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# Efficient In Vitro Platform for Multiplication, Acclimatization, and Deliver of High-NaCl-Tolerant Clones of the Halophyte *Arthrocaulon macrostachyum*

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**Abstract**
Halophytes are species able to grow and complete their life cycle under NaCl concentrations above 0.2 M. In a context of growing population and uncertain agricultural food sustainability, there is an interest on halophytes as potential source of food and fodder. However, scarce research has addressed in vitro propagation of halophytes as a tool for study, conservation, and propagation of elite germplasm. In this study, an efficient method for micropropagation of the succulent and salt accumulator halophyte *Arthrocaulon macrostachyum* has been established for the first time, using shoot tips from in vitro-germinated plant material. During shoot multiplication stage, superior genotypes were selected from explants grown in high strength and NaCl content medium and subsequently rooted and acclimatized to *ex vitro* conditions. A comprehensive characterization including determination of oxidative stress, photosynthesis performance, and mineral nutrient contents was done. This research gains insight into the physiological and biochemical characterization of halophytes during micropropagation and provides a solid platform for the production of elite *A. macrostachyum* germplasm for ulterior uses.

**Keywords** Acclimatization · Halophyte · Micropropagation · Oxidative stress · Photosynthesis · Salt tolerance

## Introduction

The Mediterranean basin is one of the main regions in terms of global biodiversity, whereas it is also among the most threatened regions by climate change, according to the International Union of Conservation of Nature IUCN (Hilton-Taylor et al. 2009). Recently, the estimated area of salt-affected soils in Europe was calculated to be over 2% of the total affected area in the world (Hassani et al. 2020). Concerning the Mediterranean area, a 25% of the irrigated area is salt affected (Ben Hamed et al. 2021a). In addition, lower water availability than the benchmark threshold of 1 dam3/person/year is reported for many Mediterranean countries (Mancosu et al. 2015). Considered as marginal lands, the salt-affected agricultural soils in the Mediterranean area could be subject of alternative solutions to mitigate this problem, as the use of salt-tolerant plants.

Halophytes are species able to grow and complete their life cycle under NaCl concentrations above 0.2 M (Flowers and Colmer 2008). A total of 350 species, mainly belonging to the orders Caryophyllales and Alismatales, have been listed (Flowers et al. 2010). Halophytes have a number of strategies to survive in saline environments, such as anatomic, morphological, and biochemical adaptations (Flowers and Colmer 2008); the latter including osmolyte accumulation, ion homeostasis, and up-regulation of antioxidant defenses (Ben Hamed et al. 2021b). In the last years, the nutraceutical and nutritional properties of halophytes have been well reported (Ben Hamed et al. 2005; Abideen et al. 2015; Ventura et al. 2015; Barreira et al. 2017). Among others, high contents of essential micronutrients, antioxidant compounds, essential fatty acids, and polysaccharides have been found (Ben Hamed et al. 2005; Ksouri et al. 2012;

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Abideen et al. 2015; Barreira et al. 2017; Duarte et al. 2019). In a scenario where a growing population has to deal with an uncertain agricultural food sustainability, there is an increasing demand of halophytes as potential source of food and fodder (Koyro et al. 2011; Centofanti and Bañuelos 2019). As a consequence, a growing body of research has been conducted in this topic worldwide (Duarte and Caçador 2021). The major number of publications to date have dealt with metabolite research and potential nutraceutical applications, followed by halophyte cultivation and sustainable agricultural solutions, halophyte ecology, and physiological- and biochemical-related research (Rodrigues et al. 2014; ElNaker et al. 2020; Ben Hamed et al. 2021a; Duarte and Caçador 2021). However, in spite of in vitro culture advantages for both fundamental and applied research purposes over traditional approaches – *e.g.*, controlled production in absence of microorganisms and higher multiplication rates of clonal plants –, very few papers, and mainly in the last decade, have addressed in vitro propagation of halophytes (Aly et al. 2002; Grigoriadou and Maloupa 2008; Joshi et al. 2012; Kulpa et al. 2020; López-Corona et al. 2019; de Jesus Raposo and de Morais 2021; Oliveira et al. 2016; Papafotiou et al. 2016; Regalado et al. 2020; Sedun et al. 2021; Singh et al. 2015; Vyas et al. 2021; Xiong et al. 2019; Yao et al. 2021). Therefore, there is still much scope for improving knowledge on halophyte in vitro culture, especially concerning multiplication of species with limited sexual and vegetative propagation, production of bioactive compounds, and propagation of endangered species (Gulzar et al. 2020; Custódio et al. 2023). Moreover, in vitro techniques are pointed out as the most suitable for screening of stress-tolerant genotypes in comparison to conventional screening tools (Rai et al. 2011; Singh et al. 2020). Major research on these topics would facilitate the integration of halophytes into a sustainable production system, by which a large number of plant clones could be provided along the year independently on seasonal variations and seed productivity.

