Journal of Integrative Plant Biology 2006, **48** (10): 1185–1189

NaCl and TDZ are Two Key Factors for the Improvement of *In Vitro* Regeneration Rate of *Salicornia europaea* L.

Xiu-Ling Shi[1, 2], He-Ping Han[1], Wu-Liang Shi[1] and Yin-Xin Li[1*]

(1. *Key Laboratory of Phytosynthesis and Environmental Molecular Physiology, Institute of Botany, the Chinese Academy of Sciences,* Beijing 100093, China;
2. *Graduate School of the Chinese Academy of Sciences,* Beijing 100049, China)

Abstract

The present study aimed to find out suitable conditions for the *in vitro* culture of *Salicornia europaea* L. and to develop an efficient regeneration system. *S. europaea* plants were regenerated successfully *in vitro* from callus derived from mature embryos. Via the method of 2,4-dichlorophenoxyacetic acid (2,4-D)-short-treatment on mature seeds, callus was induced from hypocotyls on the MS medium with 4.55 μmol/L *N*-phenyl-*N'*-1, 2, 3-thiadiazol-5-yl urea (TDZ) 3–4 weeks after the seeds germinated. The callus differentiated into shoots at a rate of 27.6% after subculture for one time on the same medium. When NaCl was included in the medium, shoots were formed in cluster and the shoot differentiation frequency was increased to 55.2%. The shoots were rooted when cultured on 1/2 MS medium supplemented with indole-3-butyric acid (IBA), kinetin (KN) and activated charcoal (AC). The results indicated that NaCl and TDZ played an important role in the improvement of the regeneration rate of the halophyte, *S. europaea*.

Key words: 2,4-D-short-treatment; differentiation; halophyte; NaCl; *Salicornia europaea*; TDZ.

Shi XL, Han HP, Shi WL, Li YX (2006). NaCl and TDZ are two key factors for the improvement of *in vitro* regeneration rate of *Salicornia europaea* L. *J Integr Plant Biol* **48**(10), 1185–1189.

doi: 10.1111/j.1672-9072.2006.00342.x; available online at www.blackwell-synergy.com, www.jipb.net

Salicornia europaea L., a local species in China, is an annual, succulent euhalophyte belonging to the Chenopodiaceae. It is similar in many aspects to *S. bigelovii* Torr., which is commonly recognized as a promising oilseed halophyte for seawater irrigation (Glenn et al. 1991). *S. europaea*, however, has a much wider distribution than that of *S. bigelovii*. Under seawater irrigation, the seeds of *S. europaea* have an oil content of 28.0% and protein content of 30.2% (O'Leary et al. 1985), while those of *S. bigelovii* are 26%–33% and 31%, respectively (Glenn et al. 1991). In addition, as is similar to *S. bigelovii*, the unsaturated fatty acid constitutes a large percentage of the seed oil in *S. europaea* (Zhao and Feng 2001). So, *S. europaea* could be a competitive potential oilseed crop as well. In the meantime, it is a medicinal herb with diuretic function (Zhao and Feng 2001); and its young shoots could be a vegetable. Moreover, as a euhalophyte, *S. europaea* is an ideal donor of salt tolerance related genes.

The previous studies on *S. europaea* were focused on ecology, physiology and biochemistry. There are few reports on tissue culture of *S. europaea* in the past years. Lee et al. (1992) investigated *in vitro* propagation of *S. bigelovii*, but the rate was low. The *in vitro* cultures of many halophytes belonging to the Chenopodiaceae were not very successful up to now. Although *Atriplex gmelini* plants were regenerated from hypocotyl explants (Uchida et al. 2003), the regeneration rate was poor. Maybe some special conditions are needed in the *in vitro* cultures of Chenopodiaceae plants. Finding out the favorable conditions for *in vitro* culture of *S. europaea* could also be helpful for the regeneration of other halophytes.

