

In Vitro Approach to the Multiplication of a Halophyte Species Forage Shrub *Atriplex halimus* L. and *in Vitro* Selection for Salt Tolerance

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ABSTRACT

A successful and detailed *in vitro* multiplication system for micropropagation of the forage shrub *Atriplex halimus* L. has been developed. Explants excised from adult shrubs were surface-disinfected before being placed onto Murashige and Skoog (MS) basal medium containing a combination of plant growth regulators (PGRs) at different concentrations: N⁶-benzyladenine (BA) or kinetin at 2.22 or 4.44 μM each with indole-3-butyric acid (IBA) at 0.49 μM and gibberellic acid (GA₃) at 0.58 μM. A 7.2-fold multiplication rate was achieved every 4 weeks on MS medium supplemented with 4.44 μM BA, 0.49 μM IBA and 0.58 μM GA₃. Rooting was achieved with 73% efficiency within 2-4 weeks on agar-gelled MS basal medium free of PGRs. Rooted plantlets were gradually acclimatized to field conditions over 5-6 weeks with 65% efficiency. MS medium was supplemented with increasing concentrations of NaCl ranging between 25 and 1000 mM to study the effects on multiplication and to select high salt-tolerant clones *in vitro*. *In vitro* shoots could tolerate up to 600 mM NaCl with optimal growth at 200 mM, while higher concentrations of NaCl affected growth negatively. Growth and shoot number decreased with increasing NaCl concentration; all plantlets died at 1000 mM NaCl. A few clones survived high concentrations of NaCl (600 mM) which are being propagated under the same stress conditions for further studies. This method has the potential to produce masses of plantlets within a short time to expand its cultivation in dry and saline areas. Doing so would contribute to alleviating desertification and providing fodder for livestock, mainly during the dry season.

Keywords: N⁶-benzyladenine (BA), cytokinin, kinetin, micropropagation, shoot formation, tissue culture

Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; 2iP, isopentenyl-adenine; BA, N⁶-benzyladenine; GA₃, gibberellic acid; ha, hectare; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog; NAA, α-naphthalene acetic acid; NN, Nitsch and Nitsch; PGR, plant growth regulator; WPM, medium, McCown Woody Plant Medium (1980)

INTRODUCTION

Desertification, as defined by the UN Convention to Combat Desertification (UNCCD), is a process of land degradation in arid, semi-arid and dry sub-humid areas resulting from various factors, including climate variation and human activities. 30% of the earth's land is threatened by desertification and 500,000 ha turning to desertification annually. This costs the world 52 billions dollars a year (UNCCD 2002). There are more than 110 countries that are jeopardized by desertification (Harba 2006). The area of degraded lands in Arab countries is about 316.6 million ha, mainly because of salinity which affects about 134.1 million ha (Wardeh 2005). The Syrian Arab Republic is located on the Eastern coast of the Mediterranean Sea with an area of 18,517,071 ha and a population of about 20.376 million (Anon 2009). Dry land in Syria accounts for about 10 million ha, about 55% of the entire country, with annual rainfall of < 200 mm per year for the last 20 years (Wardeh 2005). Desertification is threatening large areas in Syria mainly due to salinity, which has expanded to the irrigated areas in the Eastern part of the country (Harba 2006). Therefore, it is crucial to prevent salinity and desertification in any way possible. The presence of salt in soil is one factor that increases arid lands areas in the region. The salinity that covers a large area and which is remarkably amplified with severe drought represents the main edaphic constraint limiting plant growth (Abbad *et al.* 2004). One way to reha-

bilitate and reclaim these soils is by planting salt-tolerant forage species. Among these species is the genus *Atriplex*, which has recently raised interest where some 100,000 ha have been planted in the Mediterranean basin. Species of the genus *Atriplex* are known for their high tolerance to aridity and salinity (Le Houerou 1992). Using halophytes to produce forage in saline lands is the best economic solution (Khan and Duke 2001). Halophytes can play a role in soil reclamation in degraded lands (Le Houerou 1992). *Atriplex* are the dominant plants in arid and semi-arid lands of the world, especially when soil is combined with salinity (Ortiz *et al.* 2005). *Atriplex halimus* L. is a member of the *Chenopodiaceae* and one of the major species used in combating desertification due to its salt tolerance, which is defined as a plant's ability to grow under saline stress (Munns 2002). In a semi-arid climate, desertification becomes a serious problem with the progressive reduction of vegetation cover coupled with rapid soil erosion (Ramos *et al.* 2004). Drought resistance and salt tolerance are complex traits involving several interacting properties. *A. halimus* is an important Mediterranean xerohalophyte saltbush species highly resistant to drought (Le Houerou 2000), salinity (Bajji *et al.* 1998) and heavy metal stress (Lutts *et al.* 2004). Therefore, this study was carried out to understand the effect of increasing concentrations of NaCl on shoot growth for selection *in vitro* of highly salt-tolerant clones. The *in vitro* culture of different *Atriplex* spp. has been demonstrated (Wochok and Sluis 1979; Amato *et al.* 1990; Kenny