Recently, molecular studies determined the separation of the North American and the Eurasian *Arthrocnemum macrostachyum* into two new genera, *Arthroceras subterminale* and *Arthrocaulon macrostachyum* (Ball et al. 2017; Piirainen et al. 2017). The latter is native of salt marshes of the Mediterranean basin (Murakeözy et al. 2007; Rodrigues et al. 2014; Duarte and Caçador 2021). In the Iberian peninsula, *A. macrostachyum* occurs in inland and coastal locations in the South (Castroviejo 2012). This halophyte is a succulent species characterized by articular stems with carnose segments, reduced and stem-united leaves (Castroviejo 2012). Regarding salt tolerance, it displays a wide tolerance of soil salinity, covering concentrations from 170 to 510 mM NaCl (Redondo-Gómez et al. 2010). Due to its high $Na^+$ accumulative capacity, *A. macrostachyum* has been proposed as a candidate for phytoremediation of hypersaline and sodic soils (ElNaker et al. 2020; Munir et al. 2021). Antibacterial, hypoglycemic, antioxidant, and anti-inflammatory properties have also been determined in *A. macrostachyum* extracts (ElNaker et al. 2020).

In this study, for the first time, we have achieved efficient micropropagation of *A. macrostachyum* using shoot explants derived from in vitro-grown seedlings. During shoot multiplication stage, superior genotypes were selected from explants grown in high strength and NaCl content medium and subsequently rooted and acclimatized to *ex vitro* conditions. A comprehensive characterization including determination of oxidative stress, photosynthesis performance, and mineral nutrient contents was done. This research gains insight into the physiological and biochemical characterization of halophytes during micropropagation and provides a solid platform for the production of elite *A. macrostachyum* germplasm for ulterior uses.

## Materials and Methods

### Plant Material Collection

Vigorous plants of A. macrostachyum were collected from an inland salt marsh in the Region of Murcia (Spain) (38°3′17.16″ N, 1°12′47.2″ W) in October 2020. Out of these plants, seeds from mature and dry inflorescences were obtained. Soil samples associated to the sampling spots were also taken using a soil sample drill at a depth of 10–15 cm.

### In Vitro Seed Germination

Seeds were stored at 4 °C for two months to overcome the presence of seed dormancy. Then, seeds were disinfected by successive inmersions in a 20% commercial bleach solution containing 0.05% tween-20 for 20 min, esterilized distilled water (×3), 70% ethanol for 1 min, and esterilized distilled water (×3). Subsequently, seeds were placed onto germination (G) media, consisting of half-strength Murashige & Skoog solid (MS) (Murashige and Skoog 1962) medium with or withouth the addition of 7.5-g $L^{-1}$ NaCl (Table 1) in polypropylene boxes equipped with a filter to allow gas exchange (Duchefa Biochemie, Haarlem, the Netherlands). Boxes were incubated at 25 °C under white light fluorescence lamps (16-h photoperiod, 80 µmol $m^{-2}$ $s^{-1}$). Four weeks later, germination percentage was calculated and the most vigourous seedlings used in multiplication.

### In Vitro Multiplication, Salt Screening, and Rooting

In vitro seedlings of A. macrostachyum (3 to 5 cm long) were excised into 1.5 cm length segments and placed half embedded into multiplication (M) medium, which contains

**Table 1** Composition of media used for the in vitro germination (*G*), salt screening (*S*), regular multiplication (*M*), and rhizogenesis (*R*) of *Arthrocaulon macrostachyum*

| Component [g $L^{-1}$] | Medium | | | |
|---|---|---|---|---|
| | G | S | M | R |
| MS salts (including vitamins) | 2.2 | 8.8 | 8.8 | 3.3 |
| MES monohydrate | 0.5 | 0.5 | 0.5 | 0.5 |
| Saccharose | 30 | 30 | 30 | 60 |
| NaCl | 0; 7.5 | 0; 7.5; 15; 30; 60; 120 | 30 | |
| BAP | – | $0.001 \times 10^{-3}$ | $0.001 \times 10^{-3}$ | – |
| NAA | – | $0.002 \times 10^{-3}$ | $0.002 \times 10^{-3}$ | – |
| Kinetin | – | – | – | 0.0001 |
| IBA | – | – | – | 0.0005 |
| Casein hydrolysate | – | 0.15 | 0.15 | 0.15 |
| Sequestrene | – | 0.03 | 0.03 | 0.03 |

In all cases, 8-g $L^{-1}$ agar was added and pH was adjusted to 6. All components were obtained from Duchefa Biochemie (Haarlem, the Netherlands). Growth regulators [6-Benzylaminopurine (BAP); 1-Naphthaleneacetic acid (NAA); kinetin; and Indole-3-butyric acid (IBA)] were added from 1-g $L^{-1}$ stock solutions

30-g $L^{-1}$ NaCl (Table 1), in the same boxes and environmental conditions as for seed germination. Then, regular subcultures every fourth to fifth week were done under the same conditions, using tip and axilary node shoot segments. In parallel, the response of explants to salinity was tested in a range of concentrations from 0 to 120-g $L^{-1}$ (Table 1).

Vigorous explants having high proliferation rate (determined as new formed shoots) and stem elongation were selected along four to six subcultivation cycles in M medium. Subsequently, such clones were transferred into rooting (R) medium (Table 1) in 220-mL screw cap containers (Deltalab, Barcelona,Spain). Rhizogenesis was then followed over a 2-month period.

## Acclimatization

Plantlets were carefully washed to remove the agar and placed in pots in a esterilized mix of peat/perlite (2/1, v/v), inside an acclimatization chamber (UBBINK propagator, Northampton, UK), which displays two ventilation grills for the control of relative humidity (RH). Then, acclimatization was followed over an 8-week period. Environmental conditions were as follows: 16-h photoperiod and 25 °C under white light fluorescence. The light intensity was progresively increased from 80 µmol $m^{-2}$ $s^{-1}$ (day 0) to 140 µmol $m^{-2}$ $s^{-1}$ (from day 35 forward). In parallel, the ventilation grills were gradually open to decrease the humidity progressively. Substrate was moistened either with distilled water or a 30-g $L^{-1}$ NaCl solution from day 1. A systemic fungicide–bactericide (Beltanol-L, Probelte, Murcia, Spain) at 0.1% (v/v) was sprayed to the plantlets at days 0, 2, 5, and 7.

