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Article

In Vitro Propagation, Isolation and Expression Studies of *Suaeda edulis* Genes Involved in the Osmoprotectants Biosynthesis

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Abstract: Halophytes are an excellent choice for the study of genes conferring salt tolerance to salt-sensitive plants and, they are suitable for reclamation and remediation of saline soil. We develop an *in vitro* plant propagation protocol and studies of genes involved with GB and Pro biosynthesis in *Suaeda edulis*. Axillary buds were used as explants and cultured in different treatments on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators. The highest number of multiple shoots was on MS medium containing 1 mg/L Benzyladenine (BA) and / or 2 g/L activated carbon with 5.5 ± 06 shoots per explant. The identification and expression analysis of genes involved in glycine betaine (GB) biosynthesis were S-adenosylmethionine synthetase (*SAMS*), choline monooxygenase (*CMO*) and betaine aldehyde dehydrogenase (*BADH*), and for proline (Pro) was pyrroline 5-carboxylate synthetase (*P5CS*). These sequences shared 90–95% of identity with others plant homologous in public databases. The amino acids sequence analysis showed that all these peptides contain some of the conserved motifs of those kinds of enzymes. The qRT-PCR analysis revealed a higher expression of *SeBADH*, *SeCMO*, and, *SeP5CS* genes in the roots and leaves from plants collected in the field in contrast with from *in vitro* plants. However, the expression level of *SeSAMS* was higher only in the leaves of plants collected in the field when compared to those cultivated *in vitro*.

Keywords: Axillary buds; halophyte; proline; glycine betaine; salt-resistant

1 Introduction

The high soil salinity can cause in plants: a) osmotic unbalance, b) ionic toxicity, c) alterations in the membrane composition and d) structure and photosynthesis disruption. To overcome the harmful effects of osmotic stress, some plants can make modifications in their physiology and anatomy including a succession of mechanisms that maintain or restore the adequate metabolic activity of the plant; among these mechanisms are the osmoprotectant biosynthesis such as GB and Pro [1–3].

The GB and Pro biosynthesis is well established. For GB biosynthesis, cytoplasmic and chloroplast enzymes participate: first, the S-adenosylmethionine synthetase (SAMS) adds a methyl group to phosphoethanolamine compounds to synthesize choline, then the choline is transported to the chloroplast where the choline monooxygenase (CMO) enzyme acts to synthesize betaine aldehyde, and finally it is converted to GB by the betaine aldehyde dehydrogenase (BADH) [4]. Several studies of genes involved in the GB and Pro synthesis have been carried out in the genus *Suaeda* under conditions of salt stress: for example, *SAMS*, *CMO* and *BADH*, which have been analyzed in different species of *Suaeda* such as *S. marítima* [4], *S. salsa* [5], *S. fructicosa* [6] and *S. glauca* [7].

There are two enzymes that act in Pro biosynthesis, the first is pyrroline-5-carboxylate synthetase (P5CS), which takes glutamate as a substrate and converts into glutamate semialdehyde, which is transformed to pyrroline-5-carboxylate by spontaneous cyclization, and then the second enzyme, pyrroline-5-carboxylate reductase (P5CR) converts to Pro [8], however, it has been shown that the enzyme P5CS is the rate-limiting enzyme and is feedback-inhibited by proline [9]. These processes of salinity tolerance are carried out principally by halophyte plants [1,10] among which are those of the genus *Suaeda* Forssk. ex Scop., which include more than 110 species, usually growing in humid saline or alkaline zones, and they can be bushes or sub-shrubs, upright or prostrate, annual or perennial. One of the species of this genus is *S. edulis*, a plant of 15–110 cm high, halophyte, strictly annual, and distributed in saline-alkaline soils of the central region of Mexico. Phylogenetic studies with several *Suaeda* species, based on molecular markers of the nuclear ribosomal internal transcribed spacer (ITS) and the chloroplast rpl32-trnL intergenic, confirmed that *S. edulis* is found only in the central region of Mexico [11–13].

Since halophytes are a good source to study of the genes that conferring salt tolerance to salt-sensitive plants, then they are suitable for reclamation and remediation of saline soils [10,14]. Therefore, in this work was implemented an efficient *in vitro* protocol to *S. edulis*, to conserve the species, and as material for expression studies of *SAMS*, *BADH*, *CMO*, and *P5CS* genes. These genes closely related to salt resistance were analyzed by qRT-PCR.