Table 1 Comparison between micropropagation conditions of the present study with similar previous studies.

| <i>Atriplex</i> spp. | Explant used and method of regeneration | Basal medium + PGRs | Multiplication rate | Rooting % | References |
|------------------------|--|--|------------------------|-----------|--------------------------|
| <i>A. nummularia</i> | Nodal explants / <i>In vitro</i> shoot regeneration | MS + 0.5 mg/l BAP MS + 0.5 mg/l Kin MS + 0.5 mg/l IBA for rooting | not mentioned (N.M) | | Reddy <i>et al.</i> 1996 |
| <i>A. nummularia</i> | Axillary buds / <i>In vitro</i> shoot regeneration | MS + 0.5 mg/l TDZ + 0.5 mg/l IAA MS + 0.5 mg/l BA + 0.5 mg/l NAA 1/2 MS + phloroglucinol for rooting | N.M | 65% | Al Wasel 1998 |
| <i>A. glauca</i> | Clusters of flowers / <i>In vitro</i> regeneration | NN+0.1 mg/l IAA + 0.1 mg/l Kin NN+0.1 mg/l IAA + 0.1 mg/l 2ip WPM for rooting | 13-18 | | Kenny and Caligari 1996 |
| <i>A. canescense</i> | shoot tips / <i>In vitro</i> regeneration | MS + IAA + Kin | N.M* | | Wochok and Sluis 1979 |
| <i>A. canescense</i> | Axillary buds and leaf disc/ <i>In vitro</i> regeneration | MS + 2 mg/l BA+ 0.01 mg/l NAA MS + 0.5 mg/l IBA + 0.1 mg/l GA ₃ for rooting | 9-12.3 | 65% | Mei <i>et al.</i> 1997 |
| <i>A. nummularia</i> , | Axillary buds / | MS + 0.2 mg BA + 0.05 mg NAA | N.M | | Malan 2000 |
| <i>A. canescense</i> | <i>In vitro</i> regeneration | MS without PGRs for rooting | | | |
| <i>A. halimus</i> | Calli/ <i>In vitro</i> regeneration | MS + 2 mg/l kin + 2 mg/l 2.4D + Kin | N.M | | Zohra <i>et al.</i> 2008 |
| <i>A. canescense</i> | | | | | |
| <i>A. halimus</i> | Axillary buds - shoot tips /stem nodes <i>In vitro</i> regeneration | MS + 2.22 μM BA + 0.49 μM IBA + 0.58 μM GA ₃ MS without PGRs for rooting | 7.2 | 73.33% | Present study |
| <i>A. halimus</i> | <i>In vivo</i> propagation by cuttings | 2000 ppm IBA 4000 ppm IBA | | 70% | Amato <i>et al.</i> 1999 |

and Caligari 1996; Reddy *et al.* 1996; Mei *et al.* 1997; Alwasel 1998; Malan 2000; Zohra *et al.* 2008) and a brief comparison of micropropagation conditions between the present study with some similar previous studies is presented in **Table 1**.

On the other hand, some research has been conducted to study the effect of salinity stress on *Atriplex* spp. under *in vitro* culture conditions (Priebe and Jäger 1978; Reddy *et al.* 1996; Bajji *et al.* 1998; Abbad *et al.* 2004; Araújo *et al.* 2006; Nedjimié *et al.* 2006). Recently, biotechnology methodologies have been developed to isolate drought- and ozone-induced and salt tolerance genes from *Atriplex* spp. (Nedjimié *et al.* 2006). This long-term objective might allow for the possible isolation and use for gene transfer and genetic modification of *Atriplex* spp. for improved resistance to environmental stresses. However, application of gene transfer requires an appropriate tissue culture system.

In the present study, a method for the micropropagation of *A. halimus* was applied so that tissue-cultured plantlets can be efficiently produced for large-scale clonal multiplication and also for *in vitro* selection for clones of high salt tolerance. The final purpose of this study was to be able to extend its cultivation to areas with harsh conditions (dry and saline soils), thus contributing to the alleviation of desertification while providing feed for livestock.

