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Cultivation of *Salicornia lagascae* in High-NaCl Liquid Medium Induces a Metabolic Reprograming and Enhances Micropropagation Performance

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Abstract

This study establishes the first efficient in vitro micropropagation protocol for the halophyte *Salicornia lagascae* and provides new insights into its responses to salinity and culture system conditions. Moderate salinity enhanced explant proliferation, with 30 g L^{-1} NaCl identified as the optimal concentration, under which explants maintained stable photosynthetic performance; explants accumulated Na^+ in a concentration-dependent manner during salt tolerance screening. Liquid culture systems showed clear advantages over solid media, promoting enhanced micronutrient accumulation, improved rhizogenesis, and greater plant growth after acclimatization, supporting their potential for efficient micropropagation. Metabolomic analyses revealed that medium consistency strongly induced a metabolic reprogramming, with solid media favoring sugar and phenylpropanoid accumulation. In contrast, liquid culture triggered the accumulation of some stress-related secondary metabolites, including betaine aldehyde in shoots and quercetin derivatives in roots, alongside the secretion of signaling molecules such as 7-epijasmonic acid into the medium. Collectively, these findings demonstrate that liquid culture induces stress-adaptive mechanisms and secondary metabolic pathways, providing an efficient platform for the micropropagation and selection of high salt-tolerant *S. lagascae* clones. Despite these advantages, further field validation and optimization of liquid culture systems will be required to facilitate translation to large-scale or commercial applications.

Key message

Optimized salinity and liquid culture conditions improved biomass and rooting while triggered a metabolic reprogramming in *Salicornia lagascae*, promoting the accumulation and secretion of stress-related metabolites.

Keywords Liquid medium · Root plasticity · *Salicornia lagascae* · Salt tolerance · Secondary metabolism

Introduction

The Mediterranean basin is recognized as a significant region for global biodiversity; however, it is also one of the most vulnerable areas to the impacts of climate change. Recent assessments indicate that salt-affected soils in Europe comprise over 2% of the world's total affected land area (Hassani et al. 2020). Specifically, within the Mediterranean region, approximately 25% of irrigated lands are impacted by salinity (Ben Hamed et al. 2021). Furthermore, many Mediterranean countries experience water availability below the critical threshold of 1 dam^3/person/year (Mancosu et al. 2015).

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The intensification of irrigation practices is linked to soil salinization. Freshwater resources are severely limited. In order to satisfy increasing agricultural demands to cope with growing global population, agriculture rely on low quality water sources is increasing. The electrical conductivity (EC) of irrigation water in salinity affected countries reveals a consistent upward trend (Palmgren and Shabala 2024). Reversing soil salinization will require either the withdrawal of affected land from cultivation for a while or the implementation of phytoremediation strategies using halophytic plant species, this latter approach of increasing interest, owing to its potential to alleviate salt-induced soil degradation (Slatni et al. 2024; Jurado et al. 2024; Santin et al. 2024).

Halophytes are defined as plant species capable of thriving and completing their life cycles in environments with NaCl concentrations exceeding 0.2 M (Flowers and Colmer 2008). A total of 350 halophyte species, primarily from the orders Caryophyllales and Alismatales, have been documented (Flowers et al. 2010). These plants employ various strategies to endure saline conditions, including anatomical, morphological, and biochemical adaptations (Flowers and Colmer 2008). The biochemical adaptations encompass osmolyte accumulation, ion homeostasis, and the enhancement of antioxidant defenses (Ben Hamed et al. 2021). The Amaranthaceae family (formerly known as Chenopodiaceae) groups different species that present pharmacological and nutraceutical properties (Lefèvre and Rivière 2020). A major genus in this family is *Salicornia* L., which includes annual succulent halophyte plants whose stems and seeds are edible and a remarkable source of nutraceutical and pharmacological compounds (Cárdenas-Pérez et al. 2021; Barreira et al. 2017; Lima et al. 2020). Regarding salt tolerance, Salicornia species are capable of thriving in environments with varying salinity, from mildly saline to hyper-saline conditions (up to 1 M NaCl), making them well-suited for growth in coastal and saline ecosystems (Flowers and Colmers 2008). Recent taxonomic revision has separated *Salicornia lagascae* from the genus *Sarcocornia* (Rufo et al. 2016). *S. lagascae* is native of salt marshes and beaches in the Iberian Peninsula (Piirainen et al. 2017).

In recent years, the nutraceutical and nutritional benefits of halophytes have been extensively documented (Ben Hamed et al. 2005; Abideen et al. 2015; Barreira et al. 2017). Notably, these plants are found to contain high levels of essential micronutrients, antioxidant compounds, essential fatty acids, and polysaccharides (Ben Hamed et al. 2005; Ksouri et al. 2012; Abideen et al. 2015; Barreira et al. 2017; Duarte et al. 2019). In a context characterized by a burgeoning population facing challenges related to agricultural food sustainability, there is a notable interest on using halophytes as viable sources of food and fodder (Koyro et al. 2012; Centofanti et al. 2019). Consequently, a substantial amount of research has emerged globally on this subject (Ben Hamed et al. 2021). The majority of existing literature has focused on the investigation of metabolites and their potential nutraceutical applications, followed by studies on halophyte cultivation, sustainable agricultural practices, halophyte ecology, and research related to physiological and biochemical aspects (Rodrigues et al. 2014; ElNaker et al. 2020; Ben Hamed et al. 2021; Duarte and Caçador 2021). Despite the advantages of in vitro culture for both fundamental and applied research—such as controlled production free from microorganisms and enhanced clonal plant multiplication rates—few publications address the in vitro propagation of halophytes, particularly in the last decade (Kulpa et al. 2020; Regalado et al. 2020; Vyas et al. 2021; Yao et al. 2021; Custódio et al. 2022). Thus, there is significant potential for advancing knowledge in the area of halophyte in vitro culture, mainly regarding the multiplication of species with limited sexual and vegetative propagation, the production of bioactive compounds, and the propagation of endangered species (Gulzar et al. 2020; Custódio et al. 2022). Furthermore, in vitro methodologies are recognized as particularly effective for the screening of stress-tolerant genotypes when compared to traditional screening methods (Vuksanović et al. 2023; Wijerathna-Yapa and Hiti-Bandaralage 2023). Enhanced research in these areas could promote the integration of halophytes into sustainable production systems, thereby enabling the provision of numerous plant clones, by which a substantial quantity of plant clones could be produced throughout the year, regardless of seasonal fluctuations and seed yield.

Scarce research has provided insights into the application of liquid culture systems in plant micropropagation over traditional semi-solid media, highlighting benefits such as reduced production costs, increased multiplication rates, and improved plantlet quality (Nirmal et al. 2023; Roels et al. 2005). However, micropropagation of halophyte species in liquid medium remains unexplored. The present study marks the first successful achievement of efficient micropropagation of *S. lagascae*, or any other halophyte, using liquid culture, utilizing shoot explants obtained from in vitro-cultivated seedlings. During the shoot multiplication phase, superior genotypes were identified from explants cultured in a medium with elevated strength and sodium chloride content, which were then rooted and acclimatized to ex vitro conditions. A thorough characterization was conducted, encompassing the assessment photosynthetic performance and mineral nutrient levels. Moreover, we have conducted the first metabolomic study of in vitro halophytes comparing *S. lagascae* plantlets generated in liquid vs. solid media, which offers valuable insights into the physiological

and biochemical characteristics of halophytes during the micropropagation process.

Material and Methods

Plant Material and In vitro Introduction

Mature inflorescences of vigorous *S. lagascae* specimens were gathered from coastal salt marsh located in the Region of Murcia, Spain (37°50′32.97″ N, 0°46′6.41″ W) in November 2023. Seeds were immediately collected and preserved at 4 °C in a sealed container with silica gel for two months to induce seed dormancy breaking. Following this period, seeds were processed as previously described (Jurado-Mañogil et al. 2024). In brief, seeds underwent a disinfection process involving successive immersions in a 20% commercial bleach solution containing 0.05% Tween-20 for 20 min, followed by rinses in sterilized distilled water (three times), a one-minute immersion in 70% ethanol, and finally additional rinses in sterilized distilled water (three times). The seeds were then placed on half-strength Murashige & Skoog solid (MS) medium (Murashige and Skoog 1962) including 7.5 g L^{-1} NaCl. These were housed in polypropylene containers fitted with filters to facilitate gas exchange (Duchefa Biochemie, Haarlem, the Netherlands). After four weeks, germination percentage was calculated and the most vigorous seedlings were selected for propagation.

In Vitro Multiplication, Salinity Screening, and Culture System Comparison

In vitro seedlings, measuring between 3 to 5 cm in length, were cut into segments of 1.5 cm and positioned so that half of each segment was embedded in multiplication (M) medium in the same containers (5 segments per container) as those used for seed germination, containing the following components (Duchefa Biochemie): DKW/Juglans basal salt mixture (5.58 g L^{-1}), sucrose (38 g L^{-1}), NaCl (30 g L^{-1}), casein hydrolysate (150 mg L^{-1}), phloroglucinol (40 mg L^{-1}), sequestrene (30 mg L^{-1}), 6-benzylaminopurine (1 mg L^{-1}), 1-naphthaleneacetic acid (1 mg L^{-1}), and agar (8 g L^{-1}), pH 5.8. First, the plant material was subjected to an adaptation phase to in vitro conditions over five multiplication cycles, each consisting of a 4-week incubation period on solid medium. During this phase, explants exhibiting uniform growth and high proliferation rate (determined as new formed shoots), absence of vitrification, and minimal necrosis were selected. Shoot segments derived from the apical tip and axillary nodes of these selected explants were used for multiplication over this phase. Subsequently, the response of explants to varying salinity levels was also evaluated across a concentration range of 0 to 60 g L^{-1} on solid M medium over a 4-week period.

Finally, some explants from solid M medium containing 30 g L^{-1} NaCl were transferred into 250 mL Erlenmeyer flasks containing 100 mL of the same medium without the agar, and set into the maximum agitation speed that permitted upright explant growth (80 rpm), in order to compare explant growth and root development in comparison to explants grown on solid medium over a 42-day period. In parallel, Erlenmeyer flasks containing the same medium but in the absence of explants were subjected to the exact same conditions for their ulterior use in metabolomic analysis.

All incubations above were conducted at 25 °C under white light fluorescent lamps, maintaining a photoperiod of 16 h with a light intensity of 80 µmol m^{-2} s^{-1}.

Acclimatization

The plantlets developed during the comparison of solid vs. liquid media were meticulously rinsed to eliminate any residual agar and subsequently placed in pots filled with a sterilized mixture of peat and perlite (2:1, *v*/*v*) within an acclimatization chamber (UBBINK propagator, Northampton, UK), which is equipped with two ventilation grills to regulate relative humidity (RH). The acclimatization process was monitored during 6 weeks. The environmental parameters were set to a 16-h photoperiod and a temperature of 25 °C under white fluorescent lighting. The light intensity was gradually increased from 80 µmol m^{-2} s^{-1} on day 0 to 110 µmol m^{-2} s^{-1} on day 15 and 140 µmol m^{-2} s^{-1} from day 35 onwards. Concurrently, the ventilation grills were gradually opened to allow a progressive decrease in relative humidity, declining from 90 to 95% at day 0 to 75% by day 15, and reaching 55% from day 35 onward. The substrate was kept moist using 30 g NaCl L^{-1} water solution from day 1. Additionally, a systemic fungicide-bactericide (Beltanol-L, Probelte, Murcia, Spain) was applied to the plantlets at a concentration of 0.1% (*v*/*v*) on days 0, 2, 5, and 7.