## Nutrient Analysis

Fresh plant material (shoots and roots) and consociated soil samples from field and explants (shoot clusters) obtained at the end of the in vitro salt screening were dried to a constant weight at 60 °C for 2 days. Then, samples were ground into a fine powder using a blender. One gram of the ground powder was used to measure the different macronutrients and micronutrients, by Inductively Coupled Plasma–Optical Emission Spectrometry (ICP–OES) using a ICAP 6000SERIES spectrometer (Thermo Scientific, Madrid, Spain) according to standardized protocols (Ionomic Services of CEBAS-CSIC; Thermo Scientific, Madrid, Spain).

## Measurement of Chlorophyll Fluorescence

Chlorophyll fluorescence was determined in the plant leaves with a chlorophyll fluorimeter (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany) during the acclimatization period at days 0, 2, 5, 7, 14, 21, 28, 35, 42, 49, and 56. Plants were incubated in darkness for 20 min before the minimum and maximal fluorescence yields were determined. Kinetic analyses were done with actinic light (81 µmol quanta $m^{-2}$ $s^{-1}$ PAR) and repeated pulses of saturating light at 2700 µmol quanta $m^{-2}$ $s^{-1}$ PAR for 0.8 s, at intervals of 20 s. The following parameters were also analyzed: effective PSII quantum yield [Y(II))]; non-photochemical quenching (NPQ); and coefficients of non-photochemical quenching (qN) and photochemical quenching (qP) (Acosta-Motos et al. 2019).

## Determination of Lipid Peroxidation Levels

The extent of lipid peroxidation was estimated based on the determination of thiobarbituric acid-reactive substances

(TBARS) (Barba-Espín et al. 2014), using a UV/Vis V-630 Bio spectrophotometer (Jasco, Tokyo, Japan). In brief, samples (0.2 g) were homogenated in 1-M perchloric acid (1/10, w/v) followed by centrifugation at 12,000 × g for 10 min. The resulting supernatant was mixed with 0.5% thiobarbituric acid in 1-M perchloric acid (1/4, v/v) and incubated at 90 °C for 20 min. Subsequently, samples were centrifuged at 10,000 × g for 5 min, and supernatant was used to determine TBARS based on the extinction coefficient 155 $mM^{-1}$ $cm^{-1}$. Samples consited of shoot clusters at the end of the in vitro salt screening and plantlets (roots and aerial part) during the acclimatization to ex vitro conditions.

### Statistical Analysis

Statistical analysis were done with at least four biological replicates. Data were compared using a two-way analysis of variance (ANOVA) followed, when suitable, by a Duncan post hoc test ($p \leq 0.05$), using the SPSS Statistics software version 27 (IBM, Endicott, NY, USA).

## Results and Discussion

In the present work, we have explored in vitro culture as a tool for micropropagation and physiological characterization of elite germplasm, in terms of salt tolerance, of *A. macrostachyum*.

Firstly, disinfected seeds were germinated in vitro on G medium in the absence or the presence (7.5-g $L^{-1}$) of NaCl (Fig. 1). The highest germination rate (78%) was achieved at 28 days in the absence of salt, whereas, at the same day, 7.5-g $L^{-1}$ NaCl caused germination to reach 57% (Fig. 1A). Moreover, early seedling growth and vigor was visually superior in the absence of NaCl (Fig. 1B). Salinity tolerance of *A. macrostachyum* seeds have been reported to reach 1M (Ajmal Khan and Gul 1998); however, it is well reported that seed germination of most halophytes is optimal in fresh water and is reduced in the presence of salts, although salinity is not necessarily toxic at this stage as seeds may recover and germinate when transferred to less saline conditions (Chadwick 1981; Ungar 2001). More recently, germination