2 Material and Methods

2.1 In vitro Propagation

Samples of 10 cm sections of plants were collected from saline-alkaline soil [12] with ECe of 4.2 dS m^{-1}, pH of 9.2 and corresponding also with a primary type salnity; in according with the most widely accepted definition (FAO 1997), a saline soil is that has an electrical conductivity of the saturation extract (ECe) of 4 dS m^{-1}. The vegetal material was transferred in coolers to the laboratory, then rinsed twice with sterile water and washed carefully with benzalkonium chloride 1% (Antibenzil®). The internodal regions with axillary buds were used as explants for *in vitro* propagation. These explants were disinfected under axenic conditions as follows: 10 min in a solution of 20% Tween and 10% Antibenzil®; 30 s in a solution of 50% ethanol; and 10 min in a solution of 0.35% colloidal silver (Mycrodin®), and 1% sodium hypochlorite, rinsed for 1 min with sterile distilled water between each solution. Once the explants were disinfected, they placed in glass containers with 20 mL basal medium MS [15]. Ten explants per bottle were placed with three repetitions each one. The treatments were the following: 1) MS medium, 2) MS at pH 8.0, 3) MS with $CaCl_2$ 0.66 g/L, 4) MS with Benzyladenine (BA) 1 mg/mL at pH 8.0, 5) MS plus activated charcoal 2 g/L of, and 6) MS plus BA 1 mg/mL. They were incubated at 25°C and in the light (16 h light/8 h dark). The explants with the best response were established on MS without plant growth regulators (PGRs) for rooting. The response data of the explants to the different treatments were taken at 15 d and 30 d. Roots and leaves taken from this material were subsequently used to perform the expression analyses of the genes of interest. The results were compared by variance analysis (ANOVA) followed by the Tukey test. A value of $p < 0.05$ was considered significant statistically.

2.2 DNA Extraction and PCR Amplification

The extraction of DNA was carried out as was previously described [16]. Specific oligonucleotides were designed with DNAMAN program [http://www.lynnon.com/dnaman.html] for each of the genes starting from conserved regions corresponding to *Suaeda* species and genera of the Chenopodiaceae and Amaranthaceae family previously reported in the NCBI website [http://www.ncbi.nlm.nih.gov/].

The oligonucleotides used were: fSAMS 5-CATGCACTAAGACTAACATGGTC-3 and rSAMS 5-GATGACGGGCTTGATGACA-3; fBADH 5-TCTCAGGCAGCCTATTGGTGT-3 and rBAHD 5-CACTCTTTGCTGTCGATATGTAC-3; fCMO 5-AGAAGTGAATTCCCCATGGAAT-3 and rCMO 5- TTCTCAATTGGCATCACATATCT-3; fP5CS 5-CAGTTTGGATATGGCGAAGCAGAT-3 and rP5CS 5-GAGCAAAACCCAACCCACGAC-3;

For the gene amplification, GoTaq® Flexi DNA Polymerase (Promega Corp.) commercial kit was used along with 10 ng of DNA, 0.4 mM of each oligonucleotide and 0.2 mM of each dNTP, in a final volume of 25 µl. The amplification conditions were: initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 52–58°C for 1 min and 72°C for 1 min, ending with a final extension at 75°C for 5 min.

2.3 Bioinformatic Analysis

The partial sequences of *SAMS*, *BADH*, *CMO*, and *P5CS* of *S. edulis* were subjected to a bioinformatics analysis starting with the search of homologous sequences in the NCBI database using the basic local alignment search tool (BLAST) algorithm [http: //blast.ncbi.nlm.nih.gov/Blast.cgi]. The deduced amino acid sequences were obtained by "Translate tool" from ExPASy Proteomics Server (http://www.expasy.ch/). The search for the conserved domains was done through the NCBI's Conserved Domain Architecture Retrieval Tool (CDART) platform [http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi].

The hypothetical structural modeling of the amino acid sequences was carried out through two programs: 1) CPHModels-3.2 [http://www.cbs.dtu.dk/services/CPHmodels/] to obtain the PDB file and 2) the Chimera 1.8 program [http://www.cgl.ucsf.edu/chimera/] to obtain the three-dimensional model. Phylogenetic analyses were carried out with MEGA7 software by the Neighbor-Joining method, and the evolutionary distances were computed using the Dayhoff matrix-based method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was placed next to the branches.

2.4 RNA Extraction and qRT-PCR

Roots and leaves taken from the crater plants and from *in vitro* plants were frozen and macerated separately in liquid nitrogen, then the total RNA was extracted using the PureLink® Plant RNA Reagent kit (Ambion Life Technologies, Carlsbad, CA, USA). The total RNA (1 µg) extracted from each sample was reverse transcribed to cDNA following the SuperScript™ (Invitrogen, Carlsbad, CA, USA) protocol. The cDNA concentration was adjusted to 100 ng μl^{-1} using a Nanodrop Spectrophotometer (Thermo Scientific).

For real-time quantitative reverse transcriptional PCR (qRT-PCR), primers oligonucleotides were designed from specific sequences of each gene obtained of *S. edulis*: fSeSAMS 5-AGGTAAGGAAGAATGGCACTTGT-3 and rSeSAMS 5 GAGACTGTGACCAACGATCAAAT-3; fSeBADH 5-GCAGAAGCTCTGGACAACAAGC-3 and rSeBADH 5-AGCTGTACTTAAGCCATCCGAG-3; fSeCMO 5-GCCGAGATGGTGAAGGAAAA-3 and rSeCMO 5-AGCCACCCAAACAACTGATG-3; fSeP5CS 5-GCGTTGGCAGTCATTCTTTT-3 and rSeP5CS 5-GATGTTCATGCTGCCATTGA-3; To normalize the relative quantification, the β-tubulin gene was used through the primers: fTub 5-CCTTATTCCATTCCCCAGGCTTC-3 and rTub 5-GCACAAAGGAGGTTGATGAGCAGATG-3 [17], the obtained sequence in *S. edulis* was named *SeTUB*.