S. europaea is one of the most salt tolerant higher plants. However, it does not have special salt-secreting structures like a salt gland or salt bladder. Thus, it is a good model to study

Received 8 May 2006 Accepted 7 Jun. 2006

Supported by the Hi-Tech Research and Development (863) Program of China (2003AA627010), the National Key Technologies Research and Development Program in the 10th Five-Year Plan (2005BA807B07), and the Sci-Tech Research Program of Jiangsu Province of China (BE2003383).

*Author for correspondence. Tel: +86 (0)10 6283 6258; Fax: +86 (0)10 8259 6139; E-mail: <yxli@ibcas.ac.cn>.

the cellular-based mechanisms which appear to be common to all genotypes for salt tolerance. And the elucidation of its salt tolerance mechanism is of significance for generating salt-tolerant crops via selective breeding or genetic engineering. Several stress-related genes have been isolated from this halophyte. The overexpression of these genes in the donor plant is usually needed for the functional analysis. An important step toward the goal is the setting-up of the *in vitro* regeneration system of *S. europaea*, which is the basis for the future genetic transformation and functional analysis of the genes isolated from this halophyte.

The present study aimed to find out suitable conditions for the *in vitro* culture of *S. europaea* and to develop an efficient regeneration system. The system will provide a useful tool for further investigation of salt tolerance at the cellular level, and facilitate the functional analysis of stress related genes at the molecular level for *S. europaea*. Moreover, the crop potential of *S. europaea* could be developed on the basis of the tissue culture and regeneration system reported here.

Results

The callus inducing frequencies of different explants

To select the most competent explant for callus induction, three kinds of explants, mature embryos, hypocotyls and root sections were cultured in the dark on the callus induction medium. After 3 weeks of culture, yellowish callus appeared at hypocotyl parts of the geminated seeds with an average inducing rate of 99%. In comparison, the callus inducing rates of excised hypocotyls and root sections were much lower even after 4 weeks of culture. The difference was statistically significant ($P \leqslant 0.05$) (Table 1). Therefore, we concluded that the best explant for callus inducing was mature embryo.

Plant regeneration

The callus obtained from embryos was transferred to the regeneration media containing TDZ (0.45, 2.27, 4.55 or 9.09 μmol/L) and NAA (5.37 μmol/L). The callus gradually expanded and small green shoots were observed after 3–4 weeks of culture.

Table 1. Rates of callus inducing of different explants

Type of explants[a]	Rate of explants with callus (%)
Mature embryos	99.00 ± 0.06a
Hypocotyls	36.00 ± 0.03b
Roots	10.00 ± 0.02c

Data were represented as mean ± *SE*.

[a]Means of three replicates following the different letter were significantly different at the 5% level by least significant difference (LSD) multiple comparisons.

The optimum differentiation rate of 26.7% was obtained on the shoot inducing medium SIM1. The analysis by LSD showed that the regeneration rate on SIM1 was significantly ($P \leqslant 0.05$) higher than those on the other three media (Table 2).

Short treatment with 2,4-D to improve the regeneration rate

Given the fact that 2,4-D has negative effects on *in vitro* regeneration of some plant species, we tested a short treatment with 2,4-D of 9.05, 18.10 or 36.19 μmol/L for seeds on MS medium hoping to improve the regeneration rate. Three days later, the pretreated seeds were transferred to MS medium

Table 2. Regeneration rates of callus of *Salicornia europaea* after 4 weeks of culture on different media

Media[a]	PGRs (μmol/L)	Rate of callus differentiation (%)	No. of shoots per explant
SIMI	MS+TDZ 0.45+NAA 5.37	26.70 ± 0.03a	0.40 ± 0.03a
SIMII	MS+TDZ 2.27+NAA 5.37	5.50 ± 0.04b	0.10 ± 0.06b
SIMIII	MS+TDZ 4.55+NAA 5.37	4.30 ± 0.04c	0.10 ± 0.05b
SIMIV	MS+TDZ 9.09+NAA 5.37	4.20 ± 0.03c	0.10 ± 0.07b

[a]Means following the same letter in the same column were not significantly different at the 5% level by least significant difference (LSD) multiple comparisons.

