. Browning in plant tissue culture is often associated with oxidative stress and the accumulation of phenolic compounds, which may

not have been mitigated in the absence of AC or calcium (Gaspar et al. 1996). Recent studies emphasize the importance of balancing plant growth regulators, such as IBA, and nutrient supplements to prevent tissue damage and ensure successful regeneration in vitro (Singh et al. 2020).

Acclimatization

The establishment of in vitro-produced plants in soil and to the ex vitro conditions is a critical step, and thus, the acclimatization to the ex vitro environment must be as gradual as possible (Din et al. 2020). In this study, only plantlets obtained from the rooting treatment with MS medium supplemented with 10 mM CaCl_2 were selected for acclimatization. This treatment was chosen because it resulted in the highest rooting percentage and produced vigorous plantlets with well-developed roots and overall healthy morphology, increasing their likelihood of surviving under ex vitro conditions. The different stages of plantlet acclimatization, from early transfer to substrate through full establishment, are illustrated in Fig. 6. In the control group, 9.8% of the plants survived the acclimatization process, while the paraffin-treated group showed a slightly higher survival rate of 13%. Although this indicates a potential protective effect of paraffin, the difference was not substantial enough to demonstrate a clear benefit under the tested conditions. Paraffin acts as a film-forming anti-transpirant, creating a thin, transparent barrier on the leaf surface that reduces water loss through transpiration without significantly hindering gas exchange. This mechanism helps mitigate dehydration during the early ex vitro phase, when plantlets are particularly susceptible due to underdeveloped cuticles and impaired stomatal function (Suneetha et al. 2020).

The low survival rate during acclimatization may be related to insufficient ventilation, suboptimal substrate characteristics, and osmotic stress caused by the abrupt shift from in vitro to ex vitro conditions. High humidity and poor aeration may have impaired root function and increased leaf dehydration. These factors should be further investigated to improve plant establishment and survival.

To improve acclimatization success, future work should explore complementary strategies that address both physiological stress and environmental adjustment. A key approach involves optimizing the substrate, particularly by using slightly acidic formulations, more similar to in vitro culture conditions, to reduce transplant shock. Incorporating slow-release fertilizers or organic matter can also ensure a steady nutrient supply and promote consistent growth (Neri et al. 2022). Additionally, the inoculation with beneficial microorganisms, such as mycorrhizal fungi, may enhance root development and nutrient uptake, supporting better establishment (Dewir et al. 2023). The use of anti-transpirants or



Fig. 6 Representative stages of *L. algarvense* acclimatization from in vitro to ex vitro conditions. (A) Early stage after transferring to substrate, showing establishment of plantlets in individual cells of plastic cuvettes. (B) Intermediate stage, approximately 4 weeks after transfer, with visibly healthy shoots and signs of adaptation. (C and D) Detail of shoot development and root growth within the substrate during the

same intermediate phase. (E) Advanced stage, 8 weeks after transfer, showing a fully acclimatized plant with robust vegetative growth. (F) Final stage, after 150 days of growth under greenhouse conditions, with mature, well-established plants. The sequence illustrates the full acclimatization process and successful transition from in vitro culture to soil-based conditions

protective coatings, such as paraffin, which showed limited effectiveness in this study, could be optimized or replaced by alternatives like biodegradable polymer-based films (e.g., chitosan), natural wax emulsions, or stomatal-closing agents such as abscisic acid analogues. These options may offer improved permeability to gases, reduced interference with photosynthesis, and greater environmental compatibility due to their biodegradability (Pospíšilová et al. 2007). Moreover, raising CO₂ concentrations during early acclimatization may improve photosynthetic efficiency, reduce transpiration, and accelerate biomass accumulation, especially in C3 plants—a phenomenon known as the CO₂ fertilization effect (Pospíšilová et al. 2007). Among these strategies, substrate optimization and CO₂ enrichment appear particularly promising for *L. algarvense*, a halophytic species with sensitive root systems and high responsiveness to osmotic

balance. Together, these approaches have the potential to significantly enhance plant survival and growth during the critical transition from in vitro to ex vitro conditions.

Conclusions

The in vitro establishment, multiplication, and rooting protocols for the endemic and medicinal halophyte species *Limonium algarvense* were successfully developed from the shoot tips of 4-leaf seedlings. The small clusters of plants gave rise to new healthy vegetative shoots which were continuously multiplied to preserve living germplasm and produce large numbers of plants for restoration, conservation, and distribution of a “Near Threatened” species of medicinal importance. Although the overall protocol was effective,

survival under ex vitro conditions must be improved to increase the number of plants capable of successfully establishing themselves in soil. Still, the in vitro multiplication of the Iberian endemism *L. algarvense* represents a promising strategy for both conservation and sustainable use. Given its recognized medicinal and bioactive potential, the development of a reliable micropropagation protocol reduces the pressure on natural populations and enables a controlled and sustainable supply of plant material for future commercial or pharmaceutical applications. Overall, this work provides a potential foundation for future reintroduction programs or for the species' integration into local nursery production systems, contributing simultaneously to biodiversity preservation and bioeconomic valorization.

Author contributions MJR designed and supervised the whole study; MJR, LC, LL, and CM performed the experiments and contributed to data analysis and interpretation; and LC and MJR wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability Data will be made available on request.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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