Chlorophyll Fluorescence

Chlorophyll fluorescence measurements were conducted on shoots using a chlorophyll fluorimeter (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany). Prior to assessing the minimum and maximum fluorescence yields, the plants were kept in darkness for 20 min. Kinetic analyses were performed under actinic light conditions (81 µmol quanta m^{-2} s^{-1} PAR), supplemented by repeated applications of saturating light at 2700 µmol quanta m^{-2} s^{-1} PAR for 0.8 s, with intervals of 20 s between pulses. Then, the following variables were evaluated to assess PSII performance: effective PSII quantum yield [Y(II)] was used to estimate

the proportion of absorbed light energy utilized for photochemistry under light-adapted conditions; non-photochemical quenching (NPQ) assessed the regulated dissipation of excess excitation energy as heat; photochemical (qP) and non-photochemical (qN) quenching coefficients were determined to estimate the fraction of open PSII reaction centers and the overall capacity for non-photochemical energy dissipation, respectively; the electron transfer rate (ETR) light curves were generated by exposing light-adapted samples to increasing actinic light intensities (Acosta-Motos et al. 2019).

Nutrient Analysis

Shoots and roots were subjected to drying at a consistent temperature of 60 °C for a duration of two days until a constant weight was achieved. Subsequently, the dried samples were processed into a fine powder utilizing a blender. For the analysis of macronutrients (Ca, K, Mg, Na, P and S) and micronutrients and trace elements (Al, As, B, Be, Bi, Cd, Co, Cr, Cu, Fe, La, Li, Mn, Mo, Ni, Pb, Rb, Sb, Se, Si, Sr, Ti, Tl, V and Zn), one gram of the powdered material was employed in Inductively Coupled Plasma–Optical Emission Spectrometry (ICP–OES), utilizing an ICAP 6000 SERIES spectrometer (Thermo Scientific, Madrid, Spain), in accordance with established protocols provided by Ionomic Services of CEBAS-CSIC.

Metabolomic Analysis

Analyses were conducted on shoots and roots of in vitro plantlets cultivated in flasks (liquid medium) and containers (solid medium), as well as in the liquid incubation medium that was not used for micropropagation but maintained under the same environmental conditions, at the end of the incubation period (42 days).

Shoot and roots samples (1 and 0.5 g of fresh material, respectively) were extracted with 50% methanol at a ratio of 1:20 (*w*/*v*), while liquid medium samples were extracted with 100% methanol at a ratio of 1:1 (*v*/*v*). Both types of extracts were vigorously vortexed and centrifuged for 15 min at 13,500 × g, then filtered through PTFE 0.45 µm filters (Agilent Technologies, Santa Clara, CA, USA). Non-targeted metabolomics analysis was performed utilizing ultra-performance liquid chromatography (UPLC) coupled with quadrupole-time-of-flight mass spectrometry in positive ionization mode (Jurado-Mañogil et al. 2023; Barba-Espín et al. 2024). Detailed UPLC conditions and analytical procedures are provided in the Supplemental Materials and Methods.

The metabolomics data were analysed using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca), with the data undergoing normalization through logarithmic transformation (log 10) and Pareto scaling. Putative metabolite identification was achieved by accurate mass matching and MS/MS fragmentation spectra comparison against the Human Metabolome Database (http://www.hmdb.ca/) as reference. In both shoots and roots, putative identification focused of the top 25 most variable features (m/z) selected from heatmap analysis (*t*-test/ANOVA; distance measure, Euclidean; clustering algorithm, Ward) was performed. For the comparison between control liquid medium (placed in the growth chamber without explants) and liquid medium used for explant cultivation, the top 150 most variable features were examined, as the top 25 features yielded only a single identifiable compound. Displaying the top 150 variables features by heatmap analysis led us to select 33 upregulated compounds for putative identification. Because only a limited subset of features could be annotated, formal multiple testing correction was not applied; instead, statistical ranking was used to prioritize features for identification. Metabolite annotations were assigned at Metabolomics Standards Initiative (MSI) Level 2 (putatively annotated compounds) based on accurate mass and MS/MS fragmentation similarity without confirmation using authentic standards.

Furthermore, a metabolic pathway analysis was carried out using the Mummichog algorithm (Li et al. 2013); by means of pairwise comparisons, this approach match mass features to existing pathway databases to highlight differentially expressed metabolic pathways. The metabolite annotation was based solely on accurate mass; therefore, metabolite identities correspond to MSI Level 4. For this analysis, primary ions were enforced, a molecular weight tolerance of 5 ppm was established, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) library using *Arabidopsis thaliana* as a reference organism was used (Jurado-Mañogil et al. 2023; Barba-Espín et al. 2024).

Statistical Analysis

Four to five biological replicates per treatment were used, each biological replicate consisting of a pull of the five explants incubated in the same container/flask. For data not related to metabolomics, differences among treatments were assessed by one-way analysis of variance (ANOVA), followed by Tukey´s post hoc test when appropriate ($p \leq 0.05$). Statistically significant differences among groups are indicated by different letters, while non-significant comparison remains unmarked. These analyses were performed using SPSS Statistics software version 27 (IBM, Endicott, NY, USA).

Results and Discussion

S. lagascae Micropropagation and Selection of High Salt Tolerant Explants

In this work, *S. lagascae* was successfully propagated under in vitro conditions. Firstly, seeds were germinated in vitro (67% germination rate) and vigorous seedlings selected for propagation (data not shown), as reported in *Arthrocaulon macrostachyum* (Jurado-Mañogil et al. 2024), a halophyte closely related to *S. lagascae*. Then, the plant material was adapted to micropropagation conditions over five multiplication cycles on solid M medium. Subsequently, a screening was performed on the same solid medium but varying NaCl concentration from 0 to 60 g L^{-1} (Fig. 1A). Intermediates concentrations (30 and 45 g L^{-1}) yielded a significantly higher explant proliferation rate, determined as the percentage of explants forming at least 5 new shoots, compared to the absence of NaCl, whereas explant biomass and viability remained unchanged (Fig. 1B). In the context of conservation of halophytic plant species through tissue culture techniques, NaCl has been identified as a pivotal factor influencing micropropagation success (Martini and Papafotiou 2020). Concerning mineral nutrient content, a progressive accumulation of Na in the explant alongside the NaCl concentration in the medium was observed (Table 1). These results agree with recent findings in micropropagated *A. macrostachyum* (Jurado-Mañogil et al. 2024), where

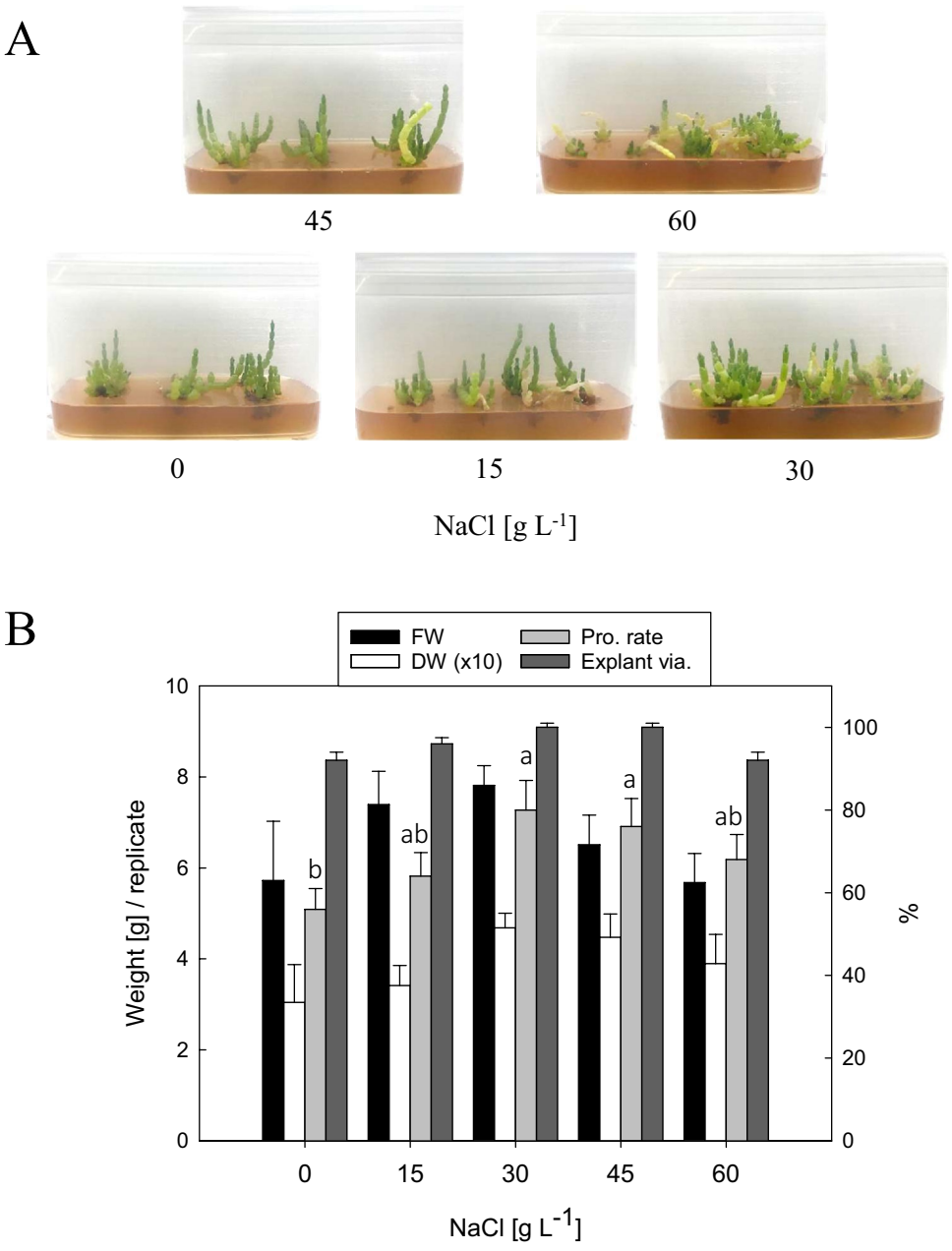


Fig. 1 In vitro response of *Salicornia lagascae* to increasing NaCl concentrations (0 to 60 g L^{-1}) in solid medium after 4 weeks of incubation. (**A**) Visual appearance of the explants. (**B**) Biomass [fresh weight (FW) and dry weight (DW) per replicate], proliferation rate (defined as the percentage of explants forming at least five new shoots per culture box), and percentage of explant viability per box. DW values were multiplied by 10 for graphical representation, as indicated. FW and DW are shown on the left y-axis. Proliferation rate and explant viability are shown on the right y-axis (%). Data represent mean ± standard error ($n \geq 5$). For FW and DW, each replicate consisted of a pooled sample of five explants from a single box. Different letters indicate significant differences according to Tukey's Multiple Range Test ($p < 0.05$). Statistical lettering is only shown where significant differences were found