PGRs, plant growth regulators; SIM, shoot inducing medium.

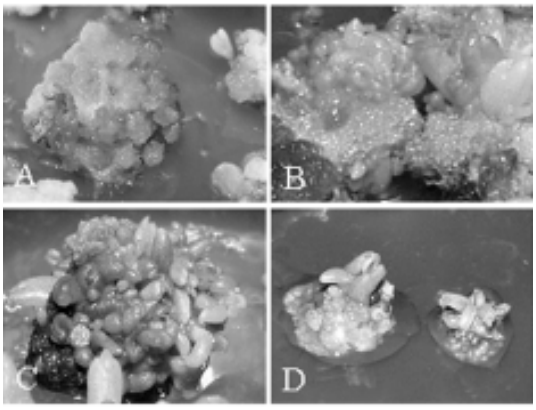

Figure 1. The regeneration of shoots by a short treatment with 2,4-D.

(A) The callus induced on MS medium containing TDZ after a short treatment with 2,4-D for three days.

(B) The shoot buds emerging from the callus.

(C) Shoots in cluster obtained on MS medium containing TDZ and NaCl.

(D) Shoots on NaCl-free MS medium containing TDZ.

with 4.55 µmol/L TDZ but without 2,4-D. After 3 weeks of culture, embryogenic callus (Figure 1A) appeared from hypocotyls parts of seedlings with suppressed root growth. One week later, the callus was incubated on MS containing 4.55 µmol/L TDZ. Only a few shoots (Figure 1D) were obtained after 3–4 weeks culture. The callus differentiation rates for treatment 1 (9.05 µmol/L 2,4-D) and treatment 2 (18.10 µmol/L 2,4-D) were significantly higher ($P \leqslant 0.05$, Table 3) than treatment 3 (36.19 µmol/L 2,4-D) on MS medium with 4.55 µmol/L TDZ. Compared with the differentiation rate (26.7%) on MS with TDZ and NAA, that (27.6%) via the 2,4-D-short-treatment was slightly higher (increased by 0.9%).

Differentiation of shoots in cluster via the 2, 4-D-short-treatment in combination with additional NaCl

Although we succeeded in inducing shoot buds from callus of *S. europaea*, the inducing rate was low and the average number of regenerated shoots per explant was limited. Since *S. europaea* is a euhalophyte, salt may have a positive effect on its regeneration. So we added NaCl to the medium. Shoot spots in cluster were obtained two weeks after incubation on SIM-A medium (MS + 4.55 µmol/L TDZ + 170 mmol/L NaCl) (Figure 1B). Subculture of the shoots on the medium resulted in proliferation of shoots (Figure 1C). On SIM-B medium (MS+ 4.55 µmol/L BA + 170 mmol/L NaCl) multiple shoots were also obtained. The differentiation rate and number of shoots per explant on SIM-A medium were significantly higher ($P \leqslant 0.05$) than those on SIM-B and SIM-C medium (Table 4). The highest differentiation rate was 55.2% and the average number of shoots per explant was 4.6 after 4 weeks culture on NaCl-included SIM-A medium. It was obvious that use of TDZ and NaCl increased the differentiation rate significantly.

Rooting of the shoots

Single shoots about 1–2 cm long were isolated and cultured for rooting on rooting media (RM) which are 1/2MS medium containing 0.05% AC with or without plant growth regulators. The highest rate of rooting after 3 weeks of culture was 25% on RM5 supplemented with IBA and KN (Table 5).