MATERIALS AND METHODS

Initiation of cultures

The current study was carried out at the Biotechnology Department, General Commission for Scientific Agricultural Research (GCSAR), Damascus, Syria. Plant material used in this study was obtained from a shrub of *A. halimus* grown in a desert area at Altalila Reservation near Palmyra, Syria.

Shoot tips, axillary buds, and stem nodes were taken from rapidly growing new branches during spring. These explants were washed with water and detergent, they were then shaken for 1 min in 70% ethanol followed by immersion in 15, 25 and 50% commercial Clorox (5.25% sodium hypochlorite) for 10, 15 and 25 min. Finally, explants were rinsed three times with sterile distilled water and transferred to multiplication medium.

Culture media

Murashige and Skoog (MS, 1962) basal medium, used throughout this research, supplemented with 3% sucrose (HiMedia-laboratories Pvt. Limited, India) and solidified with 0.7% agar (HiMedia).

Medium was dispensed into culture tubes (2.5 × 20) cm containing 15 ml medium, which were covered with aluminum foil, and sterilized by autoclaving at 121°C for 21 min. The pH was adjusted to 5.7 before autoclaving.

1. Initiation medium

This consisted of basal MS medium with half-strength macroelements. Three explants were cultured in each glass jar (200 ml) containing 30 ml medium.

2. Multiplication medium

At the end of the 5th subculture, shoots 2-3 cm long were harvested from 4-weeks old proliferating cultures and cultured on MS basal media with six combinations of hormonal supplements to the basic medium to try and optimize shoot induction (**Table 2**). Subculturing was performed every 4 weeks on MS medium supplemented with 4.44 μM BA until sufficient shoots formed so as to be able to evaluate the efficacy of media. Basal medium was supplemented with different combinations of plant growth regulators (PGRs): N⁶-benzyladenine (BA) (Duchefa Biochemie, The Netherlands) or kinetin (Kin) (Duchefa Biochemie) at 2.22 or 4.44 μM each with indole-3-butyric acid (IBA) (Duchefa Biochemie) at 0.49 μM; all of these PGR combinations were in turn supplemented with 0.58 μM gibberellic acid (GA₃) (Duchefa Biochemie) (**Table 2**).

Explants were subcultured every 4 weeks, at which point data was calculated (after fifth subcultures from culturing on multiplication media with 30 days intervals at the end of the 4th week). The number and length of shoots produced per single explant of 1-2 cm length was recorded (the length was measured from the surface of the medium to the top of longest shoot).

3. Rooting medium

For rooting, shoot tips 2-3 cm long were excised from multiplication cultures and transferred to rooting media with MS basal medium free of PGRs (as control), 3% sucrose and solidified with 0.7% agar or supplemented with IBA at 0.49 or 2.45 μM. Rooting percentage number and length of the longest root, were recorded after 30 days after culture on rooting media (**Table 3**).

4. Media for *in vitro* selection for salt tolerance

MS basal medium was supplemented with increasing concentrations of NaCl (25, 50, 100, 150, 200, 300, 400, 500, 600, 1000 mM) to study the effect of salt on multiplication and selection *in*

in vitro of shoots which tolerate high concentrations of salt. Three explants of 1-2 cm long were cultured per 200-ml glass jar containing 30 ml medium. Cultures were maintained for 4 weeks in a growth room after which the number and length of shoots which were still alive were recorded in terms of the number and length of new shoots produced as affected by salt concentration in the medium.

Culture conditions

Three explants per 200-ml glass vessel containing 30 ml of MS medium supplemented with 4.44 μM BA were subcultured every 4 weeks for four subcultures until there were sufficient suitable shoots (around 400 shoots) for starting experiments to evaluate media for shoot formation. The shoot cultures were maintained in a growth room at $25 \pm 1^\circ\text{C}$ and a 16-h photoperiod provided by Philips fluorescent lamps giving an average light intensity of ca. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux at the surface of culture vessels to assess shoot induction responses. All media were adjusted to pH 5.7 with 1 N KOH or 1 N HCl prior to autoclaving at 121°C , 1.4 kg/cm^2 for 20 min.

Acclimatization

Rooted plantlets were then acclimatized gradually to field conditions by transplanting into pots with a mixture of 2: 1 (v/v) peat moss: perlite (local origin Mahran, Syria) and were covered with plastic bags and acclimatized gradually to field conditions for a 4-week period.