Table 1 Variations in mineral nutrients in in vitro explants of *Salicornia lagascae* under NaCl screening (0 to 60 g L^{-1})

	g/100 g DW						mg Kg^{-1} DW						
	Ca	K	Mg	Na	P	S	Fe	Mn	B	Zn	Mo	Sr	Cu
0	0.75 a	2.5 a	0.16 a	5.1 d	0.27 a	0.56 a	289.5 a	208.8 a	28.5 a	105.6 a	8.2 a	28.5 a	4.3 a
15	0.55 b	2.3 a	0.11 b	11.7 c	0.18 b	0.42 b	219.9 b	146.2 b	20.9 b	70.4 b	4.5 ab	20.9 b	2.3 b
30	0.44 c	1.9 b	0.08 c	16.0 b	0.13 c	0.29 c	135.5 c	113.5 c	16.3 c	48.5 c	4.1 ab	16.3 c	1.5 c
45	0.37 cd	1.9 b	0.06 cd	16.3 ab	0.09 cd	0.25 c	134.2 c	96.8 cd	13.9 cd	40.9 cd	2.6 b	13.9 cd	1.5 c
60	0.31 d	1.9 b	0.06 d	18.1 a	0.12 c	0.23 c	63.6 d	82.5 d	11.5 d	34.2 d	2.3 b	11.5 d	1.2 c

Different letters indicate significant differences according to the Tukey's Multiple Range Test ($p \leq 0.05$)

NaCl accumulation in the explant was proportional to the external NaCl concentration (0 to 120 g L^{-1} NaCl), 30 g L^{-1} NaCl being optimal for explant proliferation. On the other hand, the content in Ca, K, Mg, P and S exhibited, overall, a decline as salinity increased. In this sense, Lima et al. (2020) reported a drop in the concentrations of key macronutrients such as K, Ca, and Mg in *Salicornia ramosissima* with rising salinity levels. Regarding micronutrients levels, increasing NaCl concentration in the culture media resulted in a progressive decline, with the lowest contents observed at 60 g L^{-1} NaCl (Table 1). Similarly, in *A. macrostachyum* and *S. europaea* irrigated with saline water under greenhouse conditions, a reduction in mineral nutrient content was also observed (Ushakova et al. 2005; Redondo-Gómez et al. 2010).

During the salt tolerance screening, chlorophyll fluorescence was also recorded (Figs. 2 and 3). After 4 weeks of growing in the presence of NaCl, no significant differences were observed in the photochemical parameters [Y(II) and qP] (Fig. 2A and B), indicating that PSII photochemistry remained stable throughout the tested NaCl range and confirming the in vitro high salt tolerance of *S. lagascae*. In contrast, non-photochemical quenching parameters exhibited a non-linear response to salinity: values decreased at low salinity (15 g L^{-1}), increased at intermediate salinity (30–45 g L^{-1}), and declined again at the highest salinity (Fig. 3). This pattern reflects a dynamic regulation of energy dissipation, with moderate salinity promoting enhanced photoprotective heat dissipation. Such dynamic pattern of energy dissipation mechanism has been documented in other plant species under salt stress conditions, such as lettuce (Shin et al. 2020) and the halophyte *Mesembryanthemum crystallinum* (He et al. 2022). Overall, stable Y(II) and qP together with increased NPQ and qN indicate regulated photoprotection rather than photoinhibition. Such fluorescence signatures reflect a balanced partitioning between photochemical energy use and non-photochemical energy dissipation, minimizing PSII damage and sustaining efficient photosynthetic performance (Zuo 2025), suggesting that 30–45 g L^{-1} NaCl represents near-optimal salinity for *S. lagascae*.

At 45 g L^{-1} NaCl, the decline in ETR at high irradiance (Fig. 2D) is consistent with this interpretation and likely reflects down-regulation of linear electron transport to facilitate excess energy dissipation, while PSII efficiency remained stable. At 30 g L^{-1}, explants showed similar chlorophyll fluorescence parameters to those at 45 g L^{-1}, but maintained slightly higher ETR under high light, suggesting that linear electron transport is maximized under 30 g L^{-1} while maintaining effective photoprotection (Figs. 2 and 3). Such regulation prevents over-reduction of the electron transport chain and limits oxidative stress under high irradiance, thereby maintaining an optimal balance between

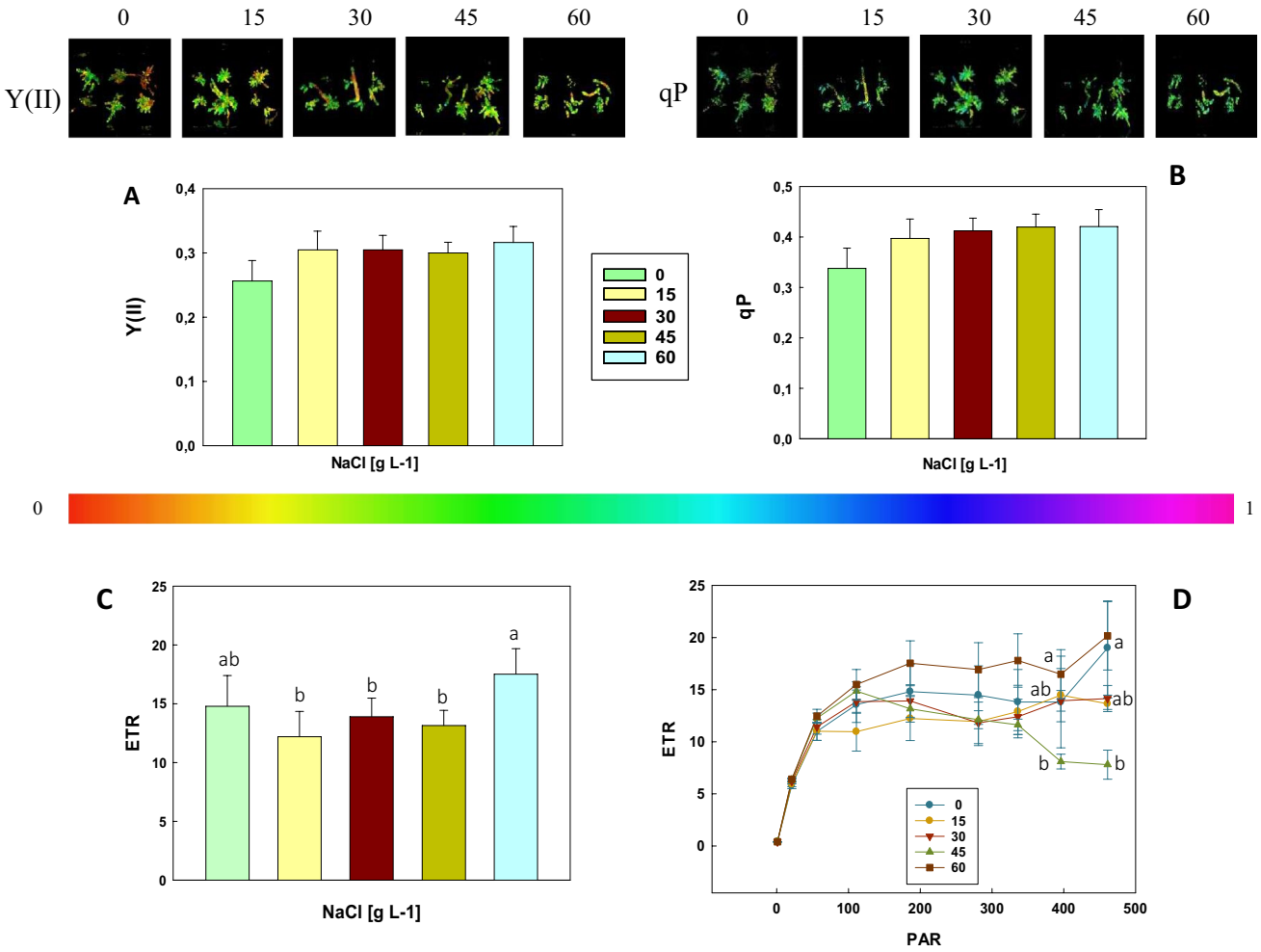


Fig. 2 Evolution of photochemical [Y(II) (**A**) and qP (**B**)] values and the electron transport rate (ETR) (**C** and **D**) of in vitro plants of *Salicornia lagascae* under NaCl screening (0 to 60 g L^{-1}) in solid medium after 4 weeks of incubation. At the top of the figure, representative images of Y(II) and qP are shown. Data represent the mean ± the standard error, $n = 4$. Different letters indicate significant differences according to Tukey's Multiple Range Test ($p < 0.05$). Statistical lettering is only shown where significant differences were found (Color figure online)

energy capture, utilization, and dissipation, and supporting sustained metabolic activity and morphogenesis. Similar patterns have been reported in halophytes such as *Sarcocornia fruticosa* and *Salvadora persica* under moderate salinity (Rangani et al. 2016; Redondo-Gómez et al. 2006). Notably, at the highest irradiance, plants grown at 0 and 60 g L^{-1} NaCl exhibited the highest ETR values compared with the other salinity treatments (Fig. 2C and D). In the absence of salt, halophytes may experience ionic disequilibrium, whereas high salinity increases the energetic costs of ion homeostasis and stress defense. In both cases, electrons may be diverted toward alternative sinks rather than carbon assimilation, involving adjustment of electron flow and energy dissipation, as reported for other halophytes (He et al. 2021).

Overall, based on the results of the screening assay, 30 g L^{-1} NaCl was selected as the optimal concentration for the micropropagation of *S. lagascae* explants in subsequent experiments.