Discussion

In earlier experiments, the callus induced on the media with 2,4-D and BA or KN was shown to be incapable of regeneration. It is possible that the callus, while maintaining on inducing media, lost its differentiation potential (Gairi and Rashid 2004). Lack of regeneration may be related to loss of totipotency that often takes place soon after callus initiation (Heyser et al. 1983). So on the one hand, TDZ and NAA were used to induce callus and shoot differentiation; on the other hand, the method of a short treatment of 2, 4-D was used in later experiments.

Table 3. The effect of a short treatment with 2,4-D on callus differentiation after 4 weeks of culture on the MS medium containing 4.55 µmol/L TDZ

Treatment	2,4-D (µmol/L)	Ratio of differentiated callus pieces (%)	Number of shoots per callus piece
1	9.05	27.60 ± 0.03a	1.30 ± 0.10a
2	18.10	27.20 ± 0.02a	1.20 ± 0.16a
3	36.19	18.20 ± 0.05b	0.80 ± 0.18b

Data were represented as mean ± *SE*. Means of three replicates following the different letter within the same column were significantly different at the 5% level by least significant difference (LSD) multiple comparisons.

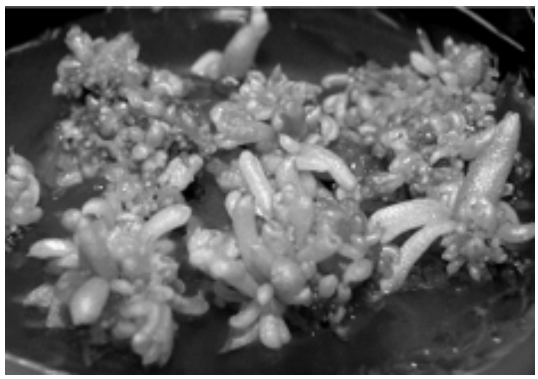

Figure 2. The proliferation of shoots on the NaCl-included subculture medium.

Table 4. The effect of NaCl on callus differentiation after 4 weeks of culture on the regeneration media

No. of media	Media	Rate of differentiated callus pieces (%)	Number of shoots per callus piece
SIM-A	MS + TDZ 4.55 µmol/L + NaCl 170 mmol/L	55.20 ± 0.01a	4.60 ± 0.20a
SIM-B	MS + BA 4.55 µmol/L + NaCl 170 mmol/L	36.10 ± 0.06b	2.10 ± 0.13b
SIM-C	MS + TDZ 4.55 µmol/L	27.60 ± 0.03c	1.30 ± 0.10c

The concentration of 2, 4-D in the short treatment was 9.05 µmol/L. Data were represented as mean ± *SE*. Means of three replicates following the different letter within the same column were significantly different at the 5% level by least significant difference (LSD) multiple comparisons. SIM, shoot inducing medium.

Table 5. Rate of rooting shoots of *Salicornia europaea* after 3 weeks of culture on rooting media

Media[a]	PGRs (μmol/L)	Rate of rooting shoots (%)[b]
RM1	0	0
RM2	NAA 2.69	0
RM3	IBA 0.49	0
RM4	IBA 2.46	12.5 ± 0.6
RM5	IBA 2.46 + KN 0.46	25.0 ± 1.0

Data were represented as mean ± *SE*.

[a]The basal medium was 1/2 MS medium supplemented with AC 0.05% (w/v). RM, rooting medium; PGRs, plant growth regulators.

[b]Rooting rate of shoots was defined as the rate of the number of rooting shoots to that of shoots inoculated for rooting per flask. Mean ± *SE* of three independent experiments with three replicates using 32 shoots in one experiment.

Thidiazuron, a non-purine phenylurea derivative, is widely used for plant organogenesis (Bhagwat and Lane 2004; Malik and Saxena 1992) and somatic embryogenesis (Dolendro et al. 2003; Murthy et al. 1996). The *in vitro* regeneration of *S. europaea* was successfully achieved when we used it. Given the possible suppression of the shoot regeneration by 2,4-D, we tried the method of a short treatment of 2,4-D on mature embryos on MS medium. Shoots were successfully regenerated at a frequency of 27.6% on MS medium supplemented with 4.55 μmol/L TDZ. This was in accordance with the results of Gairi and Rashid (2004).