Experimental design and statistical analyses

Data of all experiments were recorded after 4 weeks from culture on the experimental media. Thirty replicates were used per proliferation treatment and 20 replicates per rooting treatment. Each culture jar was a repetition in a randomized block experimental design, in which the six different media were compared. For each treatment, 30 explants were used with 3 explants/jar and 10 replications. Significance was determined at $P = 0.05$ according to Duncan's multiple range test by analysis of variance (ANOVA-2) using the statistical evaluation program SPSS v. 15. All experiments were repeated three times.

RESULTS AND DISCUSSION

Disinfection and culture initiation

In vitro contamination is a serious problem with tissue culture initiation. Trees in the field have been growing in open conditions for extended periods of time and their organs are frequently contaminated both externally and internally by various microorganisms (Campbell 1965). Most of the organisms that are encountered are of no particular importance to the plant *in vivo*, but can result in contamination when cultured *in vitro* because bacteria and fungal spores grow rapidly on the rich culture medium. Commercial Clorox (5.25% sodium hypochlorite) efficiently surface-disinfected explants. Immersion in 15% commercial Clorox for 25 min was the best method for surface disinfection with 79% efficiency decontamination of which 92% were survived (Fig. 1). Higher concentrations were too strong, caused bleaching with resulting death of explants. Kenny and Caligari (1996) used 5% commercial Clorox to surface sterilize *A. glauca* flower clusters. In our study, in contrast, we used 15% Clorox because *A. halimus* is a woody plant, unlike *A. glauca*. In addition, we used axillary buds which are harder than flowers. Mei *et al.* (1997) used 100% commercial bleach for 20 min to sterilize Axillary buds and leaf disc of *A. canescens*.

Effects of PGRs on shoot organogenesis and shoot multiplication *in vitro* of *A. halimus*

Adventitious shoots originating along the cut base of explants and were clearly visible after 3-4 weeks' culture.

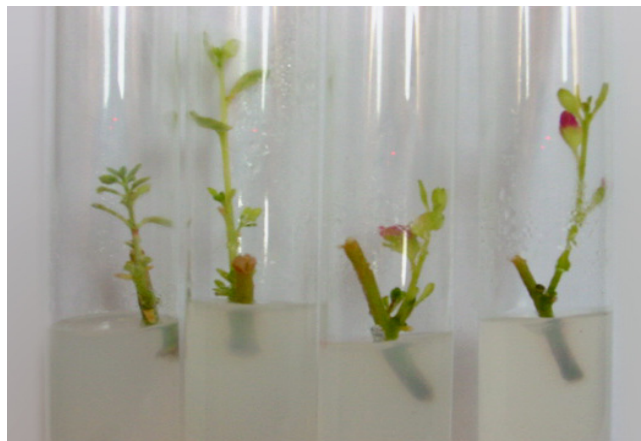


Fig. 1 Initial culture of *Atriplex halimus* L.



Fig. 2 Effect of best combination of growth regulators and best concentrations on *in vitro* proliferation of *Atriplex halimus* L.

Considering both the percentage of explants producing shoots and the number of shoots/explant, best shoot multiplication was achieved on media supplemented with 4.44 μM BA, 0.49 μM IBA and 0.58 GA_3 with a multiplication rate of 7.2 shoots/ explant. There was a significant reduction in shoot multiplication rate when BA was replaced with Kin, which resulted in a multiplication rate of 2.1 (Table 2, Fig. 2).

Results presented in Table 2 demonstrate the effects of a combination of PGRs at different concentrations on the number and length of shoots. The best multiplication rate was achieved on medium containing 4.44 μM BA + 0.49 μM IBA + 0.58 μM GA_3 with an average of 7.26 new shoots/explant every 4 weeks (Fig. 2). On other hand, control medium gave the best average shoot length (2.08 cm). The results presented here confirm earlier observations of Malan (2000) who reported that the best medium for shoot multiplication and elongation of *A. canescens* was MS with 0.20 mg/l BA + 1 mg/l NAA while MS medium with 0.05 mg/l BA + 0.05 mg/l NAA was best for elongation. Our results also agree with those of Mei *et al.* (1997) who produced shoots from both leaf discs and axillary buds. In their study, the best medium for shoot production was 2 mg/l BA and 0.01 mg/l NAA while the best medium for shoot elongation was PGR-free MS: the number of shoots generated ranged from 0.7 to 9.1/explant. Reddy *et al.* (1996) found that MS medium with 0.5 mg/l BA or 0.5 mg/l Kin was best for micropropagation of *A. nummularia*.