Comparison of Micropropagation in Solid and Liquid Media

In plant tissue culture, medium formulation comprises mineral salts, organic supplements, growth regulators, and gelling agents, all of which critically influence culture performance (Gamborg and Phillips 1995). Incorporating gelling agents into the culture medium increases its viscosity, thereby supporting explant development while avoiding submersion in the medium (Klimaszewska et al. 2000). Conversely, numerous studies have reported that liquid culture systems can enhance plant growth and development compared with solid media (Kuria et al. 2008; Mbiyu et al. 2012; Rezali et al. 2017). Agar, due to its inert nature

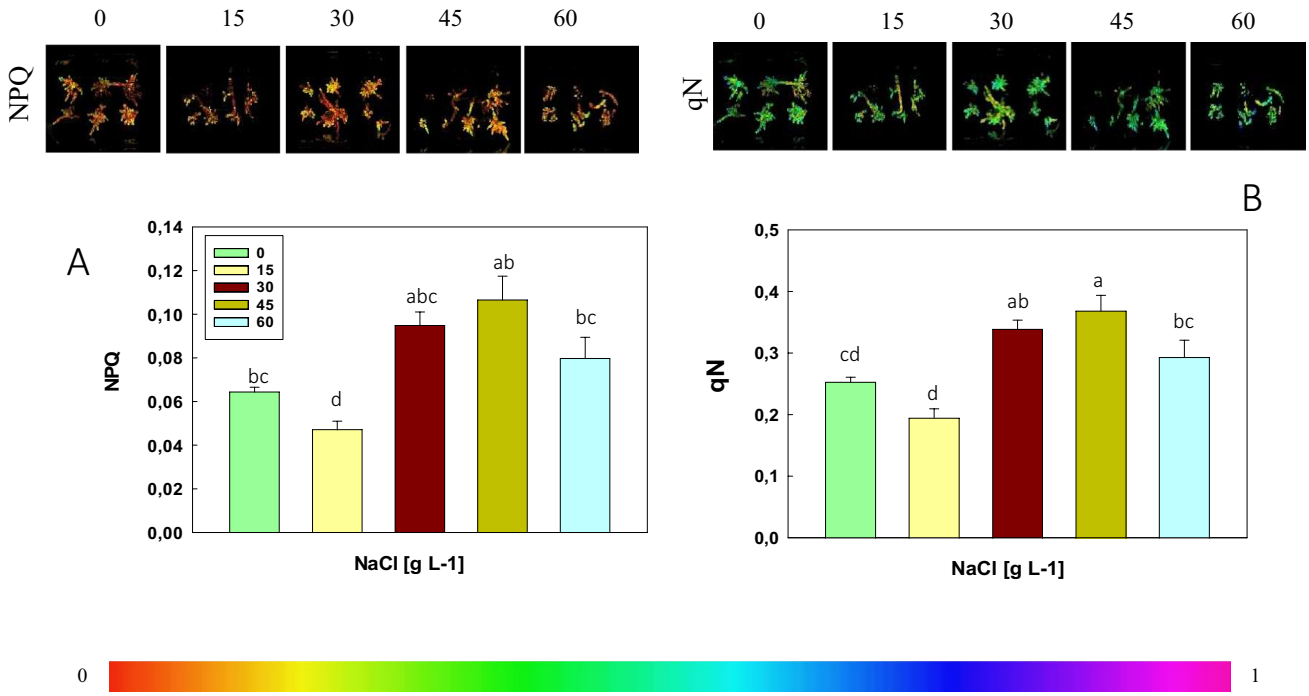


Fig. 3 Evolution of non-photochemical quenching [NPQ (**A**) and qN (**B**)] values of in vitro plants of *Salicornia lagascae* under NaCl screening (0 to 60 g L^{-1}) in solid medium after 4 weeks of incubation. At the top of the figure, representative images of NPQ and qN are shown. Data represent the mean ± the standard error, $n = 4$. Different letters indicate significant differences according to Tukey's Multiple Range Test ($p < 0.05$) (Color figure online)

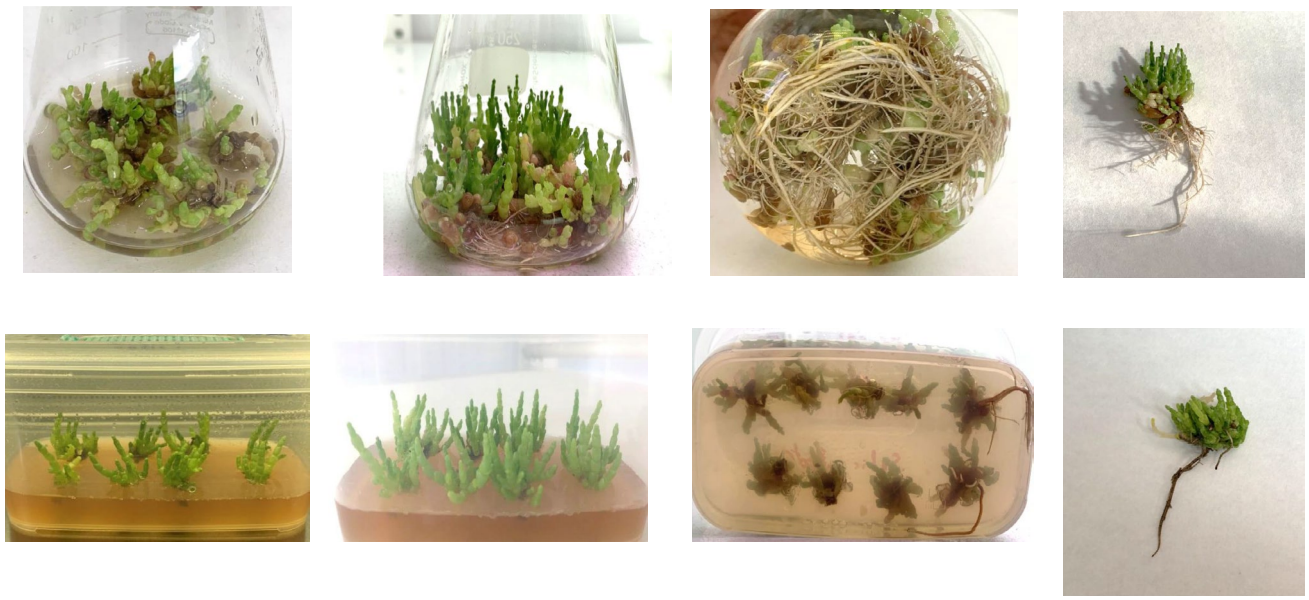


Fig. 4 In vitro response of *Salicornia lagascae* to NaCl screening in liquid (top images) and solid (bottom images) media after 4 weeks of incubation

that prevents interference with plant metabolism, remains the most commonly used gelling agent in in vitro cultures (Ozel et al. 2008); however, it is use has also been associated with adverse effects on culture growth and differentiation (Mbiyu et al. 2012). On the other hand, numerous studies have reported that liquid systems may enhance plant growth and development compared with solid media (Kuria et al. 2008; Mbiyu et al 2012; Rezali et al. 2017). In this work, we compared *S. lagascae* explants micropropagated in solid and liquid media containing 30 g L^{-1} NaCl (Fig. 4), representing, to our knowledge, the first implementation of this approach. No significant differences were observed in

macronutrients contents (Supplemental Table 1); however, explants grown in liquid medium exhibited higher concentrations of the micronutrients Mn, Zn and Cu (Supplemental Table 1).

Rhizogenesis in vitro was observed in nearly 100% of explants cultivated in liquid medium, whereas in solid medium the percentage was about 55% (Supplemental Fig. 1). This may be attributed to the more direct root contact with the culture medium compared to the solid medium, potentially leading to faster explant growth, as reported (Mbiyu et al. 2012). In this sense, Kuria et al. (2008) suggested that nutrient assimilation was favoured in liquid medium due to increase in the nutrient incorporation rate. Moreover, visually, a higher root development and a more profuse root branching could be observed under liquid medium conditions (Fig. 4). During acclimatization, the gradual exposure of explants to increasing light intensities may induce wilting as a consequence of reduced photosynthetic efficiency (Kumar and Rao 2012). Given that rooting is a critical step for acclimatization to *ex vitro* conditions, this study provides evidence that micropropagation of *S. lagascae* in liquid medium constitute a novel and efficient approach for the rapid production of large numbers of plants. Subsequently, the acclimatization of explants derived from solid and liquid media to *ex vitro* conditions was monitored through the assessment of chlorophyll fluorescence parameters and plant development (Fig. 5; Supplemental Fig. 2). During acclimatization, the gradual exposure of explants to increasing light intensities may induce wilting as a result of reduced photosynthetic efficiency (Kumar and Rao 2012). In this sense, acclimatization of *A. macrostachyum* to *ex vitro* resulted in a decline of both photochemical [Y(II) and qP] and non-photochemical (NPQ and qN) quenching parameters measured in the first week of the process, followed by a progressive recovery thereafter (Jurado-Mañogil et al. 2024). A similar trend was observed in *S. lagascae*, which displayed the lowest levels of both photochemical [Y(II) and qP] and non-photochemical quenching (NPQ and qN) variables, as well as of ETR, at day 7 of acclimatization. Thereafter, a progressive increase in photochemical quenching parameters and ETR was observed until day 42 of acclimatization, and until day 35 for non-photochemical quenching parameters (Fig. 5A; Supplemental Fig. 2). Although no remarkable differences in chlorophyll fluorescence parameters were detected between plants micropropagated in liquid and solid media (Supplemental Fig. 2), those cultured in liquid media exhibited greater growth by the end of the acclimatization period (Fig. 5B).

Comparative Metabolomic Profile: Micropropagation in Solid vs. Liquid Media

In order to gain deeper knowledge on how the medium's physical consistency affected plant metabolism, we performed a metabolomic comparative analysis of *S. lagascae* shoots and roots micropropagated in solid and liquid media.

Heatmap analysis revealed distinct patterns for several features in *S. lagascae* shoots, with nine of them putatively identified, most of which were secondary metabolism compounds (Table 2). The intensity of the m/z 457.369, 575.141, 793.403, 485.113, 325.114 and 667.225, were significantly higher in solid media than liquid media, while the m/z 369.352, 102.128 and 147.091 were upregulated in shoots from explants growing in liquid media (Supplemental Fig. 3A). Among the putatively identified metabolites, the upregulation of betaine aldehyde in shoots from explants micropropagated in liquid medium (Table 2) is noteworthy, as betaine is a well-known metabolite involved in plant osmotic adjustment and stress responses (Jarin et al. 2024). In this sense, in halophytes, betaine accumulation as an organic osmolyte has been well documented under various stress conditions (Lokhande and Suprasanna 2012; Ren et al. 2014). Liquid culture systems provide increased exposure and mass transfer of dissolved salts to plant tissues compared with solid media, leading to more uniform and sustained contact with stress agents (Zheleznichenko et al. 2023). Under our experimental conditions, a potential enhanced availability of NaCl in liquid medium could be promoting a higher accumulation of compatible osmolytes in the explant.

Metabolic profiling of *S. lagascae* roots revealed a diverse array of primary and secondary metabolites, with eleven compounds putatively identified (Table 2). Among the top 25 differentially expressed metabolites according to heatmap analysis, only the m/z 597.141, annotated as quercetin 3-O-glucosyl-xyloside (Table 2), was upregulated in liquid media (Supp. Fig. 3B). During abiotic stress conditions, flavonoids, such as quercetin, act as antioxidants and signalling molecules, mitigating ion toxicity and oxidative stress (Akram et al. 2024). Likewise, the halophyte *Glaux maritima* exposed to NaCl concentrations from 75 to 300 mM accumulated quercetin derivatives (Pungin et al. 2023). Moreover, glycosylated forms of flavonoids, including quercetin, are considered storage forms that enhance solubility and stability in plant tissues, facilitating their accumulation and release in response to stress conditions (Behr et al. 2020). The accumulation of quercetin 3-O-glucosyl-xyloside induced by liquid media supports the hypothesis that flask agitation is perceived as a stress condition.