On the basis of 2,4-D pretreatment, shoots in cluster were formed and the differentiation rate was increased to 55.2% (Table 4) by addition of NaCl to the medium. When the shoots were subcultured on the NaCl-included SIM-A medium, they proliferated rapidly and plenty of shoots were obtained (Figure 2). However, the regenerated shoots grew slowly on the NaCl-free medium. Therefore, NaCl played an important role on the basis of TDZ in the regeneration of *S. europaea*. The results were in accordance with those in *Mesembryanthemum crystallinum* (Cushman et al. 2000; Wang and Lüttge 1994) and in rice (Binh and Heszky 1990). It remains to be investigated how NaCl affects shoot bud differentiation in halophytes.

In conclusion, we have developed a system for *S. europaea* regeneration from mature embryos. The results of this study demonstrated that NaCl and TDZ were two key factors for the *in vitro* culture of the *S. europaea*. They enhanced its regeneration rate at a significant difference level. In combination with the promotion of callus differentiation by NaCl in *Mesembryanthemum crystallinum* (Cushman et al. 2000) and shoots inducing of many plant species by TDZ (Bhagwat and Lane 2004; Malik and Saxena 1992), we can infer that NaCl and TDZ possibly have a positive effect on the *in vitro* cultures of other halophytes.

Materials and Methods

Plant materials

Seeds of *Salicornia europaea* L. were collected by Han-Cai Liu from Dafeng, Jiangsu Province in the east part of China.

Culture media and growth conditions

Basal medium consisted of Murashige and Skoog (1962) salts and vitamins, 3% sucrose and 0.8% agar. The pH was adjusted to 5.9 prior to autoclaving (121 °C for 20 min). All plant growth regulators were added to the medium before autoclaving. Cultures were placed in a culture room at (25±1) °C, under cool white fluorescent light with a light intensity of 3 000 lx and a 16-h photoperiod for plant regeneration, and darkness for both callus induction and aseptic culture of seedlings.

Callus cultures

Mature seeds of *S. europaea* were surface-sterilized with 70% ethanol for 30 s, then immersed in 10% (v/v) sodium hypochloride solution for 15 min, and rinsed four times in sterile distilled water. After a culture of 3 weeks on MS medium, the hypocotyls and roots were cut from the seedlings. Sterilized seeds, hypocotyls and roots were incubated on the callus induction medium (0.45 μmol/L MS+TDZ + 5.37 μmol/L NAA) and cultured in dark at (25±1) °C for 4 weeks.

Plant regeneration conditions

Shoot differentiation tests were conducted on the differentiation media in a culture room at (25±1) °C, under cool white fluorescent light with a light intensity of 3 000 lx and a 16-h photoperiod.

Treatment with 2,4-D

In order to avoid the differential suppression of 2, 4-D during a long-time culture, a method of a short treatment with 2, 4-D for embryos was tested. The formation of embryogenic callus was achieved by treating the seeds for 3 d on media supplemented with three levels of 2, 4-D (9.05, 18.10 or 36.19 μmol/L), followed by culture of the seeds on MS medium containing 4.55 μmol/L thidiazuron (TDZ) for 4 weeks. Then the callus was

transferred to MS medium containing 4.55 μmol/L TDZ or BA with or without additional NaCl.

Data analysis

The experiments were conducted in a completely randomized design. The callus induction rate was defined as the ratio of the number of explants with callus formed per flask to that of explants surviving. The differentiation rate was defined as the ratio of the number of callus pieces with regenerated shoots per flask to that of the callus pieces inoculated for differentiation. The experiments were repeated three times. Variance of analysis (ANOVA) and least significant difference (LSD) multiple comparisons were performed for analysis of the callus induction rate and the differentiation rate.

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(Managing editor: Wei Wang)