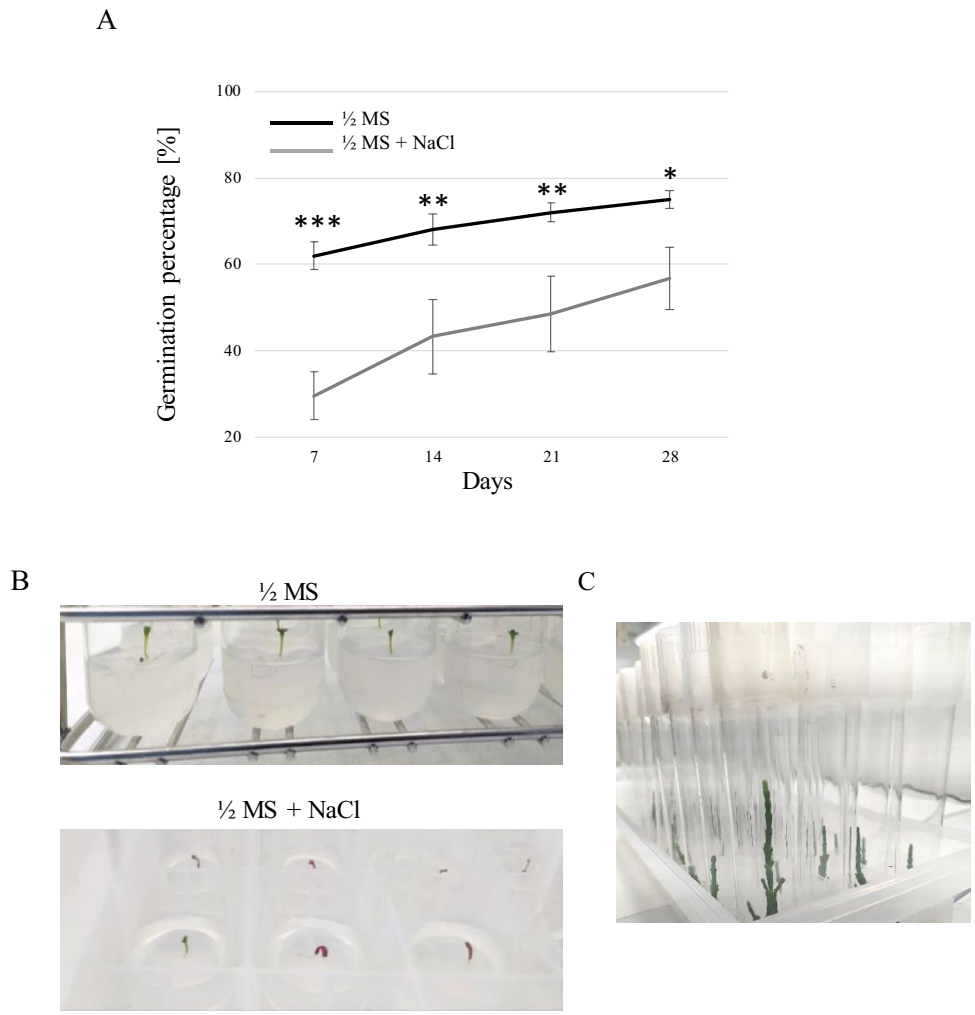



**Fig. 1** In vitro germination and seedling growth of *Arthrocaulon macrostachyum*. **A** Seed germination percentage in the presence or absence of NaCl during 28 days. Data represent the mean ± standard error, $n = 5$. **B** Seedlings at day 10 in the presence or absence of NaCl. **C** Seedlings at day 40 in the absence of NaCl utilized for multiplication. Asterisks denote significant differences between pairwise comparisons (½ MS *vs*. ½ MS + NaCl) at the 99.9% (***), 99% (**), or 95% (*) level of probability from two-way ANOVA

experiments in *Salicornia* spp. and *Sarcocornia* spp. showed that the percentage germination was superior at lower salinities and temperatures of 20/10 °C (day/night) (Singh et al. 2014). This has been related to ecology aspects, since seed germination in European coastal halophytes occurs in early spring, when temperatures are lower and salinity is reduced by high soil moisture content (Ajmal Khan and Weber 1986).

Therefore, our results are in accordance with the higher germination and seedling growth for halophyte species found at low salinity.

Shoots derived from in vitro-grown seedlings are frequently used in micropropagation of different species, including halophytes (Binh et al. 1989; Wang et al. 2005; Sun and Hong 2009; Reyes-Vera et al. 2010). In this study, forty-day-old seedlings in the absence of NaCl were utilized for multiplication, by placing 1.5 cm length segments into M medium, which had a high salinity and osmotic pressure due to its content of NaCl (30-g $L^{-1}$), sucrose (30-g $L^{-1}$), and MS salts (double strength). To our knowledge, no previous research conducted micropropagation on a medium having a similarly high nutrient concentration. This medium also contains BAP and NAA, as growth regulators frequently used in the micropropagation of halophyte and glycophyte species, at concentrations previously tested (Joshi et al. 2012; Acosta-Motos et al. 2019; Lee et al. 2019; Custódio et al. 2023). Moreover, based on preliminary trials (data not show), casein hydrolysate, as a growth adjuvant, and sequestrene, as an iron chelate form (Ahmad and Anis 2005; Clapa et al. 2018; de Jesus Raposo and de Morais 2021), were added to the medium.

A gradual habituation of the explants to the in vitro conditions was achieved over the successive multiplication cycles; at the end of the third subculture on M medium, a stable multiplication behavior of the explants was achieved, characterized by a high number of explants forming new shoots and a proliferation rate ≥ 5 (Fig. 2).

During ex situ conservation of wild plants from salt marshes using tissue culture, NaCl has been found to be a critical factor for in vitro propagation (Martini and Papafotiou 2020). In this study, the response of explants to salinity (0- to 120-g $L^{-1}$) was tested in terms of average biomass per explant and lipid peroxidation levels (Fig. 3). In this sense, the average biomass per explant was statistically equivalent for most of the salt concentrations, whereas only the highest concentrations (60- and 120-g $L^{-1}$ NaCl) led to a significant decrease in the biomass, of about 75% with respect to the explants grown in the absence of NaCl (Fig. 3A). The levels of lipid peroxidation are a useful target for assessment of oxidative stress in plants. In halophytes, lipid peroxidation has been mostly evaluated by measuring TBARS in in vivo studies (Ozgur et al. 2013). In this sense, the damage produced by salinity was found to vary with the species, the saline levels (from 8.8- to 58-g $L^{-1}$ mM),