Complementarily, the profile of compounds exuded into the liquid medium was investigated. Roots play key roles

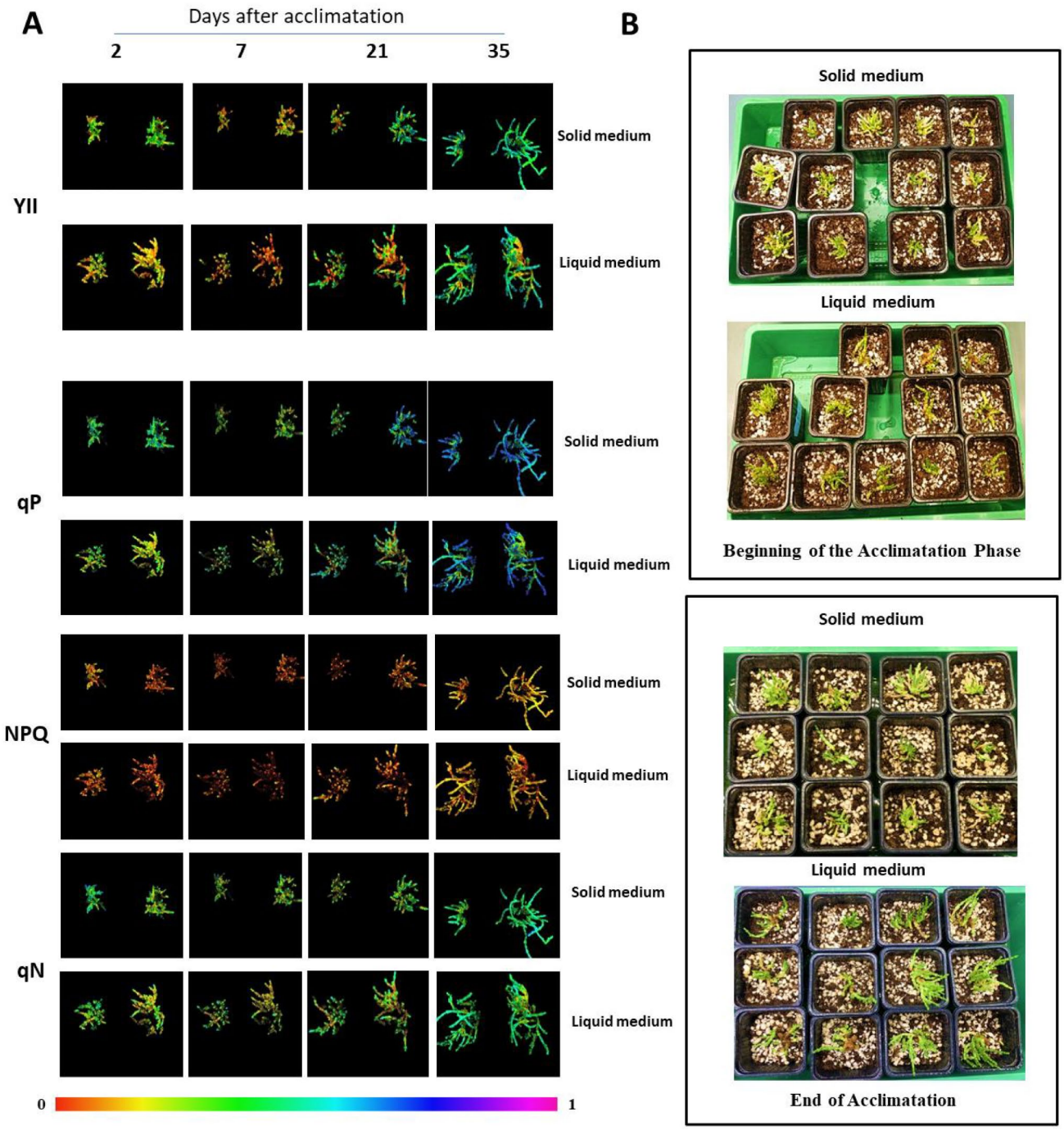


Fig. 5 Evolution of photochemical [Y(II) and qP] and non-photochemical quenching (NPQ and qN) parameters of in vitro plants of *Salicornia lagascae* after 35 days of acclimatation. A comparison between explants micropropagated in liquid and solid media was performed. **A**: Colored images of apical shoot segments representing fluorescence intensity from black (0) to magenta (1) for the different variables at selected time points. **B**: Images of acclimatization cultured in liquid and solid media from the beginning of the acclimatation phase to the end

in the plant-environment interactions by releasing chemical signals into the rhizosphere, with potential ecological implications. Root-secreted signals are part of intricate networks, whose comprehensive characterization may have practical applications in fields such as agriculture and forestry (Wang et al. 2021). For the putative identification of secreted metabolites in the medium, and in view of the superior micropropagation performance observed in liquid medium, the analysis focused on metabolites that were upregulated in liquid medium used for micropropagation relative to liquid

Table 2 Putative identification of selected features in shoots and roots from in vitro explants of *Salicornia lagascae* based on accurate mass and MS/MS fragmentation pattern comparison using the Human Metabolome Database (MSI Level 2)

m/z	Putative identification	Molecular formula	Adduct	Error	Family
Shoots cultivated in Liquid medium versus Solid medium					
457.369	Betulinic acid	$C_{30}H_{48}O_3$	M+H	0	Triterpenoids
575.141	Mulberrofuran P	$C_{34}H_{22}O_9$	M+H	12	2-arylbenzofuran flavonoids
793.403	Betavulgaroside II	$C_{41}H_{60}O_{15}$	M+H	3	Triterpenoids
485.113	Pyranodelphinin B	$C_{24}H_{23}O_{12}$	M+H-H2O	8	Flavonoid glycosides
325.114	D-Fructose,6-O-alpha-D-glucopyranosyl-	$C_{12}H_{22}O_{11}$	M+H-H2O	0	Fatty acyl glycosides
369.352	5alpha-Cholest-8(14)-en-3beta-ol	$C_{27}H_{46}O$	M+H-H2O	1	Cholastane steroids
102.128	betaine aldehyde hydrate	$C_5H_{14}NO_2$	M+H-H2O	4	Quaternary ammonium salts
147.091	3,4-Dimethylpyrrolo[1,2-a]pyrazine	$C_9H_{10}N_2$	M+H	1	Pyrrolopyrazines
667.225	Maltotetraose	$C_{24}H_{42}O_{21}$	M+H	6	Oligosaccharides
Roots cultivated in Liquid medium versus Solid medium					
133.101	Carvone	$C_{10}H_{14}O$	M+H-H2O	0	Monoterpenoids
130.050	D-Glutamic acid	$C_5H_9NO_4$	M+H-H2O	1	Amino acids
205.159	tetradeca-8,10,12-trienoic acid	$C_{14}H_{22}O_2$	M+H-H2O	3	Fatty acids
121.049	L-Erythrulose	$C_4H_8O_4$	M+H	0	Monosaccharides
219.079	Cysteinyl-Proline	$C_8H_{14}N_2O_3S$	M+H	0	Dipeptides
221.190	Isobornyl 2-methylbutyrate	$C_{15}H_{26}O_2$	M+H-H2O	1	Monoterpenoids
154.086	p-Octopamine	$C_8H_{11}NO$	M+H	2	Benzenoid
121.101	(3Z,6Z)-3,6-Nonadienal	$C_9H_{14}O$	M+H-H2O	5	Medium-chain aldehydes
203.150	Symmetric dimethylarginine	$C_8H_{18}N_4O_2$	M+H	2	Amino acids
112.051	1H-Imidazole-4-carboxamide	$C_4H_5N_3O$	M+H	1	2-heteroaryl carboxamides
597.141	Quercetin 3-O-glucosyl-xyloside	$C_{26}H_{28}O_{16}$	M+H	2	Flavonoid glycosides

Comparison established: cultivation in liquid medium *versus* solid medium

Table 3 Putative identification of upregulated features in the cultivation liquid media of *S. lagascae* based in accurate mass and MS/MS fragmentation pattern comparison using the Human Metabolome Database (MSI Level 2)

m/z	Putative identification	Molecular formula	Adduct	Error	Family
Polyphenolic compounds and Terpenoids					
503.335	Ganolucidic acid B	$C_{30}H_{46}O_6$	M+H	5	Triterpenoids
485.113	Pyranodelphinin B	$C_{24}H_{23}O_{12}$	M+H-H2O	8	Flavonoid glycosides
217.158	Procurcumenol	$C_{15}H_{22}O_2$	M+H-H2O	2	Sesquiterpenoids
Lipids					
575.106	phosphatidic acid	$C_{31}H_{61}O_8P$	M+H-H2O	9	Glycerophospholipids
556.175	diacylglycerol	$C_{28}H_{45}NO_8S$	M+H	7	Diradylglycerols
878.507	Oxidized phosphatidylethanolamine	$C_{48}H_{73}NO_{12}$	M+Na	5	Glycerophospholipids
Other organic compounds					
543.545	5-Hexatriacontanone	$C_{36}H_{72}O$	M+Na	0	Fatty Acyl Ketones
109.101	1,5-octadien-3-ol	$C_8H_{14}O$	M+H-H2O	5	Fatty alcohols
182.009	2-(Methylthio)benzothiazole	$C_8H_7NS_2$	M+H	0	Benzothiazoles
196.169	4-Methyl-2-pentyl-5-propyloxazole	$C_{12}H_{21}NO$	M+H	1	Oxazoles
233.115	7-Epijasmonic acid	$C_{12}H_{18}O_3$	M+Na	2	Jasmonates

Comparison established: Cultivated liquid media *versus* non-cultivated liquid medium

medium maintained under identical environmental conditions but without explants (Table 3; Supplemental Fig. 3C). Eleven metabolites were identified in the liquid medium and classified into three groups: flavonoids and terpenoids, lipids, and other organic compounds (Table 3). Among the putatively identified metabolites, the accumulation of epijasmonic acid in the media is noteworthy (Table 3). Jasmonic acid has been found in root exudates of various species and is known to play an important role in triggering allelopathic plant responses under different environmental conditions (Kong et al. 2018). In fact, 7-epijasmonic acid has been described as key inducible signaling molecule in plants, known for activating the transcription of numerous genes regulating several developmental processes and wound-induced defense mechanisms (Mueller 1997). In this sense, the presence in the medium of flavonoids and terpenoids may also be linked to the well-known regulatory roles of jasmonates in tissue culture, therefore stimulating

the biosynthesis and formation of those compounds (Wasternack and Song 2017). Although several signaling compounds such as strigolactones, jasmonic acid and loliolide have been identified in root exudates, many others remain to be identified (Wang et al. 2021).

The pathway analysis revealed a stimulation of the "Phenylpropanoid biosynthesis" pathway and of sugar metabolism ("Galactose metabolism" and "Starch and sucrose metabolim" pathways) in shoots cultivated in solid medium compared to liquid medium (Table 4; Fig. 6). Given the high osmotic pressure of the medium utilized (30 g L^{-1} NaCl and 38 g L^{-1} sucrose) and the low water availability of solid media (George et al. 2008), the observed increase in sugar-related metabolites may contribute significantly to osmotic adjustment by lowering the osmotic potential, as reported (Ashraf et al. 2025). Halophytes have developed specialized physiological mechanisms for osmotic adjustment, including the accumulation of soluble carbohydrates, which help maintain cellular water balance and turgor pressure under salinity and drought conditions (Mehta et al. 2024). Furthermore, in *S. europaea*, salinity tolerance was correlated with the stimulation of the biosynthesis of sugars and phenolic compounds, while in *Haloxylon salicornicum* seedlings sugar metabolism significatively contributed to salinity tolerance (Panda et al. 2021). Those results agree with the stimulation of sugar metabolism and phenylpropanoid biosynthesis in explants micropropagated in solid media, and aligns with other studies demonstrating that plants accumulate secondary metabolites as an adaptive response to various stress conditions (Bhattacharya and Kundu 2020).