Table 2 Effect of PGRs combination s on the number and length of shoots produced *in vitro* in *A. halimus* L. within 4 weeks of culture.

| Treatment | Combination of PGRs (Conc. in μM) | Average № of shoots (\pm SEM)* | Average shoot length (cm) (\pm SEM)* |
|-----------|---|-----------------------------------|---|
| 1 | MS + 2.22 BA + 0.49 IBA + 0.58 GA ₃ | 4.73 \pm 0.15 b | 1.05 \pm 1.25 b |
| 2 | MS + 4.44 BA + 0.49 IBA + 0.58 GA ₃ | 7.26 \pm 0.32 a | 1.0 \pm 2.05 b |
| 3 | MS + 2.22 Kin + 0.49 IBA + 0.58 GA ₃ | 2.96 \pm 0.16 bc | 1.07 \pm 0.61 b |
| 4 | MS + 4.44 Kin + 0.49 IBA + 0.58 GA ₃ | 2.13 \pm 0.27 c | 1.06 \pm 0.28 b |
| 5 | MS + 0.49 IBA + 0.58 GA ₃ | 2.36 \pm 0.32 c | 1.16 \pm 0.55 b |
| 6 | MS medium Control (Free of PGRs) | 4.4 \pm 0.89 bc | 2.08 \pm 1.22 a |
| LSD 0.05 | | 2.53 | 0.59 |

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$).
Data are the means of 30 replications

Table 3 Effect of IBA concentration on rooting parameters *in vitro* in *A. halimus* L. within 4 weeks of culture on rooting media.

| Treatment | IBA concentration (μM) | Rooting % (\pm SEM)* | Average № of roots (\pm SEM)* | Average root length (cm) (\pm SEM)* |
|-----------|-------------------------------------|-------------------------|----------------------------------|--|
| 1 | 0.49 | 46.67 \pm 0.15 b | 1.93 \pm 0.60 a | 1.14 \pm 0.09 a |
| 2 | 2.45 | 20 \pm 0.1 c | 2.33 \pm 0.57 a | 0.64 \pm 0.16 b |
| 3 | Control (free of PGRs) | 73.33 \pm 0.251 a | 3.33 \pm 1.02 a | 1.63 \pm 0.87 a |
| LSD 0.05 | | 12.56 | 1.4 | 0.49 |

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$).
Data are the means of 20 replications

Cytokinins such as TDZ and BAP have considerable effects in inducing regeneration in most woody plants (Korban *et al.* 1992; DeBondt *et al.* 1996). BA has been the most commonly used cytokinin for proliferation of many plants (Murai *et al.* 1997). A high concentration of cytokinin with low concentrations of auxins results in a high proliferation efficiency in many plant species (Pierik 1987).

In the present study, however, although induction of shoots was observed in most media tested, BA proved to be more efficient than Kin in shoot induction (**Table 2**).

Our results demonstrate that an optimum combination of PGRs plays a key role in the successful induction of shoot organogenesis *in vitro*. The number of shoots/explant was influenced by the type and concentrations of PGRs used. The number of newly produced shoots varied between 2.1 and 7.2 (**Table 2**). No abnormality, necrosis or chlorosis was observed during culture. Most explants produced shoots and green shoot meristems were seen on a range of media containing BA or Kin and also on control medium free of PGRs.

Multiple shoot induction rate and organogenic response varied significantly to a greater extent according to the explant type and concentrations of PGRs used. Kenny and Caligari (1996) recorded that NN medium was more appropriate than MS medium in their study on *A. glauca*. The IAA/2iP combination at 0.1/0.1 mg/l was better than IAA/Kin at 0.1/0.1 mg/l and gave the best response with this species. Zohra *et al.* (2008) found that MS medium with Kin and 2,4-D 2-2 mg/l was best for *in vitro* shoot induction from the calli of *A. halimus* and *A. canescens*. However, our study showed that shoots could be induced in only 4 weeks unlike the study of Mei *et al.* (1997) who noted that shoot induction in *A. canescens* took at least 2 months. In our study, however, shoot formation could be developed without an intervening callus phase. Moreover, the type of explant and culture medium with specific PGR concentrations influenced organogenesis considerably. We demonstrated that shoot tips and nodal explants can be used for clonal propagation on optimum culture medium.