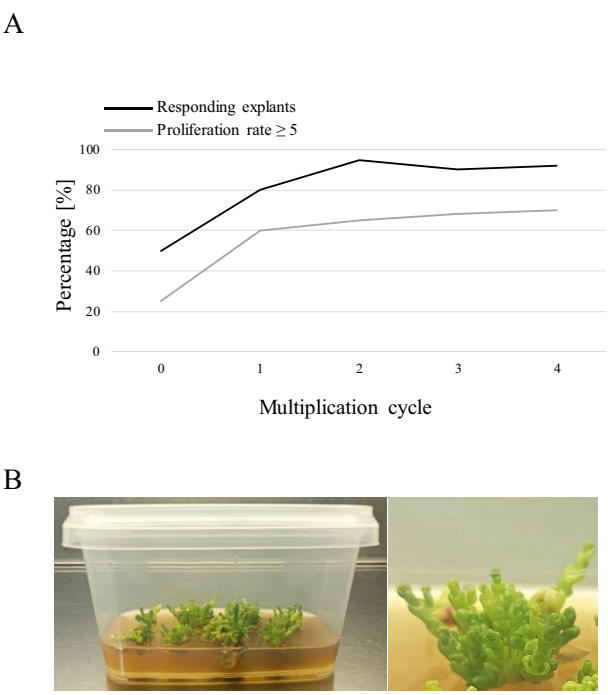



**Fig. 2** Proliferation of *Arthrocaulon macrostachyum* shoots after four weeks of culture in multiplication medium. **A** Percentages of responding explants and explants forming at least 5 new shoots, along the successive multiplication cycles. **B** View of several shoot clusters in polypropylene boxes and detail of a shoot cluster

and the duration of the treatment (Ozgur et al 2013). In this study, lipid peroxidation was estimated to be lower at intermediate concentrations (15- and 30-g $L^{-1}$ NaCl), whereas it was highest in 120-g $L^{-1}$ NaCl, followed by 60- and 0-g $L^{-1}$ NaCl (Fig. 3B). Moreover, the extent of lipid peroxidation was positively associated to a reduced development of the explants and the presence of necrosis symptoms (data not shown). This may indicate that intermediate NaCl concentrations do not induce a damaging oxidative burst; on the contrary, the presence of this salt levels can be necessary for an optimal micropropagation, as it is for growth stimulation of in vivo plants (Ben Hamed et al. 2021a).

Additionally, a mineral nutrient analysis was conducted in the explants in response to the different salt concentrations (Table 2; Supplementary Table 1). The analysis of the macronutrients showed an increase of Na level with the NaCl concentration, ranging from 24.5- to 216-g $kg^{-1}$ dry weight (DW). On the other hand, overall, the rest of the macronutrients analyzed decreased their concentration with the NaCl level, this effect being more pronounced at salt concentrations above 30-g $L^{-1}$ (Table 2). Likewise, the content of most of the micronutrients detected were reduced along with the increase in NaCl concentration (Supplementary Table 1). There are a lack of data reporting mineral nutrient composition in halophytes in response to increasing salinity in vitro.

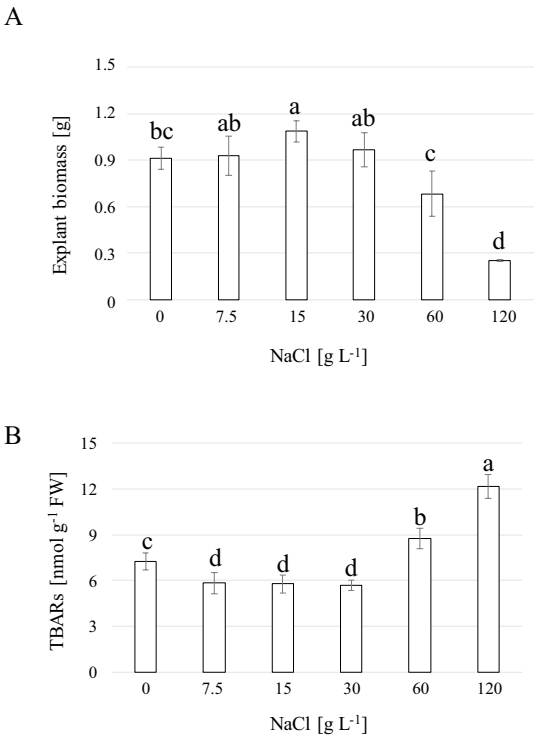



**Fig. 3** Physiological response of *Arthrocaulon macrostachyum* explants to different NaCl concentrations in the medium after three weeks of incubation in vitro. **A** Biomass per explant cluster. **B** Determination of thiobarbituric acid-reactive substances (TBARS). Data represent the mean ± the standard error of 4 replicates, each one consisting of the pool of explants of a box. Different letters indicate statistical significance according to Duncan's test ($p \leq 0.05$)

However, it is well reported that nutrient availability and uptake by plants are influenced by salinity; in this sense, deficit or imbalance of nutrients can be provoked by competition with $Na^+$ and $Cl^-$ in glycophytes and halophytes (Flowers and Colmer 2008; Ehtaiwesh 2022). Accordingly, the concentrations of $K^+$, $Ca^+$, and $Mg^+$, among other nutrients, descended in *Salicornia ramosissima* in function of salinity (Lima et al. 2020). Similarly, NaCl irrigation reduced the contents of mineral nutrients of *A. macrostachyum* and *Salicornia europaea* plants grown under greenhouse conditions (Ushakova et al. 2005; Redondo-Gómez et al. 2010).