Amplification reactions were carried out in 96-well plates in a StepOne™ Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Each RT-PCR reaction contained 10 µl SYBR Green I Master Mix, 100 ng of cDNA and 0.5 pM of forward and reverse primers in a final volume of 20 µl. The program parameters for amplification were 50°C 2 min, 95°C 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curves were generated by a cycle of 95°C during 15 s, 60°C during 1 min and 95°C during 30 s. The data were analyzed using the Applied Biosystems StepOne™ Real-Time PCR System Software (Applied Biosystems, Mulgrave, AUS).

2.5 Statistical Analysis

The qRT-PCR was performed with two biological replicas to increase the trustworthy of gene expression analysis. For each biological replica the samples were evaluated in triplicate. To detect differences in the relative quantification of each gene, a single factor analysis of variance (ANOVA) was performed. The significance of the differences between each value obtained was determined by the Tukey test. Differences with $p \geq 0.05$ were considered significant, and all analyses performed with the Minitab v16 software (State College, PA, USA).

3 Results and Discussion

3.1 In vitro Propagation

The explants response in culture medium was different among treatments. For example, in treatment 1, 95% of explants survived but with slow growth and some necrotic leaves (Fig. 1a). In treatment 2, 96% of the explants showed thickness in the stem with few leaves and in 30 d 100% of the plants were necrotic, etiolated and died (Fig. 1b). In treatment 3, after 15 d the seedlings showed more growth; however, the leaves began to etiolate (Fig. 1c) and at 30 d 95% died. In treatment 4, all explants came to have small leaves but later they were etiolated (Fig. 1d) and 30 d later 97% of them died. In treatments 2 to 4, an attempt was made to have an *in vitro* environment with some characteristics of the natural habitat of the plant as a high pH and high concentration of $CaCl_2$; however, all explants under these conditions died. At this respect, is known, that most *in vitro* plants can tolerate pH ranges of 4.2 to 7.2 and that above pH 8 they die [18].

On the other hand, in treatments 4, 5 and 6 the effect of BA was analyzed because it is the cytokinin that has been most used in tissue culture for the proliferation of many plants [19]. In treatments 5 and 6; 5.5 (± 06) shoots per explant were generated (Figs. 1e and 1f) and after 30 d they were transferred to MS medium without PGR and greater proliferation of shoots, and root was shown (Figs. 1g and 1h). However, with a combination of BA and pH 8, the plants died (treatment 4). In treatment 5, which contained activated charcoal, greater sprouting, and even root generation was observed; regarding this result, that one of the effects of activated charcoal is the irreversible adsorption of inhibitory compounds in the culture medium and decreases in the amount of toxic metabolites and phenolic exudation, in addition to releasing natural substances that promote growth, alteration and darkening of the culture media and absorption of vitamins, metals, ions and plant growth regulators [20].

As far as the authors know, there are no reports in the literature about *S. edulis in vitro* propagation. Therefore, this report presents a simple and rapid protocol for seedling micropropagation, demonstrating that it is not necessary to combine others PGRs to induce a large number of buds or to use other means for rooting. *S. edulis* establishment allowed us to compare the behavior of genes associated with salinity in plants growing *in vitro* with plants growing in saline soil.

3.2 Bioinformatics Analysis

GB is present in almost all kingdoms and confers resistance against abiotic stress such as salinity, drought, and cold. In higher plants, GB is synthesized in two oxidative enzymatic steps of choline,

