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Establishment of a cell suspension culture of the halophyte *Cakile maritima*

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Abstract: *Cakile maritima* is a member of the Brassicaceae family also known as sea rocket. It is an annual succulent halophyte frequent in coastal dune vegetation in Mediterranean regions and Atlantic coasts from North Africa to the north of Europe. This halophyte presents a complex survival strategy at high salinity and its seeds contain up to 40% of an oil which could be suitable for biofuel production and other industrial applications. However, data concerning the cellular mechanisms allowing this plant to resist salinity are still lacking. Cell suspension cultures offer an *in vitro* system convenient for cell biology studies and biotechnological methods are still not developed for this putative crop. The present paper reports initiation of *C. maritima* cell suspension cultures from callus obtained from aerial parts of seedlings. The establishment of a suspension culture which preserves its salt resistance provides an opportunity to gain insights into *C. maritima* biology.

1. Introduction

The world's cultivated lands are increasingly affected by drought and salinity (Barrett-Lennard, 2000). Halophytes growing in highly saline soils could thus serve as a resource for the identification and development of new crop systems for marginal saline soils (Debez *et al*., 2011; Ben Hamed *et al*., 2013). The actual yield of halophytes remains largely unknown since their domestication is still limited, yet the economic potential of some of these plants has been positively assessed by various groups (Aronson, 1989; Ashour and Thalooth, 1993; Abdelly *et al*., 2006). Numerous Brassicaceae species are current and emerging biodiesel crops: in addition to the oil-rich seed, the ability of Brassicaceae species to grow on marginal land with minimal inputs make them particularly attractive and potentially viable for this application. Among halophyte species studied for their potential as oleaginous plants, the Brassicaceae *Cakile edentula* (O'Leary *et al*., 1985), *Crambe abysinnica* (Mandal *et al*., 2002) or *Cakile maritima* (Ghars *et al*., 2005) have been reported to contain high amounts of oil. *Cakile maritima*, the sea rocket, is an annual succulent halophyte frequently found from the Black Sea coasts to the Mediterranean region, and from the Atlantic coasts of North Africa to the north of Europe (Clausing *et al*., 2000). Tunisian accessions of *C. maritima* contain up to 40% seed-oil (Ghars *et al*., 2005). Plant growth, harvest index, silique number and seeds produced per fruit segment is maximal at 100 mM NaCl (Debez *et al*., 2008) but *C. maritima* can survive at up to 800 mM NaCl (Ellouzi *et al*., 2013) and successfully reproduces till 500 mM NaCl salinity (Debez *et al*., 2004). Seed-oil content did not seem to be affected by salinity, although erucic acid level could increase (Debez *et al*., 2006). These facts highlight the need to better understand the basis of adaptation to saline environments, as well as traits associated with oil production itself. In previous works we described some aspects of the response of *C. maritima* to salt stress (Debez *et al*., 2004, 2006, 2008; Ellouzi *et al*., 2011) and our data point out that *C. maritima* adopts a complex survival strategy at high salinity, however numerous data concerning the cellular mechanisms allowing this plant to resist salinity are still lacking.

Suspension culture cells offer an *in vitro* system that is widely used in plant biology as a convenient tool to investigate a wide range of phenomena. It consists in a model system as suspension culture provide a ready source of a homogenous cell type and avoids the complications of multicellular tissue types *in planta* (Moscatiello *et al*., 2013). Suspension culture cells were recently used in various studies and models concerning plant responses to salinity, such as proteomic studies (Chen *et al*., 2012; Liu *et al*., 2013), metabolomic studies (Liu *et al*., 2013) or transcriptomic studies (Matsuura *et al*., 2010; Bae *et al*.,

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2012). It is also a convenient means to study transport system regulations and oxidative responses to various biotic and abiotic constraints (Kadono *et al*., 2010; Baz *et al*., 2012; Yukihiro *et al*., 2012; Tran *et al*., 2013), comprising salinity (Cessna *et al*., 2007; Wang *et al*., 2010; Pons *et al*., 2011; Queirós *et al*., 2011).

Here, we report the development of a cell suspension culture of *C. maritime*; growth performance was evaluated on control and NaCl cultured cells to validate the biological system. These suspension culture cells could be a valuable tool to gain further insights into halophyte studies and their potential applications.

2. Materials and Methods

Establishment of callus of Cakile maritima

In this study we used *Cakile maritima* seeds harvested in the Raoued region in the north of Tunisia. Callus cultures were initiated from the aerial part of 14-day-old, light-grown seedlings. Seeds were submerged in 70% ethanol for 1 min, then rinsed with sterile distilled water, submerged in chlorine bleach for 10 min and then rinsed three times (5 min each) with sterile distilled water. The seeds were placed in petri-dishes containing Murashige and Skoog medium including vitamins (MS) (Murashige and Skoog, 1962), supplemented with 30 $g.L^{-1}$ sucrose, 8 $g.L^{-1}$ agar. The pH was adjusted to 5.8 with KOH.