Complementarily, plants from which seeds were originally collected and consociated soil samples were also subjected to the same nutrient mineral analysis (Supplemental Tables 2 and 3), with the aim of establishing a comparison between the nutrient profile of in vivo and in vitro conditions. The shoots and the roots were collected in the flowering period. In this regard, overall, macronutrients analyzed showed a higher concentration in the aerial part shoots, with the exception of $Mg^{2+}$, whose levels were markedly higher in the soil. The analysis showed a high content of Na in the aerial shoots and its concentration being several times lower in the roots (Supplemental Table 2). This is in accordance with the salt tolerance mechanism of *A. macrostachyum*, whose $Na^+$ accumulation relies mainly in the vacuoles (Khan et al. 2005; Redondo-Gómez et al. 2010). Interestingly, $Na^+$ contents in the shoot (81.2-g $kg^{-1}$ DW) and soil (15.5-g $kg^{-1}$ DW) resemble the corresponding levels found in the explants (86.2-g $kg^{-1}$ DW) when exposed to 15-g $L^{-1}$ NaCl in the medium (Table 2), which suggest that micropropagation in the present study have been conducted at higher NaCl concentration (30-g $L^{-1}$) than the observed in field conditions. Concerning micronutrients (Supplemental Table 3), soil Al and Fe were detected at very high concentrations (14- and 8.6-g $kg^{-1}$ DW, respectively); however, these levels were not reflected in a high accumulation in the plant, which may result from a low availability of these elements in the soil. Halophytes have stronger metal accumulation capacity than glycophytes (Peng et al. 2022). In this sense, the accumulation of Pb and Ni was nearly 10 times higher in the root than in the shoot, which is in accordance with the reported ability of certain halophytes for immobilizing heavy metals in the root system (Caparrós et al. 2022). In addition, Cu and $Zn^{2+}$ were accumulated at equivalent concentrations in the plant and in the soil (Supplemental Table 3). These results suggest an uninvestigated potential of *A. macrostachyum* in heavy metals phytoremediation.

Subsequently, vigorous explant clusters that had been multiplied in 30-g $L^{-1}$ NaCl for four to six cycles were

**Table 2** Macronutrients detected by Inductively Coupled Plasma–Optical Emission Spectrometry in *Arthrocaulon macrostachyum* explants

| NaCl [g L−1] | Macronutrients [g kg−1 dry weight] | | | | | |
|---|---|---|---|---|---|---|
| | Ca | K | Mg | Na | P | S |
| 0 | 3.58 ± 0.08a | 25.9 ± 0.5ab | 0.88 ± 0.02a | 24.5 ± 1.3e | 2.31 ± 0.09a | 3.03 ± 0.07a |
| 7.5 | 2.36 ± 0.06b | 30.0 ± 0.39a | 0.79 ± 0.01b | 76.0 ± 3.7d | 2.44 ± 0.15a | 3.21 ± 0.04a |
| 15 | 1.79 ± 0.47bc | 25.6 ± 1.3ab | 0.55 ± 0.05c | 86.2 ± 9.1d | 1.77 ± 0.03b | 2.92 ± 0.33ab |
| 30 | 1.68 ± 0.01bc | 23.2 ± 2.7bc | 0.54 ± 0.04c | 142 ± 9c | 1.43 ± 0.04c | 2.44 ± 0.15bc |
| 60 | 1.58 ± 0.17bc | 18.9 ± 0.75c | 0.43 ± 0.01d | 180 ± 18b | 1.09 ± 0.03d | 1.96 ± 0.08c |
| 120 | 1.24 ± 0.04c | 6.92 ± 0.47d | 0.28 ± 0.02e | 216 ± 5a | 0.55 ± 0.05e | 1.32 ± 0.08d |

Data are presented as the mean ± standard error, $n = 4$. Different letters denote statistical significance according to Duncan's test ($p \leq 0.05$)

transferred to R medium, where rooting was followed over an 8-week period (Fig. 4). As a result, over 75% and 80% of explants were rooted after 45 and 60 days, respectively (Fig. 4A). This is the first study reporting in vitro rooting of *A. macrostachyum*; on the other hand, in vitro rhizogenesis have been reported for *Salicornia* spp. (Joshi et al. 2012; Lee et al. 2019). After 60 days, plantlets having a profuse root system (Fig. 4B) were used for the acclimatization to *ex vitro* conditions. In this sense, a 90% of plantlets survived after 8 weeks of acclimatization, regardless of whether plants were subjected to water or NaCl irrigation (data not shown). Similarly, high acclimatization percentages have been reported for other halophytes such as *Limoniastrum monopetalum* (Martini and Papafotiou 2020).

During the acclimatization process, plantlets are usually subjected to light intensities progressively higher, which might induce leaf wilting. Moreover, the transfer to *ex vitro* conditions may lead to a transient decrease in photosynthetic parameters (Kshitij 2012). On the other hand, in many halophytes, photosynthesis was shown to be unaffected by salinity, or even stimulated at low salt concentrations (Kurban et al. 1999; Parida et al. 2004). In this work, the evolution of chlorophyll fluorescence variables was monitored (Figs. 5 and 6), in order to associate the state of photosynthesis with the progression of the acclimatization. Visually, fluorescence data were transformed onto a false color system in which plants were colored from black (0) to magenta (1) (Fig. 5A). In this sense, decreased levels of both photochemical [Y(II) and qP] and non-photochemical quenching (NPQ and qN) variables, as well as of ETR, could be noted until day 7, followed by a progressive increase until the end of the experiment. Moreover, no substantial differences were observed between NaCl and water irrigation for any of the variables measured (Figs. 5 and 6).