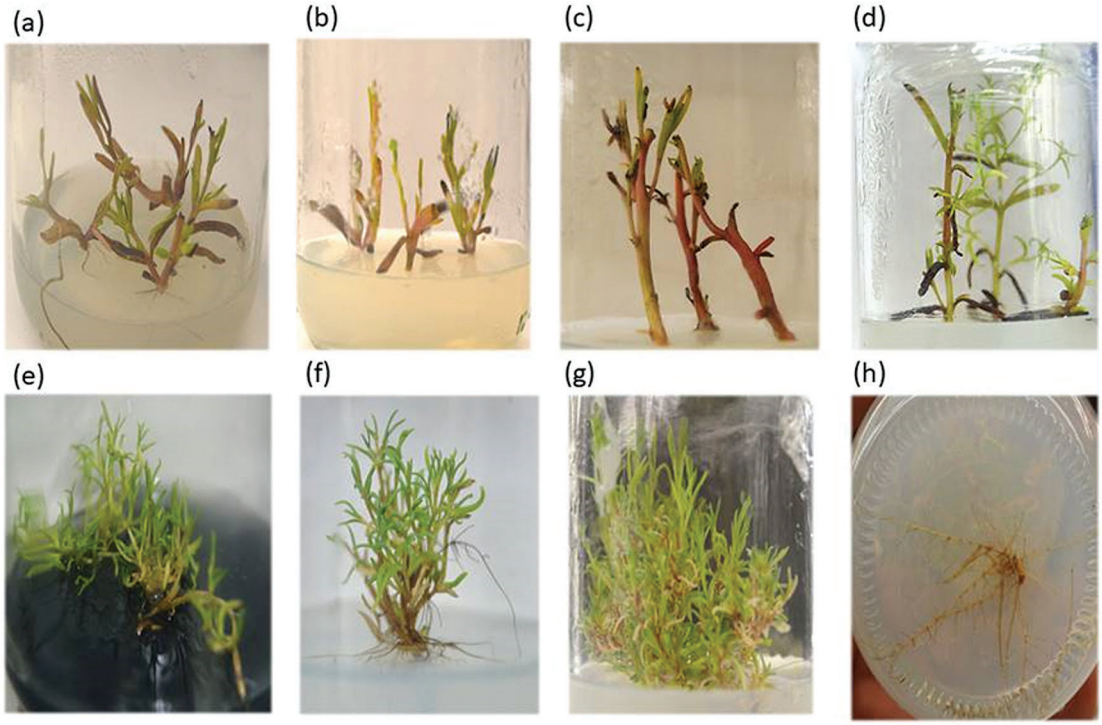

Figure 1: *In vitro* propagation of *S. edulis* process. In all the treatments, MS was used as a basal medium. (a-f) Seedlings at fifteen days, (a) seedlings in treatment 1; MS medium (b) seedlings in treatment 2; MS pH 8. 0, (c) seedlings in treatment 3; MS pH 8 plus BA 1 mg/ml, (d) seedlings in treatment 4; MS plus $CaCl_2$, (e) seedlings in treatment 5; MS plus activated carbon, (f) seedlings in treatment 6; MS plus BA 1 mg/ml, (g) seedlings from treatments 5 and 6 in MS after 30 days, (h) seedling roots in MS medium

catalyzed by CMO and BADH, respectively [4,21]. Generally, the gene studies for GB synthesis begins with CMO. However, in this work was decided to start with the gene that codes for SAMS, since this enzime catalyzes the formation of S-adenosyl methionine (SAM) and is a critical molecule for different metabolic processes such as DNA methylation, ethylene, polyamides, proteins, and lipids synthesis, and also plays a crucial role in the protection of plants against various abiotic stresses [22,23].

The corresponding sequences for these genes identified in *S. edulis* were named *SeSAMS*, *SeBADH*, *SeCMO*, and *SeP5CS*. Sequence analysis revealed that *SeSAMS* was 470 bp. It was deduced to encode a 153 amino acids polypeptide and showed a 97% similarity with *Suaeda salsa* and 91% with *Malus domestica*. Besides, SeSAMS sequence contains the central conserved (GAGDQG) SAMS motifs (Fig. 2a) that are presents in all plant SAMS; the protein sequence of SAMS has three conserved motifs: 1) the methionine binding GHPDK is at the amino terminus, 2) GAGDQG is at the central ATP-binding, and 3) is GGGAFSGK, which forms a P-loop for the phosphate-binding region [24]. The evolutionary history of the protein shows an important closeness with SAMS from *S. salsa* (Fig. 2b). To predict SeSAMS tertiary structure, we constructed a comparative model of its three-dimensional structure using Chimera software, which shows the GAGDQG domain and its splice with 4KTT (Fig. 2c).

For *SeCMO*, was obtained a fragment of 456 bp, and the putative translatable region was 152 amino acids (Fig. 3a). SeCMO showed 100% similarity to CMO of *Suaeda maritima* and *Suadea liaotungensis* and 88 % to *Salicornia europaea*. The phylogenetic analyses showed that the deduced amino acids