Rooting

Proliferated shoot tips (2-3 cm length) were excised and rooted readily on MS medium. Rooting was observed from the cut ends of the shoots within 30 days. All of the developing roots were physically vigorous and healthy. Results in **Table 3** present rooting data: best rooting rate (73.33%) with an average number of roots of 3.3 and the best average stem length being 1.63 cm were achieved on MS medium (**Fig. 3**) while 46% rooting efficiency could be recorded on medium containing 0.49 μM IBA. Our study agrees with the findings of Malan (2000) who noted that MS basal

**Fig. 3** Rooting of *Atriplex halimus* *in vitro*.

medium without any PGRs was the best medium for rooting. Al-Wasel (1998), who studied the micropropagation of *A. nummularia*, noted that $\frac{1}{2}$ -MS without PGRs with phloroglucinol (162 mg/l) was the best rooting media. In contrast, Mei *et al.* (1997) produced *A. canescens* roots using 0.5 mg IBA and 0.1 mg/l GA₃ in MS medium with 65% success. In several species, the utilization of high concentrations of IBA produces callus and abnormal roots that affect the survival of explants during acclimatization (Welander 1983; Yepes and Aldwinckle 1994). In the current study, high concentrations of auxins did not give good results and callus formation was induced at the bases of shoots. Best rooting percentage was better on medium free of PGRs or containing low concentration of auxin (**Table 3**, **Fig. 3**). Amato *et al.* (1990) recorded best rooting percentage of 67% using IBA at 2000 mg/l although increasing IBA concentration to 4000 mg/l resulted in decreased rooting to 40%. However, in our study, 73% rooting efficiency could be obtained on media without any auxin while use of IBA at 0.49 and 2.45 μM (equiv. 0.1 and 0.5 mg/l) decreased rooting efficiency to 46.6 and 20%, respectively.

Shoot tips and axillary buds from 2-year-old shrubs were excised and used for micropropagation because some studies mentioned that shoot age influences rooting positively probably because of the greater quantity of reserve material, or because of bud dormancy (Amato *et al.* 1990).

Acclimatization of rooted plantlets

During *in vitro* culture, plantlets grow under very special conditions, these conditions result in the formation of plantlets of abnormal morphology, anatomy and physiology. After transfer from the *in vitro* to the *ex vitro* conditions, the plantlets have to correct the above-mentioned abnormalities. However, plantlets need gradual changes in environmental conditions to avoid desiccation losses and photoinhibition (Kozai *et al.* 1991). Al-Wasel (1998) reported a 55% acclimatization efficiency for micropropagated *A. nummularia*, which is in agreement with the findings of our study but 65% efficiency for *A. halimus* in which rooted plantlets were acclimatized to ambient conditions within 2 months and were later established under greenhouse conditions (Fig. 4) and finally in the field under natural conditions (Fig. 5). Mei *et al.* (1997) also reported 65% survival of acclimatized shoots of *A. canescens*. Rooted *A. canescens* plantlets were also successfully acclimatized by Malan (2000) but without any reference to the acclimatization efficiency obtained.

Salt tolerance of *A. halimus*

Results of the present study indicate that *A. halimus* is a salt-tolerant species also in *in vitro* conditions, where an increase in shoot number could be recorded in media containing up to 200 mM NaCl, while higher concentrations of NaCl (400-500-600 mM) inhibited plant growth *in vitro* (Table 4). This result is in agreement with Khan *et al.* (1999) who recorded little inhibition in seedling growth of *A. griffithii* in media containing up to 180 mM NaCl, while 360 mM NaCl inhibited plant growth.

Our study indicated that *A. halimus* is a highly salt-tolerant species that survived at 600 mM NaCl (salinity of seawater). NaCl at 200 mM was most conducive to plant growth, where the size of leaves was bigger than the control (Fig. 6). NaCl has been used in most studies on salt tolerance in many plants. However, Nedjimi *et al.* (2006) used CaCl₂ to study salt tolerance in *A. halimus* and found that 8 g l⁻¹ CaCl₂ (about 72 mM) allowed best plant growth. Similar results were found on a study on *A. nummularia* which showed that the growth gain of plants was stimulated by 22% with NaCl at 300 mM, and then reduced significantly in the highest NaCl treatment of 600 mM NaCl with a 26% decrease in growth relative to the control. *A. nummularia* takes up Na⁺ and Cl⁻ in high amounts, but seems to store them efficiently up to an external 300 mM NaCl level only. Above this concentration, however, such a mechanism fails and growth of shoot tissues is negatively affected (Araújo *et al.* 2006). Our results also similar to those of Greenway *et al.* (1966) who reported that growth of *A. nummularia* was optimal at 100 to 200 mM NaCl. Our results also confirm the observations of Ashby and Beadle (1975) that showed that growth of *A. inflata* and *A. nummularia* was greater with NaCl at 600 mM than in controls.