Different amino acids-related pathways in the shoots were also affected by the medium in some extent, showing metabolites both upregulated or downregulated in each particular pathway (Table 4; Fig. 6). Of the metabolites belonging to the "Glycine, serine and threonine metabolism", betaine was found to be upregulated in explants micropropagated in liquid medium (Table 4), which aligns with the putative identification of betaine aldehyde in the same samples (Table 2), given that betaine aldehyde is converted to betaine by the enzyme betaine aldehyde dehydrogenase. Thus, the observed increase in these compounds may relate to the plant response to the stress condition imposed by the cultivation in liquid media. Moreover, molecular evidences revealed the involvement of betaine aldehyde dehydrogenase in abiotic stress signalling in various plant species, acting as a key regulator of betaine accumulation (Golestan Hashemi et al. 2018).

In roots, the results of pathway analysis were in agreement with the heatmap analysis, revealing marked differences between incubation in both types of medium (Table 5; Fig. 6). Specifically, different amino acid (tyrosine, arginine, phenylalanine, lysine, glycine, serine, threonine, alanine, aspartate and glutamate) metabolic pathways were downregulated in roots grown in liquid medium. Studies in in vitro plant cell suspension cultures demonstrate that exposure to abiotic stress such as high salinity induces profound metabolic reprogramming, including significant changes in amino-acid related metabolites and central carbon metabolism pathways. For example, salt-treated Arabidopsis root suspension cells showed differential regulation of branched-chain and aromatic amino acids, consistent with stress-driven reallocation of metabolic fluxes away from basal amino-acid biosynthesis toward stress adaptation processes (Chun et al. 2019). Such perturbations are in line with our observations in roots incubated in liquid medium, in which basal amino acid-related pathways ("Arginine and proline metabolism", "Glycine, serine and threonine metabolism", and "Alanine, aspartate and glutamate metabolism") were downregulated, whereas an accumulation of betaine aldehyde and quercetin 3-O-glucosyl-xyloside (Table 2), and an alteration of the "Phenylpropanoid biosynthesis" pathways were observed (Table 5).

In the liquid medium used for explant cultivation, pathway analysis showed the downregulation of sugar-related pathways (Table 6; Fig. 6), consistent with the consumption of available carbohydrates to supply energy and carbon skeletons required for cell proliferation and explants growth. Moreover, agitation of liquid medium may enlarge its contact surface with the explant, thereby favoring nutrient uptake (Thorpe et al. 2007), biomass accumulation, and hence the observed depletion of sugar-related compounds. Likewise, different pathways related to amino acids metabolism were affected, whereas the "C5-branched dibasic acid metabolism" (linked to amino acid and fatty acid metabolisms) and "alpha-Linolenic acid metabolism" (linked to fatty acid metabolism and jasmonic acid biosynthesis) pathways were upregulated in liquid medium used for explant cultivation (Table 6; Fig. 6). The stimulation of metabolites belonging to the linolenic acid metabolism, including jasmonic acid (Table 6), agrees with the putative identification in the medium of 7-epijasmonic acid (Table 3). Jasmonates regulate diverse physiological processes, including modulation of root development and secondary metabolite production. Moreover, jasmonates are key regulators of plant responses to environmental stress conditions. They contribute to the coordination of growth and defense responses, thereby enabling plants to adaptively allocate resources under different conditions (Chini et al. 2016). The presence of jasmonates and other compounds in the micropropagation medium (Tables 3 and 6) may have a dual role: mediating stress responses induced by the immersion in the liquid, and/or acting as signaling molecules altering root architecture plasticity. In a previous study (Fattorini et al. 2018), exogenous methyl jasmonate enhanced auxin-induced

Table 4 The most affected metabolic pathways in in vitro shoots of *S. lagascae* according to the Mummichog algorithm (MSI Level 4; metabolite annotation based solely on accurate mass)

Pathway	Pathway total	Hits	Adduct	KEEG	Compound name	Molecular formula	m.z	log2 (FC)
Phenylpropanoid biosynthesis	46	11	M+H	C05610	Sinapoyl aldehyde	$C_{11}H_{12}O_4$	209.081	-2.9
			M-H2O+H	C01494	Ferulate	$C_{10}H_{10}O_4$	177.054	-1.3
			M-H2O+H	C00852	Chlorogenate	$C_{16}H_{18}O_9$	337.093	-2.2
			M-H2O+H	C12204	5-Hydroxyconiferaldehyde	$C_{10}H_{10}O_4$	177.054	-1.3
			M-H2O+H	C00482	Sinapate	$C_{11}H_{12}O_5$	207.065	-2.3
			M+H	C00811	4-Coumarate	$C_9H_8O_3$	165.055	-2.9
			M+H	C12206	Caffeyl alcohol	$C_9H_{10}O_3$	167.07	-2.4
			M+H	C10945	Caffeic aldehyde	$C_9H_8O_3$	165.055	-2.9
			M+H	C02646	4-Coumaryl alcohol	$C_9H_{10}O_2$	151.075	-3.2
			M+H	C10434	5-O-Caffeoylshikimic acid	$C_{16}H_{16}O_8$	337.093	-2.2
			M+H	C05838	cis-2-Hydroxycinnamate	$C_9H_8O_3$	165.055	-2.9
Phenylalanine metabolism	11	4	M+H	C05332	Phenethylamine	$C_8H_{11}N$	122.096	1.1
			M+H	C00166	Phenylpyruvate	$C_9H_8O_3$	165.055	-2.9
			M-H2O+H	C05852	2-Hydroxyphenylacetate	$C_8H_8O_3$	135.044	1.4
			M+H	C07086	Phenylacetic acid	$C_8H_8O_2$	137.06	-2.2
Galactose metabolism	27	6	M+Na	C01613	Stachyose	$C_{24}H_{42}O_{21}$	689.213	-1.9
			M-H2O+H	C00089	Sucrose	$C_{12}H_{22}O_{11}$	325.114	-2.6
			M-H2O+H	C05402	Melibiose	$C_{12}H_{22}O_{11}$	325.114	-2.6
			M-H2O+H	C05400	Epimelibiose	$C_{12}H_{22}O_{11}$	325.114	-2.6
			M-H2O+H	C01235	alpha-D-Galactosyl-(1->3)-1D-myo-inositol	$C_{12}H_{22}O_{11}$	325.114	-2.6
			M-H2O+H	C00243	Lactose	$C_{12}H_{22}O_{11}$	325.114	-2.6
Alanine, aspartate and glutamate metabolism	22	5	M+H	C00041	L-Alanine	$C_3H_7NO_2$	90.055	-2.6
			M-H2O+H	C00232	Succinate semialdehyde	$C_4H_6O_3$	85.028	-2.9
			M+H	C00064	L-Glutamine	$C_5H_{10}N_2O$	147.076	-2.5
			M-H2O+H	C00025	L-Glutamate	$C_5H_9NO_4$	130.050	2.3
			M-H2O+H	C00042	Succinate	$C_4H_6O_4$	101.023	-2.1
Arginine and proline metabolism	34	6	M-H2O+H	C00555	4-Aminobutyraldehyde	C_4H_9NO	70.065	-3.8
			M+H	C05936	N4-Acetylaminobutanal	$C_6H_{11}NO_2$	130.086	2.1
			M-H2O+H	C05938	L-4-Hydroxyglutamate semialdehyde	$C_5H_9NO_4$	130.05	2.3
			M+H	C04281	L-1-Pyrroline-3-hydroxy-5-carboxylate	$C_5H_7NO_3$	130.05	2.3
			M+H	C00148	L-Proline	$C_5H_9NO_2$	116.071	-2.8
			M-H2O+H	C00025	L-Glutamate	$C_5H_9NO_4$	116.071	-2.8
Glycine. serine and threonine metabolism	33	5	M+H	C00213	Sarcosine	$C_3H_7NO_2$	130.05	2.3
			M+Na	C00143	5.10-Methylenetetrahydrofolate	$C_{20}H_{23}N_7O_6$	90.055	-2.6
			M+H	C00719	Betaine	$C_5H_{11}NO_2$	480.158	2.7
			M+H	C00168	Hydroxypyruvate	$C_3H_4O_4$	118.086	-1.8
			M-H2O+H	C00109	2-Oxobutanoate	$C_4H_6O_3$	85.028	-2.9
Starch and sucrose metabolism	22	4	M-H2O+H	C00185	Cellobiose	$C_{12}H_{22}O_{11}$	85.028	-2.9
			M-H2O+H	C00089	Sucrose	$C_{12}H_{22}O_{11}$	325.114	-2.6
			M-H2O+H	C01083	alpha.Alpha	$C_{12}H_{22}O_{11}$	325.114	-2.6
			M-H2O+H	C00208	Maltose	$C_{12}H_{22}O_{11}$	325.114	-2.6