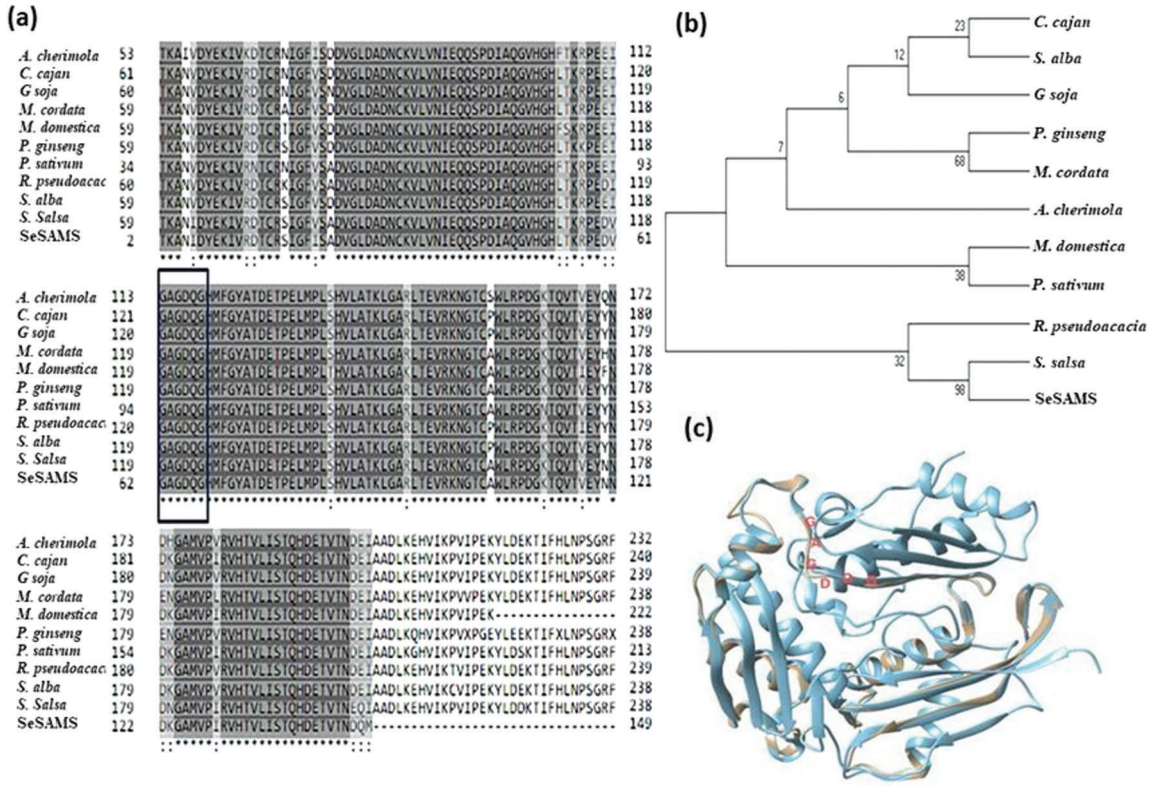

Figure 2: Alignment, evolutionary history, and hypothetical structural modeling of *SeSAMS*. (a), SeSAMS with SAMS from *Annona cherimola* ABC24692.1, *Cajanus cajan* AEY85025.1, *Glycine soja* KHN28175.1, *Macleaya cordata* OVA13537.1, *Malus domestica* BAI66450.1, *Panax ginseng* ACD92982.1, *Pisum sativum* AAA58772.1, *Robinia pseudoacacia* AIT39705.1 *Sonneratia alba* AGJ71754.1 and *Suaeda salsa* AAG42490.1; the amino acid residues conserved in all sequences are shown by asterisks, and conservative substitutions are shown by dots (residues that are associated with the putative ATP-binding site are shown by a box). (b), Evolutionary relationships of SeSAMS with taxa, inferred using the Neighbor-Joining method with MEGA7. The optimal tree with the sum of branch length = 0.25799755 is shown. (c), ProB domine in SeSAMS and its splice with chain B from transferase 4KTT

sequence protein was closely with the CMO of *S. liaotungensis*, although it also showed evolutionary closeness to *S. maritima* (Fig. 3b). SeCMO has conserved putative motif (G/DX3-4DX2HX4-5 H) for the coordination of mononuclear non-heme Fe. Multiple alignments of several CMO and CMO-like proteins present two conserved motifs, 1) for the coordination of the Rieske-type [2Fe-2S] cluster CXHX15-17 CXH El, and 2) for the coordination of mononuclear non-heme Fe, G/DXDX2HX4-52 H [25]. The comparative model constructed to predict the three-dimensional structure of SeCMO and its splice with chain A from oxidoreductase IWQL are shown in Fig. 3c, highlighting the CESVQKGLE conserved region located in the ring hydroxylating alpha subunit (catalytic domain).

For *SeBADH* we obtained a fragment of 252 bp, and the translatable region is 84 amino acids and contains part of the aldehyde dehydrogenase (ALDH) domain, characteristic of the ALDH superfamily (Fig. 4a). The sequence showed a 91% similarity with BADH of *Suaeda glauca* and *S. liaotungensis* and 77% with *Spinacia oloracea*. Its evolutionary history indicated that SeBADH is a unique subgroup with the BADH of *S. liaotungensis*, although it also showed closeness with that of *S. glauca* (Fig. 4b). The