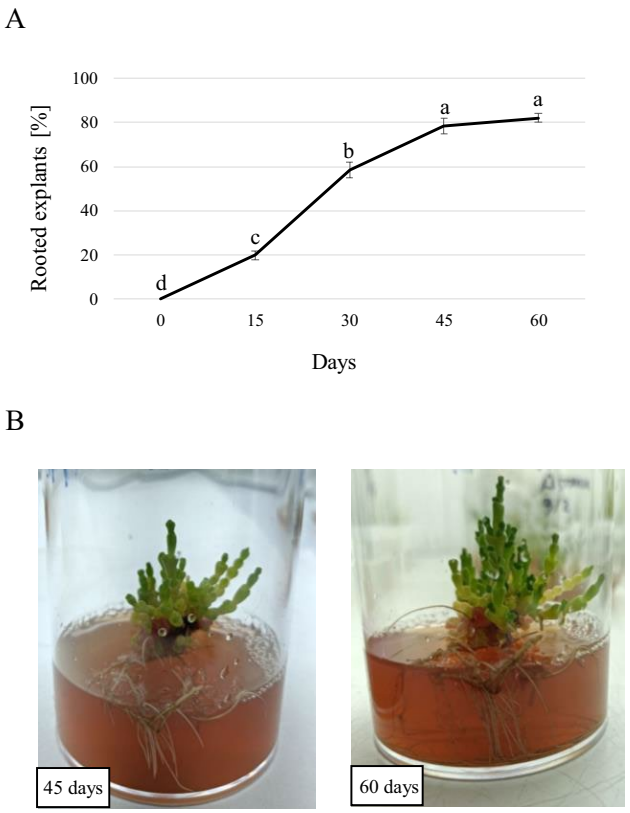


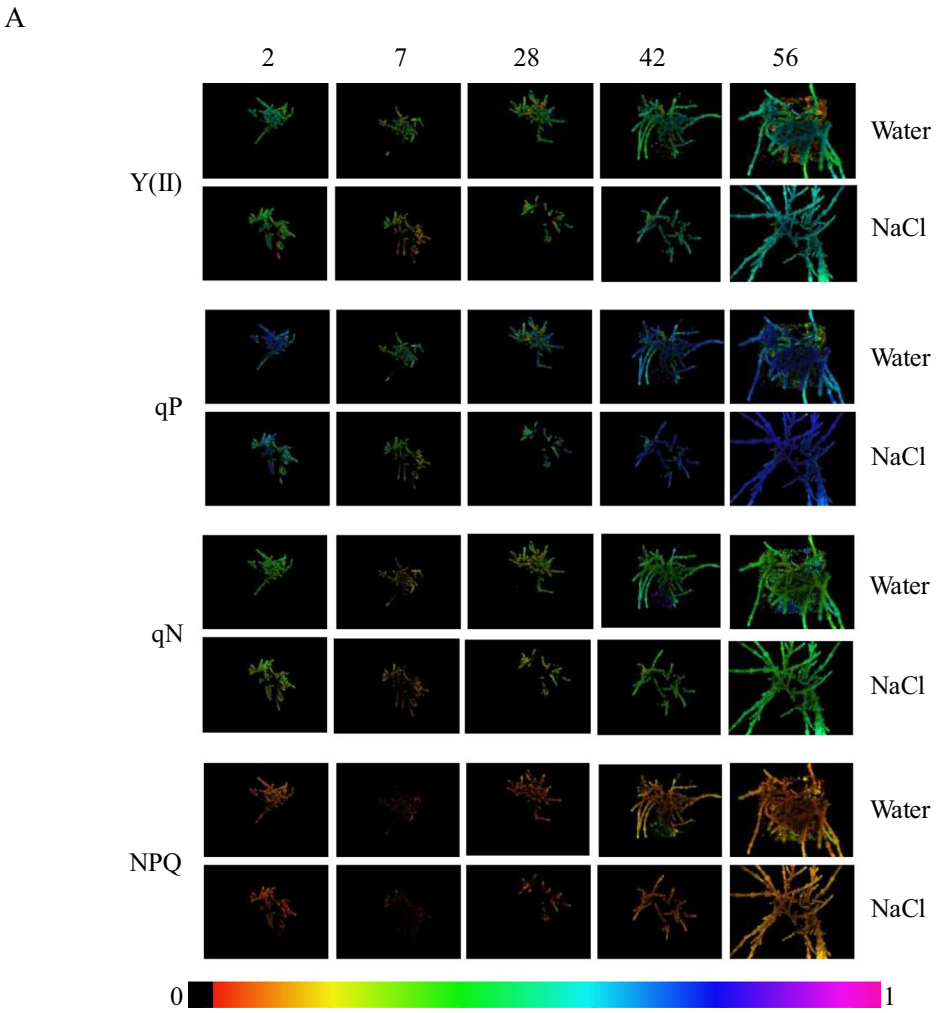

**Fig. 4** In vitro rooting of *Arthrocaulon macrostachyum* explants. **A** Percentage of rooted explants over a 60-day period. **B** Representative images of a plantlet showing root development at 45 and 60 days. Data represent the mean ± standard error of 2 experiment repetitions, each one consisting of the percentage calculated using 20 individual specimens. Different letters indicate statistical significance according to Duncan's test ($p \leq 0.05$)