Comparison: cultivation in liquid medium *versus* solid medium

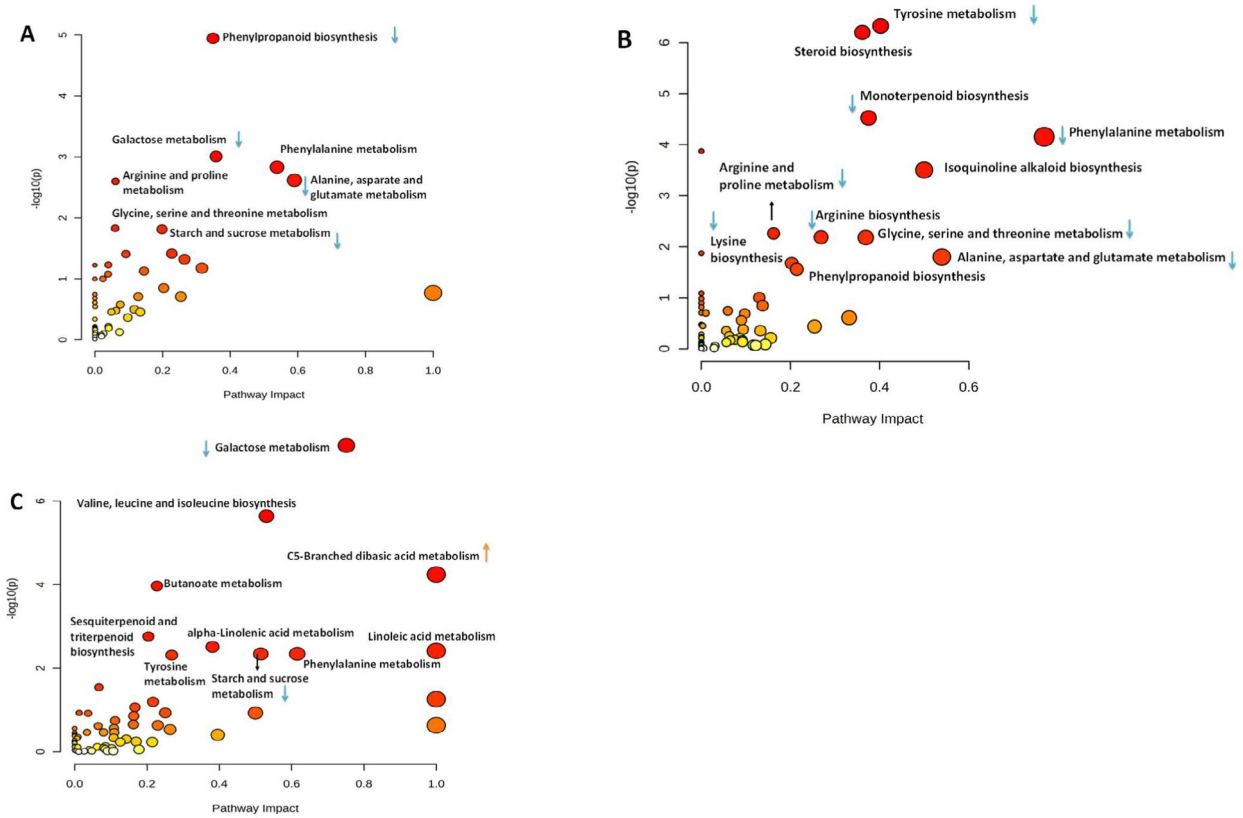


Fig. 6 Metabolic pathway analysis in *S. lagacea*. **A**: Shoots cultivated in liquid versus solid medium. **B**: Roots cultivated in liquid medium versus solid medium. **C**: Cultivated liquid medium versus non-cultivated liquid medium. Blue and orange arrows indicated downregulation and upregulation, respectively, in response to cultivation in liquid media. Black arrows link the labels to the corresponding red circles representing each pathway

adventitious root formation in in vitro cultured explants, acting through hormone crosstalk mechanisms that affect root initiation and development. This may be comparable to our experimental conditions, in which a superior root growth and the presence of 7-epijasmonic acid were jointly observed in the liquid medium. This could be related with the higher root growth observed in explants micropropagated in liquid medium over those grown in solid media (Fig. 4).

In summary, the micropropagation of *S. lagascae* explants in liquid media induced a metabolic reprogramming leading to the accumulation of stress-related metabolites and to the secretion of signaling compounds to the medium.

Conclusion

This study establishes, for the first time, an efficient in vitro micropropagation protocol for the halophyte *Salicornia lagascae* and provides new insights into its physiological and metabolic responses to salinity and culture system conditions. Moderate salinity levels were not only tolerated but also proved beneficial for explant proliferation, with 30 g L^{-1} NaCl identified as the optimal concentration. Under these conditions, explants maintained stable photosynthetic performance and activated effective energy dissipation mechanisms.

The comparison between solid and liquid culture systems demonstrated clear advantages of liquid media for *S. lagascae* micropropagation. Although macronutrient contents were largely comparable, liquid culture enhanced accumulation of certain micronutrients, promoted rhizogenesis, and resulted in greater plant growth after acclimatization. These traits are particularly relevant for large-scale propagation and successful transfer to ex vitro conditions, positioning liquid culture as a promising strategy for micropropagation.

Metabolomic analyses revealed that the physical consistency of the culture medium profoundly influences plant metabolic reprogramming. Solid media favored the accumulation of sugars and phenylpropanoid-related metabolites, likely associated with osmotic adjustment under reduced water availability. In contrast, liquid culture induced the accumulation of some stress-related secondary metabolites, including betaine aldehyde in shoots and quercetin derivatives in roots, alongside the secretion of signaling molecules such as 7-epijasmonic acid into the medium. These findings

Table 5 The most affected metabolic pathways in in vitro roots of *S. lagascae* according to the Mummichog algorithm (MSI Level 4; metabolite annotation based solely on accurate mass)

Pathway	Pathway total	Hits	Adduct	KEEG	Compound name	Molecular formula	m.z	Log2(FC)
Tyrosine metabolism	17	9	M-H2O+H	C05576	3,4-Dihydroxyphenylethyleneglycol	$C_8H_{10}O_4$	153.054	-1.9
			M-H2O+H	C05577	3,4-Dihydroxymandelaldehyde	$C_7H_6O_3$	151.039	-2.1
			M+H	C03758	Dopamine	$C_8H_{11}NO_2$	154.086	-3.7
			M+H	C04043	3,4-Dihydroxyphenylacetaldehyde	$C_8H_8O_3$	153.054	-1.9
			M-H2O+H	C01179	3-(4-Hydroxyphenyl)pyruvate	$C_9H_7O_4$	163.039	-1.6
			M-H2O+H	C00483	Tyramine	$C_8H_{11}NO$	120.081	3.8
			M-H2O+H	C00544	Homogentisate	$C_8H_8O_4$	151.039	-2.1
			M-H2O+H	C05350	2-Hydroxy-3-(4-hydroxyphenyl)propenoate	C9H8O4	163.039	-1.6
			M+H	C03765	4-Hydroxyphenylacetaldehyde	$C_8H_8O_2$	137.060	-1.1
Steroid biosynthesis	44	14	M+Na	C15782	Delta7-Avenasterol	$C_{29}H_{48}O$	435.359	-1.9
			M-H2O+H	C15783	5-Dehydroavenasterol	$C_{29}H_{46}O$	393.352	-1.8
			M+Na	C08821	Isofucosterol	$C_{29}H_{48}O$	435.359	-1.9
			M+Na	C05442	Stigmasterol	$C_{29}H_{48}O$	435.359	-1.9
			M-H2O+H	C03845	5alpha-Cholest-8-en-3beta-ol	$C_{27}H_{46}O$	369.352	1.9
			M-H2O+H	C01189	Lathosterol	$C_{27}H_{46}O$	369.352	1.9
			M-H2O+H	C00187	Cholesterol	$C_{27}H_{46}O$	369.352	1.9
			M-H2O+H	C11508	4alpha-Methyl-5alpha-ergosta-8,14,24(28)-trien-3beta-ol	$C_{29}H_{46}O$	393.352	-1.8
			M+Na	C15776	4alpha-Methylfecosterol	$C_{29}H_{48}O$	435.359	-1.9
			M+Na	C11522	24-Methylenelophenol	$C_{29}H_{48}O$	435.359	-1.9
			M-H2O+H	C11455	4,4-Dimethyl-5alpha-cholesta-8,14,24-trien-3beta-ol	$C_{29}H_{46}O$	393.352	-1.8
			M+Na	C05108	14-Demethyllanosterol	$C_{29}H_{48}O$	435.359	-1.9
			M-H2O+H	C22120	4alpha-Carboxy-stigmasta-7,24(24(1))-dien-3beta-ol	$C_{30}H_{48}O_3$	439.358	-2.0
			M-H2O+H	C22123	Avenastenone	$C_{29}H_{46}O$	393.352	-1.8
Monoterpenoid biosynthesis	7	5	M-H2O+H	C00843	(-)-Menthone	$C_{10}H_{18}O$	137.132	-1.6
			M-H2O+H	C11393	(-)-alpha-Terpineol	$C_{10}H_{18}O$	137.132	-1.7
			M+H	C06074	Myrcene	$C_{10}H_{16}$	137.132	-1.6
			M-H2O+H	C09844	1,8-Cineole	$C_{10}H_{18}O$	137.132	-1.7
			M-H2O+H	C11389	(+)-Linaloo	$C_{10}H_{18}O$	137.132	-1.7
Phenylalanine metabolism	12	6	M-H2O+H	C00166	Phenylpyruvate	$C_9H_8O_3$	147.044	-1.8
			M+H	C00079	L-Phenylalanine	$C_9H_{11}NO_2$	166.086	-1.8
			M+H	C05852	2-Hydroxyphenylacetate	$C_8H_8O_3$	153.054	-1.9
			M-H2O+H	C02763	2-Hydroxy-3-phenylpropenoate	$C_9H_9O_3$	147.044	2.3
			M+H	C07086	Phenylacetic acid	$C_8H_8O_2$	137.060	-1.1

Table 5 (continued)

Pathway	Pathway total	Hits	Adduct	KEEG	Compound name	Molecular formula	m.z	Log2(FC)
			M+H	C00601	Phenylacetaldehyde	C_8H_8O	121.065	-2.0
Isoquinoline alkaloid biosynthesis	6	4	M-H2O+H	C01179	3-(4-Hydroxyphenyl)pyruvate	$C_9H_7O_4$	163.039	-1.6
			M-H2O+H	C00483	Tyramine	$C_8H_{11}NO$	120.081	1.2
			M+H	C03758	Dopamine	$C_8H_{11}NO_2$	154.086	-3.7
			M+H	C04043	3,4-Dihydroxyphenylacetaldehyde	$C_8H_8O_3$	153.054	1.5
Arginine and proline metabolism	32	7	M+H	C00062	L-Arginine	$C_6H_{14}N_4O_2$	175.119	-1.8
			M+H	C05936	N4-Acetylaminobutanal	$C_6H_{11}NO_2$	130.086	-1.3
			M+H	C01035	4-Guanidinobutanoate	$C_5H_{11}N_3O_2$	146.092	-2.0
			M-H2O+H	C05938	L-4-Hydroxyglutamate semialdehyde	$C_5H_9NO_4$	130.050	-2.6
			M+H	C04281	L-1-Pyrroline-3-hydroxy-5-carboxylate	$C_5H_6NO_3$	130.050	-2.6
			M-H2O+H	C00025	L-Glutamate	$C_5H_8NO_4$	130.050	-2.6
			M+H	C00148	L-Proline	$C_5H_9NO_2$	116.071	-2.9
Glycine, serine and threonine metabolism	33	7	M+H	C00049	L-Aspartate	$C_4H_7NO_4$	134.045	-1.6
			M-H2O+H	C00263	L-Homoserine	$C_4H_9NO_3$	102.055	-4.7
			M+H	C00188	L-Threonine	$C_4H_9NO_3$	120.065	-5.0
			M+H	C00213	Sarcosine	$C_3H_7NO_2$	90.055	-3.2
			M+H	C00065	L-Serine	$C_3H_7NO_3$	106.050	-1.8
			M-H2O+H	C05519	L-Allothreonine	$C_4H_9NO_3$	102.055	-5.0
			M+H	C00740	D-Serine	$C_3H_7NO_3$	106.050	-1.8
Alanine, aspartate and glutamate metabolism	22	5	M+H	C00049	L-Aspartate	$C_4H_6NO_4$	134.045	-1.6
			M+H	C00041	L-Alanine	$C_3H_7NO_2$	90.055	-3.2
			M+Na	C03794	N6-(1,2-Dicarboxyethyl)-AMP	$C_{14}H_{18}N_5O_{11}P$	486.065	-1.9
			M-H2O+H	C00026	2-Oxoglutarate	$C_5H_6O_5$	129.018	3.1
			M-H2O+H	C00025	L-Glutamate	$C_5H_8NO_4$	130.050	-2.6
Lysine biosynthesis	9	3	M-H2O+H	C00049	L-Aspartate	$C_4H_7NO_4$	134.045	-1.6
			M-H2O+H	C00263	L-Homoserine	C4H9NO3	102.055	-4.7
			M+H	C03972	2,3,4,5-Tetrahydrodipicolinate	$C_7H_7NO_4$	172.060	-4.3
Phenyl-propanoid biosynthesis	43	7	M-H2O+H	C00811	4-Coumarate	$C_9H_7O_3$	147.044	2.3
			M+H	C12208	p-Coumaroyl quinic acid	$C_{16}H_{18}O_8$	339.108	2.1
			M-H2O+H	C01197	Caffeate	$C_9H_7O_4$	163.039	-1.9
			M+H	C10945	Caffeic aldehyde	$C_9H_8O_3$	165.055	2.3
			M-H2O+H	C02666	Coniferyl aldehyde	$C_{10}H_{10}O_3$	161.060	-2.3
			M+H	C05610	Sinapoyl aldehyde	$C_{11}H_{12}O_4$	209.081	-1.8