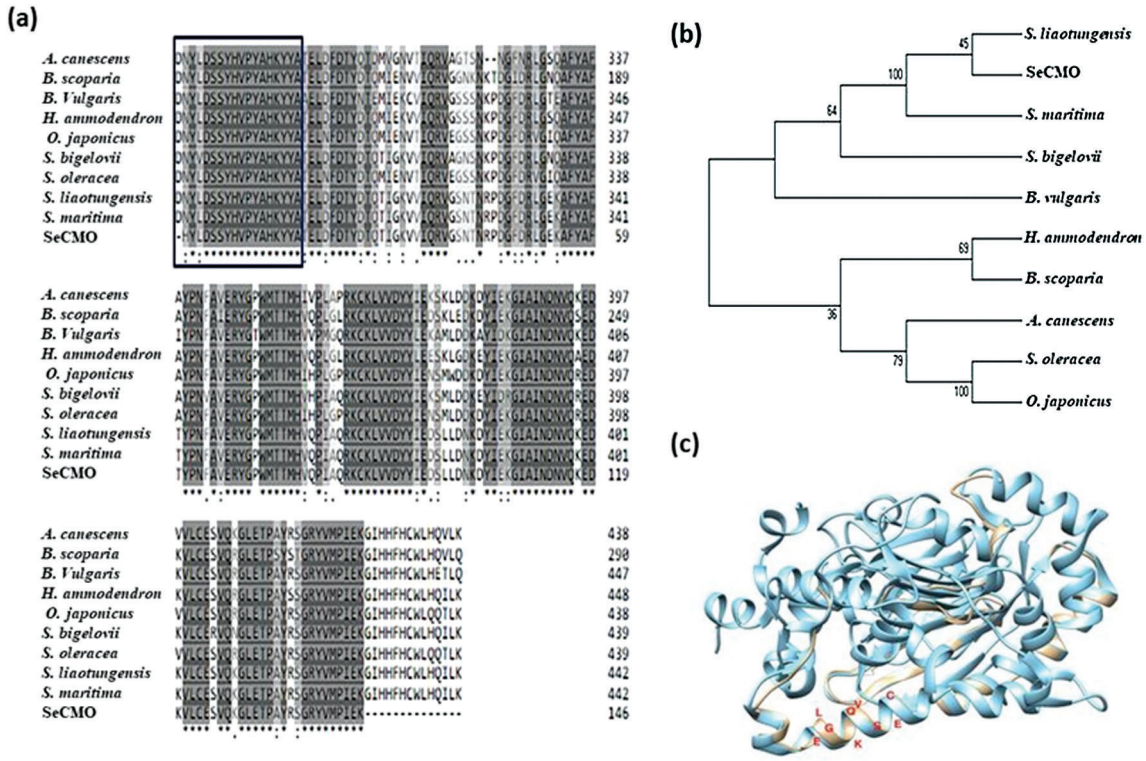

Figure 3: Alignment, evolutionary history, and hypothetical structural modeling of SeCMO. (a), SeCMO with CMO from *Atriplex canescens* AFG28558.1, *Bassia scoparia* AAQ92313.1, *Beta vulgaris* BAE07177.2, *Haloxylon ammodendron* ACX47904.1, *Ophiopogon japonicus* ABG34274.1, *Salicornia bigelovii* AJF98574.1, *Spinacia oleracea* ABN43460.1, *Suaeda liaotungensis* AAM43920.1 and *Suaeda maritima* AFW04225.1; the amino acid residues conserved in all sequences are shown by asterisks, and conservative substitutions are shown by dots. The putative consensus sequence for the coordination of mononuclear non-heme Fe (G/DX3–4DX2HX4–5 H) is shown by a box. (b), Evolutionary relationships of SeCMO with taxa, inferred using the Neighbor-Joining method with MEGA7. The optimal tree with the sum of branch length = 0.54709925 is shown. (c), SeCMO and its splice with chain A from IWQL oxidoreductase; both of them show the CESVQKGLE conserved region located in the ring hydroxylating alpha subunit (catalytic domain)

three-dimensional tertiary structure of SeBADH and its splicing with chain c from betaine aldehyde dehydrogenase 5A2D show the ALDH domain (Fig. 4c). Regarding the SeBADH fragment, its sequence was very short, and although it does not include the VTLELGGKSP decapeptide characteristic of these enzymes, it does have the highly conserved ALDH domain in this type of enzymes, and its evolutionary history grouped it in a clade that includes the BADH of *S. liaotungensis* (Fig. 4b).

The Δ1-pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) enzymes are involved in the glutamate pathway to produce proline, and studies have shown that this route is active when plants are under different types of environmental stress [8,9].

For *SeP5CS*, was obtained a fragment of 786 nucleotides, and encoding to predicted 262 amino acids polypeptide. SeP5CS showed a 69% similarity to the P5CS of *Suaeda salsa* and 61% to *Beta vulgaris*. The P5CS enzymes contain a glutamate 5-kinase (γ-GK) region corresponding to proB and followed by a gamma-glutamyl phosphate reductase (γ-PR) region corresponding to proA. SeP5CS only shows a part of