The growth of halophytes such as *Atriplex* spp. is stimulated by NaCl concentration which would inhibit the growth of non-halophytes (Osmond *et al.* 1980). It seems that growth response at moderate salinities might be largely the consequence of increased throughput of solutes required to derive cell expansion (Khan *et al.* 2001). An important finding is that plants could survive at 600 mM NaCl, which might be suitable concentration to use for selection of highly salt-tolerant plants. Higher stress with NaCl induced death in almost all shoots, while just a few could survive, which were selected and multiplied under the same stress conditions for further studies in the near future. Many studies have shown that *Atriplex* spp. such as *A. nummularia*, *A. griffithii* and *A. hortensis* could survive under highly saline conditions, with optimal growth occurring at 5 to 10 g l⁻¹ NaCl (85.5-171 mM) (Khan *et al.* 2000; Wilson *et al.* 2000; Ramos *et al.* 2004) which are in agreement with Pribe and Jäger (1978) who found that *Atriplex* spp. vary in their degree of salt tolerance, but all three species studied (*A.*



Fig. 4 *In vitro* propagated acclimatized *Atriplex halimus* (2-3 months old) grown in the green house.



Fig. 5 *In vitro* propagated *Atriplex halimus* (1 year old) under field condition.

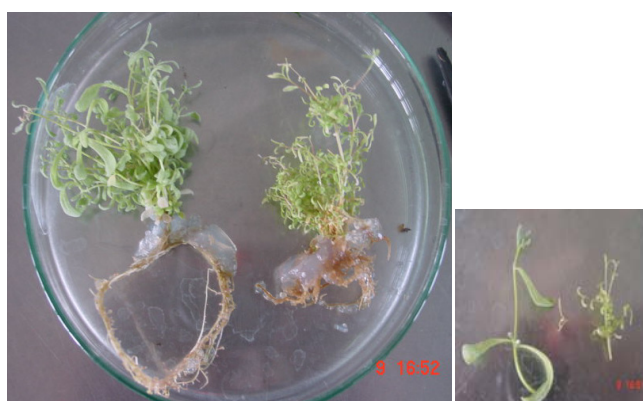


Fig. 6 Effect of NaCl on the growth and size of the leaves *in vitro* of *A. halimus* L. Left: shoots grown on medium 5 with 200 mM NaCl. Right: Control: shoots grown on medium free of NaCl.

halimus, *A. calotheca*, *A. nilens*) were able to survive at 750 mM NaCl. Storey and Wyn Jones (1979) determined that *A. spongiosa* was able to grow in medium containing 600 mM NaCl with dry mass production decreasing by 50% at 800 mM NaCl. Our study indicates that increasing NaCl concentrations had a negative effect on plant growth; there are many studies indicating that Na⁺ and Cl⁻ content in shoots and roots increased with increasing salinity (Greenway *et al.*

Table 4 Effect of NaCl concentrations on the number and length of shoots produced *in vitro* in *A. halimus* L. within 4 weeks of culture.

| Medium № | NaCl conc. (mM) | Mean shoot length (cm) (\pm SEM)* | Mean № of shoots (\pm SEM)* |
|----------|-----------------|--------------------------------------|--------------------------------|
| 1 | 25 | 3.22 \pm 0.56 a | 2.76 \pm 1.1 abc |
| 2 | 50 | 2.17 \pm 0.31 bcd | 2.60 \pm 1.7 abc |
| 3 | 100 | 2.76 \pm 0.42 ab | 3.30 \pm 0.75 ab |
| 4 | 150 | 2.63 \pm 0.40 abc | 2.53 \pm 0.2 abc |
| 5 | 200 | 1.99 \pm 0.19 cd | 3.73 \pm 1.01a |
| 6 | 300 | 2.07 \pm 0.22 d | 2.33 \pm 0.15 abc |
| 7 | 400 | 1.94 \pm 0.22 d | 3.10 \pm 0.43 abc |
| 8 | 500 | 1.57 \pm 0.46 d | 1.53 \pm 0.37 bc |
| 9 | 600 | 1.83 \pm 0.16 d | 1.46 \pm 0.46 d |
| LSD 0.05 | | 0.75 | 0.16 |

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$).