Comparison: cultivation in liquid medium *versus* solid medium

Table 6 The most affected metabolic pathways in the cultivation media of *S. lagascae* according to the Mummichog algorithm (MSI Level 4; metabolite annotation based solely on accurate mass)

Pathway	Pathway Total	Hits	Adduct	KEEG	Compound name	Molecular formula	m.z	log2 (FC)
Galactose metabolism	27	14	M-H2O+H	C05404	D-Gal alpha 1->6D-Gal alpha 1->6D-Glucose	$C_{18}H_{32}O_{16}$	487.166	-2.6
			M+H	C00492	Raffinose	$C_{18}H_{32}O_{16}$	505.177	-2.6
			M+Na	C00089	Sucrose	$C_{12}H_{22}O_{11}$	365.106	-2.2
			M+Na	C05402	Melibiose	$C_{12}H_{22}O_{11}$	365.106	-2.2
			M-H2O+H	C05400	Epimelibiose	$C_{12}H_{22}O_{11}$	325.114	-2.2
			M+Na	C01235	alpha-D-Galactosyl(1->3)-1D-myo-inositol	$C_6H_{12}O_6$	365.106	-2.2
			M-H2O+H	C00243	Lactose	$C_{12}H_{22}O_{11}$	163.060	-2.9
			M+Na	C00124	D-Galactose	$C_6H_{12}O_6$	365.106	-2.2
			M-H2O+H	C00095	D-Fructose	$C_6H_{12}O_6$	163.060	-2.9
			M-H2O+H	C00031	D-Glucose	$C_6H_{12}O_6$	163.060	-2.9
			M-H2O+H	C00159	D-Mannose	$C_6H_{12}O_6$	163.060	-2.9
			M-H2O+H	C00031	D-Glucose	$C_6H_{12}O_6$	163.060	-2.9
			M-H2O+H	C00137	myo-Inositol	$C_6H_{12}O_6$	163.060	-2.9
			M-H2O+H	C00984	alpha-D-Galactose	$C_6H_{12}O_6$	163.060	-2.9
Valine, leucine and isoleucine biosynthesis	22	11	M+H	C02612	(R)-2-Methylmalate	$C_5H_6O_5$	149.045	4.3
			M-H2O+H	C02226	2-Methylmaleate	$C_5H_4O_4$	113.023	2.5
			M+H	C06032	D-erythro-3-Methylmalate	$C_5H_8O_5$	149.045	4.3
			M-H2O+H	C00123	L-Leucine	$C_6H_{13}NO_2$	114.091	-3.0
			M-H2O+H	C06006	(S)-2-Aceto-2-hydroxybutanoate	$C_6H_{10}O_4$	129.054	2.4
			M+H	C06010	(S)-2-Acetolactate	$C_5H_7O_4$	133.050	5.3
			M+H	C00109	2-Oxobutanoate	$C_4H_5O_3$	103.039	3.3
			M+Na	C00407	L-Isoleucine	$C_6H_{13}NO_2$	125.021	-3.0
			M-H2O+H	C14463	(R)-3-Hydroxy-3-methyl-2-oxopentanoate	$C_6H_9O_4$	129.054	2.4
			M-H2O+H	C00183	L-Valine	$C_5H_{11}NO_2$	100.076	-5.2
			M+H	C04181	3-Hydroxy-3-methyl-2-oxobutanoic acid	$C_5H_8O_{44}$	133.050	5.3
C5-Branched dibasic acid metabolism	6	5	M+H	C02612	(R)-2-Methylmalate	$C_5H_6O_5$	149.045	4.3
			M-H2O+H	C02226	2-Methylmaleate	$C_5H_4O_4$	113.023	2.5
			M+H	C06032	D-erythro-3-Methylmalate	$C_5H_8O_5$	149.045	4.3
			M+H	C06010	(S)-2-Acetolactate	$C_6H_{13}NO_2$	133.050	5.3
			M+H	C00109	2-Oxobutanoate	$C_6H_{10}O_4$	103.039	3.3
Butanoate metabolism	17	8	M-H2O+H	C00025	L-Glutamate	$C_5H_8NO_4$	130.050	-2.1
			M+H	C00232	Succinate Semialdehyde	$C_4H_6O_3$	103.039	3.3
			M+H	C00246	Butanoic acid	C_4H_8O	89.06	2.2

Table 6 (continued)

Pathway	Pathway Total	Hits	Adduct	KEEG	Compound name	Molecular formula	m.z	log2 (FC)
			M-H2O+H	C00989	4-Hydroxybutanoic acid	$C_4H_8O_3$	87.044	3.4
			M+H	C00164	Acetoacetate	$C_4H_6O_3$	103.039	3.3
			M-H2O+H	C00042	Succinate	$C_4H_6O_4$	101.023	-4.3
			M+H	C06010	(S)-2-Acetolactate	$C_5H_7O_4$	133.05	5.3
			M+H	C0263	2-Hydroxyglutarate	$C_5H_8O_5$	149.045	4.3
Sesquiterpenoid and triterpenoid biosynthesis	24	8	M-H2O+H	C03428	Presqualene diphosphate	$C_{30}H_{52}O_7P$	569.318	-1.8
			M+H	C03461	2-trans,6-trans-Farnesal	$C_{15}H_{26}O$	221.19	6.1
			M+Na	C09684	Humulene	$C_{15}H_{24}$	227.176	-2.6
			M+Na	C09629	beta-Caryophyllene	$C_{15}H_{24}$	227.176	-2.6
			M+Na	C16814	gamma-Bisabolene	$C_{15}H_{24}$	227.176	-2.6
			M+Na	C19740	(+)-alpha-Barbatene	$C_{15}H$	227.176	-2.6
			M+Na	C19908	(+)-beta-Chamigrene	$C_{15}H_{24}$	227.176	-2.6
			M+Na	C20724	(+)-Thujopsene	$C_{15}H_{24}$	227.176	-2.6
alpha-Linolenic acid metabolism	26	8	M-H2O+H	C01226	12-OPDA	$C_{18}H_{28}O_3$	275.201	5.1
			M+Na	C08491	(-)-Jasmonic acid	$C_{12}H_{18}O_3$	233.115	3.1
			M-H2O+H	C04672	(9Z,15Z)-(13S)-12,13-Epoxyoctadeca-9,11,15-trienoic acid	$C_{18}H_{28}O_3$	275.201	5.1
			M+H	C04780	8-[(1R,2R)-3-Oxo-2-{(Z)-pent-2-enyl}cyclopentyl]octanoate	$C_{18}H_{30}O_3$	295.227	5.0
			M+H	C08492	3-Hexenol	$C_6H_{12}O$	101.096	3.3
			M-H2O+H	C16324	9,10-EOT	$C_{18}H_{28}O_3$	275.201	5.1
			M-H2O+H	C19757	(Z)-3-Hexen-1-ol acetate	C8H14O	125.096	4.3
			M+H	C21924	9-Hydroxy-12-oxo-15(Z)-octadecenoic acid	$C_{18}H_{32}O_4$	313.238	-3.9
Linoleic acid metabolism	4	3	M-H2O+H	C01595	Linoleate	$C_{18}H_{31}O_2$	263.237	1.3
			M-H2O+H	C04717	(9Z,11E)-(13S)-13-Hydroperoxyoctadeca-9,11-dienoic acid	$C_{18}H_{32}O_4$	295.227	-3.9
			M-H2O+H	C14827	9(S)-HPODE	$C_{18}H_{32}O_4$	295.227	-3.9
Phenylalanine metabolism	12	5	M+H	C00079	L-Phenylalanine	$C_9H_{11}NO_2$	166.086	-2.8
			M+H	C05332	Phenethylamine	$C_8H_{11}N$	122.096	3.7
			M-H2O+H	C00423	trans-Cinnamate	C_6H_5CH	131.049	-1.9
			M-H2O+H	C05852	2-Hydroxyphenylacetate	$C_8H_8O_3$	135.044	5.1
			M+H	C07086	Phenylacetic acid	$C_8H_8O_2$	137.06	2.2
Starch and sucrose metabolism	22	7	M+Na	C00185	Cellobiose	$C_{12}H_{22}O_{11}$	365.106	-2.3
			M-H2O+H	C00095	D-Fructose	$C_6H_{12}O_6$	163.06	-2.9
			M+Na	C00089	Sucrose	$C_{12}H_{22}O_{11}$	365.106	-2.2
			M-H2O+H	C00031	D-Glucose	$C_6H_{12}O_6$	163.06	-2.9
			M+Na	C01083	alpha,alpha-Trehalose	$C_{12}H_{22}O_{11}$	365.106	-2.3

Table 6 (continued)

Pathway	Pathway Total	Hits	Adduct	KEEG	Compound name	Molecular formula	m.z	log2 (FC)
			M-H2O+H	C00208	Maltose	$C_{12}H_{22}O_{11}$	325.114	-2.3
			M-H2O+H	C00721	Dextrin	$C_{18}H_{32}O_{16}$	487.166	-3.6
Tyrosine metabolism	17	6	M+H	C00544	Homogentisate	$C_8H_7O_4$	169.049	-2.0
			M+H	C00082	L-Tyrosine	$C_9H_{11}NO_3$	182.081	-3.8
			M+H	C05577	3,4-Dihydroxymandel Aldehyde	$C_8H_8O_4$	169.049	-2.0
			M+Na	C00164	Acetoacetate	$C_4H_5O_3$	125.021	3.3
			M-H2O+H	C04043	3,4-Hydroxyphenylacetaldehyde	$C_8H_8O_2$	135.044	5.1
			M+H	C03765	4-Dihydroxyphenylacetaldehyde	$C_8H_8O_3$	137.06	2.2

Comparison established: Cultivated liquid medium *versus* non-cultivated liquid medium

suggest that liquid culture conditions may be perceived as a mild stress that triggers adaptive metabolic and signaling responses, potentially contributing to enhanced rooting and growth through hormone-related regulatory mechanisms.

Despite these advantages, translation to field or commercial applications faces challenges due to the contrast between controlled in vitro conditions and the variability of natural environments. Field validation will be required to assess long-term plant performance and interactions with soil microbiota. Additionally, large-scale implementation of liquid culture will require optimization of bioreactor systems to ensure controlled aeration, nutrient delivery, and shear stress management while maintaining economic feasibility.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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