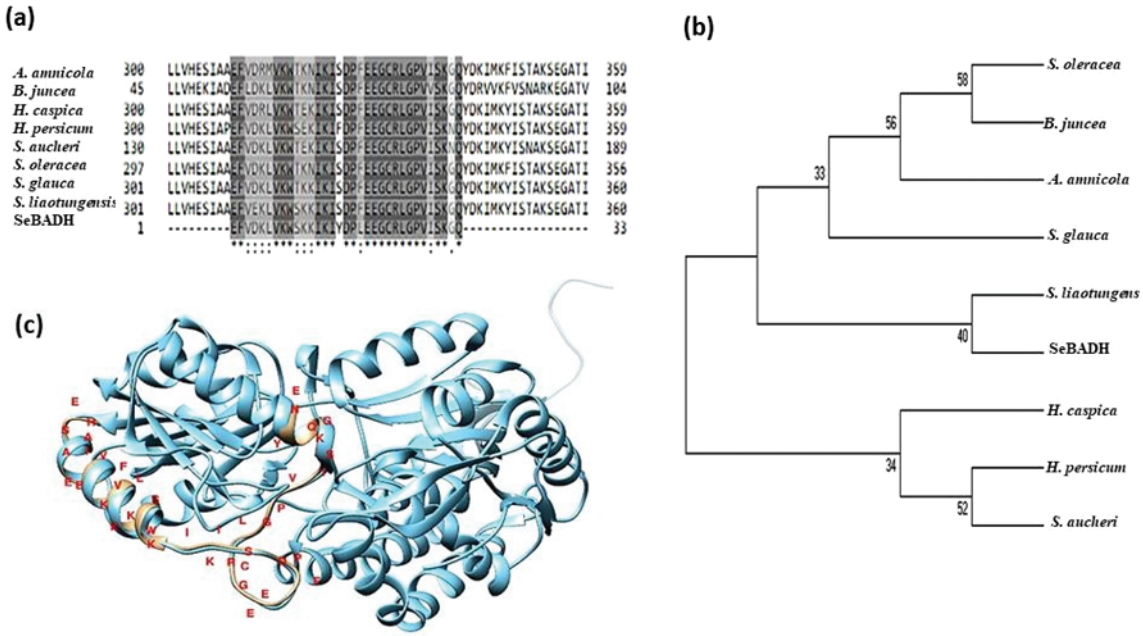

Figure 4: Alignment, evolutionary history, and hypothetical structural modeling of *SeBADH*. (a), SeBADH alignment with BADH from *Atriplex amnicola* AHH24263, *Brassica juncea* AEX55691.1, *Halostachys caspica* ABO45931.1, *Haloxylon persicum* AEW31327.1, *Salsola aucheri* AWK59869.1, *Spinacia oleracea* AAN52929.1, *Suaeda glauca* AHB08884.1 and *Suaeda liaotungensis* AAL33906.1; the amino acid residues conserved in all sequences are shown by asterisks, and conservative substitutions are shown by dots. (b), Evolutionary relationships of SeBADH with taxa, inferred using the Neighbor-Joining method with MEGA7. The optimal tree with the sum of branch length = 0.85537383 is shown. (c), ALDH-SF domine in SeBADH and its splice with chain c from 5A2D BADH

the glutamate 5-kinase domain (PLN02418), representative of delta-1-pyrroline-5-carboxylate synthase (Fig. 5a). The phylogenetic tree shows SeP5CS sub-grouped only with the P5CS from *S. salsa* (Fig. 5b). The three-dimensional comparative model of SeP5CS and its splice with the P5CS template 2H5G also show conserved amino acids (Fig. 5c)

3.3 Expression Analysis

In some studies, with *Suaeda* sp., the gene expression associated with saline stress is analyzed only in germinated seeds or plants grown in the laboratory stimulated with different concentrations of NaCl [26]. However, no study has shown gene expression in their natural habitat. In this study, was analyzed the expression of the *SeSAMS*, *SeCMO*, *SeBADH* and *SeP5CS* genes in different organs of *S. edulis* grown in its natural habitat (crater), since the soil has a pH 9.2 and a high concentration of sodium and salts, and compare it with that of seedlings grown *in vitro* (pH 5.7).

The real-time expression results showed that in leaves from crater plants, the expression levels of *SeSAMS*, *SeBADH*, and *SeCMO* are higher than *in vitro* plants (Fig. 6). Similar results were found for *BADH* and *SAMS* from *Suaeda salsa* [5] and *Suaeda aegyptiaca* [27], and *CMO* with *Solanum lycopersicum* [28] suggesting that GB could be synthesized mainly in the foliage and that *SAMS* transcription levels which are co-regulated with those of *BADH* and *CMO*. It has been observed that *BADH* high expression is associated with an increase in enzyme activity in response to salinity. Also, it has been observed that, in the genus *Suaeda* treated with NaCl, there is overexpression of genes involved