Over the whole period, a remarkable increase in plant size could also be observed, especially from day 28 (Fig. 5A), which, likewise, reflects adaptation to *ex vitro* conditions (Kshitij 2012). In most plant species, leaves grown under in vitro conditions are unable to develop further under *ex vitro* conditions, and newly formed leaves are necessary to complete plant adaptation to *ex vitro* conditions (Pospíšilová et al. 1999; Kshitij 2012). However, due to *A. macrostachyum* leaf morphology, consisting of minor fleshy scales covering the stems, leaf decay was not observed under our experimental conditions. This was also reflected on a homogeneous coloration of the whole plantlet for the different photosynthetic quenching variables (Fig. 5A).

Complementarily, the levels of lipid peroxidation, an indicator of oxidative stress, were measured in leaves and roots of plantlets subjected to acclimatization at days 0, 1, and 56 (Fig. 7). The first days of the acclimatization process are the most critical to the survival of the plantlet (Kshitij 2012). In this sense, during *ex vitro* acclimatization of *Stevia rebaudiana*, a peak of leaf lipid peroxidation was observed at day 2, followed by a decrease to initial values, which was related to a transient stress due to an excess of light and a drop in relative humidity (Acosta-Motos et al. 2019). In this study, lipid peroxidation levels increased at day 1, especially in leaves, whereas at the end of the experiment, these levels decreased to the observed at day 0. Moreover, no differences were observed between NaCl and water irrigation (Fig. 7). Two months after the finalization of the experiment, plants displayed a normal development and yet no visual differences between NaCl and water irrigation were observed (Supplemental Fig. 1).

## Conclusion

This work reports for the first time an efficient micropropagation scheme for a halophyte comprising multiplication, rooting and acclimatization to *ex vitro* conditions. NaCl concentration was found to be critical for explant multiplication,


A
2
7
28
42
56
Y(II)
qP
qN
NPQ
Water
NaCl
0
1
B
Water
NaCl
Y(II)
qP
qN
NPQ
Days
0 1 2 5 7 14 21 28 35 42 49 56

◂**Fig. 5** Evolution of photochemical [Y(II) and qP] and non-photochemical quenching (NPQ and qN) values during the acclimatization of *Arthrocaulon macrostachyum* plants to *ex vitro* conditions over an 8-week period. **A** Colored images representing fluorescence intensity from black (0) to magenta (1) for the different variables at selected time points. **B** Data recorded at the different times, representing the mean ± the standard error, $n = 4$

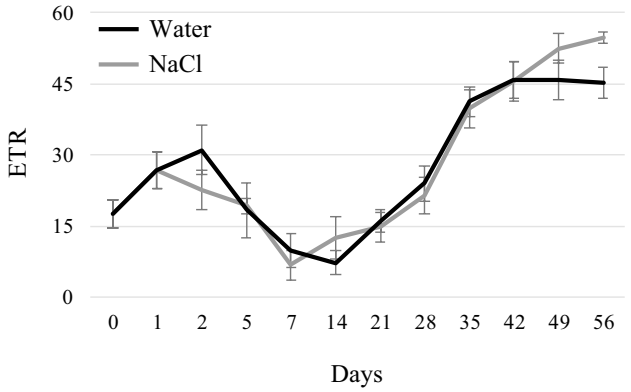



**Fig. 6** Evolution of the electron transport rate (ETR) during the acclimatization of *Arthrocaulon macrostachyum* plants to *ex vitro* conditions over an 8-week period. Data represent the mean ± the standard error, $n = 4$

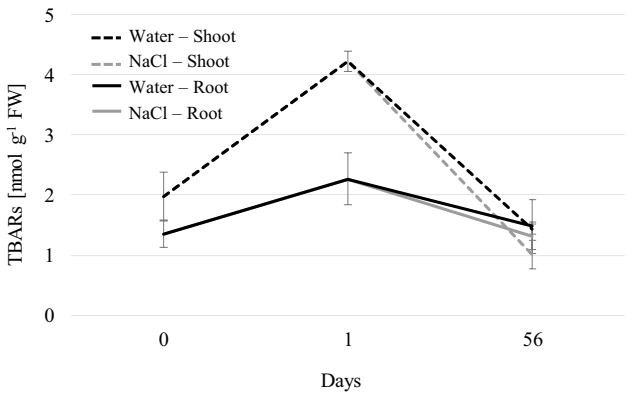



**Fig. 7** Determination of thiobarbituric acid-reactive substances (TBARS). Data represent the mean ± the standard error, $n = 4$

since the absence of NaCl and the highest contents (60- and 120-g $L^{-1}$) affected growth and nutrient contents and caused an oxidative stress in the explants. Elite germplasm was selected by its superior performance on 30-g $L^{-1}$ NaCl-containing high-strength medium (equivalent to 513-mM NaCl) over several multiplication cycles. After a profuse rooting of selected germplasm was obtained, acclimatization to *ex vitro* conditions was characterized using photosynthesis fluorescence parameters as a marker of the process. As a prospect, the obtained halophyte clones can be used for phytoremediation of salt-contaminated soils.

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## Declarations

**Conflict of interest** The authors declare no conflict of interest.



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