Data are the means of 30 replications

1966; Khan *et al.* 2000; Nedjimi *et al.* 2006). Many of the deleterious effects of Na^+ seem to be related to structural and functional integrity of membranes (Kurth *et al.* 1986). At high salinities, growth reduction might be caused by a reduced ability to make osmotic adjustment as a result of saturation of the solute uptake system (Munns 2002). Exposure to saline concentrations (400-600 mM NaCl on *Arthrocnemum macrostachyum*) has been shown to increase tissue water content of halophytes (Khan *et al.* 2005). The water potential of *A. halimus* decreased as salinity levels increase (72-108 mM CaCl_2) (Nedjimi *et al.* 2006). Also, the water potential of *A. griffithii* became increasingly negative as media salinity increase (up to 360 mM NaCl) (Khan *et al.* 2000). Deleterious effects of salinity are thought to result from low water potentials, ion toxicities, nutrient deficiencies, or a combination of these factors (Munns 2002). Growth and survival of halophytes is dependent on high levels of ion accumulation in their tissues for the maintenance of turgor and osmotic adjustment (Flowers *et al.* 1977). Halophytes are distinguished by their capacity to produce high concentrations of compatible osmolytes to tolerate salinity by increasing ion accumulation (Khan *et al.* 2000). Salt tolerance in *A. halimus* could involve a delicate balance among ion accumulation, osmotic adjustment, and maintenance of pressure potential and growth. At relatively high salinities, a significant reduction in growth occurs because of a plant's inability to make an osmotic adjustment, and specific ion toxicities can cause a significant reduction in growth (Nedjimi *et al.* 2006).

In the current study, we noticed that the plantlet leaves under high NaCl concentration turned whitish in comparison to the control. This can be ascribed to the fact that the shoots of *A. halimus* expunged excess amounts of NaCl to the outer surfaces of leaves giving them this whitish color. Many *Atriplex* species including *A. halimus* have glands on the surface of leaves where salt crystallizes without being harmful (Taiz and Zeiger 1991). Although high amounts of Na^+ accumulate outside *A. halimus* leaves in vesiculated hairs (avoidance structure) (Araújo *et al.* 2006), this protective mechanism does not alone explain the stimulation of growth recorded in the present study. Such an assumption agrees with that of Bajji *et al.* (1998) who studied *A. halimus* grown in media artificially salinized with NaCl. They reported that the physiological mechanism underlying growth stimulation of *Atriplex* plants is still unknown and that such a genus undoubtedly constitutes one of more convenient subjects for investigating the halophytic properties in the plant kingdom. Although not proven, an assumption exists where increased activity of protein synthesis leads to improved growth and is considered of great biochemical and physiological relevance within a given range of NaCl depending on the plant species (Araújo *et al.* 2006). *A. nummularia* may use the controlled uptake of Na^+ balanced by other ions, especially Cl^- , into a cell to drive water into the plant against low external water potential (Blumwald *et al.* 2000). *A. halimus* adopted two different strategies: it behaved as a salt includer at low salinity and as an excluder at high salinity, and growth stimulated in the range of NaCl

from 150 to 300 mM (Bajji *et al.* 1998) which agrees with our present study in which optimal growth occurred at 200 mM.

Our study indicates that salinity affected rooting negatively; the higher the salinity, the fewer the roots, which is unlike what Araújo *et al.* (2006) found, i.e. that salinity did not affect rooting. Just a few clones which tolerated high concentrations of NaCl were selected and are being propagated under the same stress conditions for the next step of our study for identification and isolation of salt tolerance gene(s) from these high salt-tolerant clones selected *in vitro* under high stress concentration of NaCl to be used for transformation of crops for salt and drought stress tolerance.

CONCLUDING REMARKS

An efficient system for clonal multiplication and shoot organogenesis was developed from cultured shoot tips, nodal explants and axillary buds which can be used for large-scale multiplication to contribute in alleviating desertification impact on the environment and Human. The current study demonstrates that thousands of healthy plantlets of this important forage species *A. halimus* can be successfully produced within a short period time. This highly salt-tolerant halophyte could survive at high salinity concentrations, which makes it available for distribution to be cultivated in arid and semi-arid zones of Syria and other neighbouring countries, where a major problem is insufficiency and irregularity of fodder resources. This also can contribute to combat desertification because of its ability to complete its life cycle under very high saline conditions.

Our next aim is a deep study to identify and isolate salt tolerance gene(s) from the high tolerant clones selected *in vitro* under high stress NaCl concentration to be used for transformation of crops for salt and drought tolerance.

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