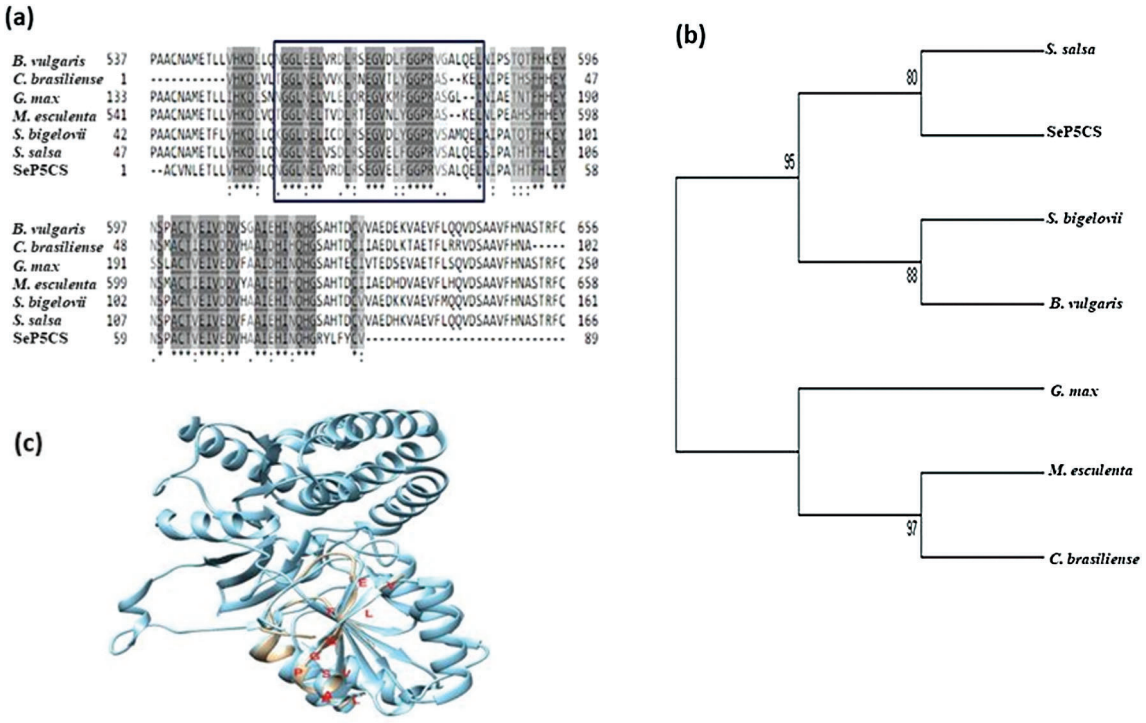

Figure 5: Alignment, evolutionary history, and hypothetical structural modeling of SeP5CS. (a), SeP5CS with P5CS from *Beta vulgaris* AQZ26218.1, *Calophyllum brasiliense* AHN15384.1, *Glycine max* CAG29643.1, *Manihot esculenta* ANU05021.1, *Salicornia bigelovii* AGN48983.1 and *Suaeda salsa* AAM28630.1; the amino acid residues conserved in all sequences are shown by asterisks, and conservative substitutions are shown by dots. The putative Leu domain is shown by a box. (b), Evolutionary relationships of SeP5CS with taxa, inferred using the Neighbor-Joining method with MEGA7. The optimal tree with the sum of branch length = 0.25799755 is shown. (c), ProB regions in SeP5CS and its splice with P5CS, 2H5G mold

in GB synthesis; for example, was found *CMO* overexpression in the shoot from *S. salsa* with NaCl 400 mM [5]. Similarly, Kuttan et al. [29] working with *S. maritima* seedlings treated with NaCl 200 mM observed *SAMS* overexpression concluding that GB accumulation is a response to salinity. These authors explain that *CMO* overexpression under saline conditions is essential in this plant for GB high accumulation in the tissue due to they noted that *BADH* expression is much lower than that *CMO* expression; however in this work, there was not a significate difference between both of them.

In addition to GB, another important osmoprotectant is Pro, and it is known that P5CS is the limiting enzyme in its synthesis [8,9]. In this study, *SeP5CS* expression levels were significantly higher in the roots and leaves from crater than *in vitro* plants (Fig. 6). Wei et al. also detected *P5CS* overexpression in roots and leaves from *Lilium regale* stimulated with 250 mM NaCl, and observed Pro accumulation, grew better in root and salinity tolerance [30]. It has also been suggested that synthesis of Pro in roots seems to be an essential parameter to halophytes differentiation, since its production in salinity tolerant plants starts with ionic stress. Under these conditions, the Pro concentrations reach higher levels than in susceptible plants [1,3,8].

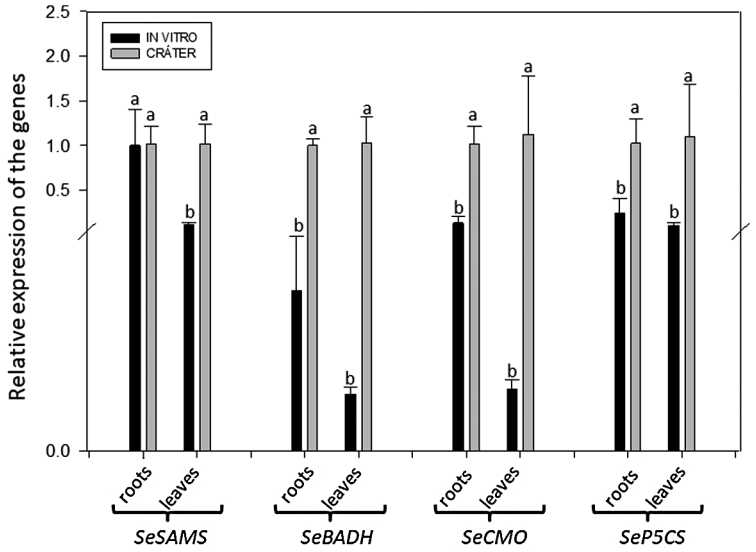

Figure 6: Quantitative RT-PCR of *SeSAMS*, *SeBADH*, *SeCMO*, *SeP5CS*, and *SeVP* in *S. edulis*. Black bars indicate the response of roots and leaves from *in vitro* culture plants, and gray bars show the response of roots and leaves from the field plants. *SeSAMS* showed expression higher in the leaves of plants from field; the expression difference between roots was not statistically significant. *SeBADH*, *SeCMO*, and *SeP5CS* showed higher expression in roots and leaves from field plants

4 Conclusion

The internodal regions with axillary buds were good material for the propagation for *S. edulis*. The use of activated charcoal after micropropagation improved the growth and development of the seedling without the need to use growth regulators. Gene expression by qRT-PCR analysis revealed higher expression of *SeBADH*, *SeCMO*, and, *SeP5CS* genes in the roots and leaves from plants collected in the field as well as from *in vitro* plants. However, the expression level of *SeSAMS* was higher only in the leaves of plants collected in the field when compared to those cultivated *in vitro*.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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