Stem segments were finely cut and then placed on a solid callus-inducing medium (CIM) containing 6.2 $g.L^{-1}$ Gamborg B5 (Gamborg *et al*., 1968) supplemented with 20 $g.L^{-1}$ glucose, 8 $g.L^{-1}$ agar and with growth regulators 9.06 µM of 2.4 D and 0.46 µM of kinetin. The pH was adjusted to 5.7 with KOH. After two to three weeks, callus appeared on the sides of the segments. When the size of callus became larger than 1 cm, they were divided and transferred to a new medium.

Establishment cell suspension cultures of Cakile maritima

Approximately 5 g of callus were transferred to 125 mL flasks containing fresh Gamborg B5 medium supplemented with 30 $g.L^{-1}$ glucose, 0.2 µM 2,4-D and 0.45 µM kinetin. The pH was adjusted to 5.7 with KOH. Flasks were incubated on a rotary shaker at 120 rpm and maintained at 22°C in the dark. Feeding of the cultures with fresh medium was done at 10-14 day intervals during which time the suspensions were allowed to settle under agitation (rotary shaker at 120 rpm). This procedure was repeated for about eight weeks. Then, the suspension was subcultured every seven days by transferring 20 mL of the culture into 50 mL of fresh medium in 250 mL Erlenmeyer flasks.

Arabidopsis thaliana *cell suspension culture conditions*

Arabidopsis thaliana L. cell suspensions were prepared from calluses of the cell line T87 generated from the ecotype Columbia plant as previously described (Tran *et al*., 2013). The suspension cells were obtained after about two months and five to six subcultures in 1 L round-bottom flasks containing 350 ml liquid Gamborg B5 culture medium (pH 5.8). Cell suspensions were sub-cultured weekly using a 1:10 dilution.

Growth evaluation

Growth of the culture was evaluated by measuring the fresh weight of cells and the density of cells using a Nageotte cell.

Cell viability

Cell viability was assayed using the vital dye neutral red. Cells (100 µL) were incubated for 5 min in 400 µL phosphate buffer pH 7 with neutral red to a final concentration of 0.001% (w/v). Cells that did not accumulate neutral red were considered dead. At least 500 cells were counted for each replicate and the procedure was repeated at least three times for each treatment.

External pH measurements

Measurements of extracellular pH were performed with pH-sensitive electrodes every 24 h for six days from 5 mL of cultured medium cells. The procedure was repeated on three independent subcultures.

Cell size

Cell images were recorded with a camera (Kappa CF11DSP) on a light microscope (Labophot-2 Nikon) and sizes were measured using image analysis software KappaImageBase-2.2SP2-Metreo (Kappa Optoelectronics GmbH, Gleichen, Germany).

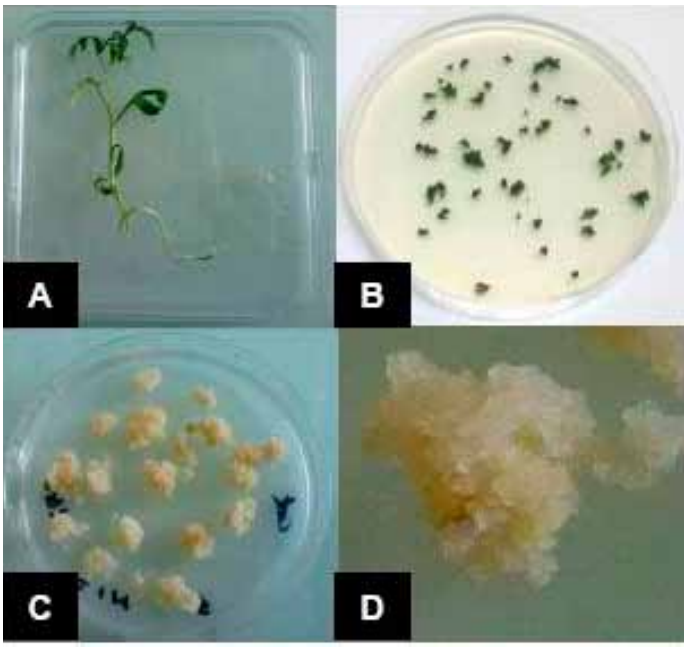


Fig. 1 - Establishment *of Cakile maritima* calluses. (A) Three-week-old *C. maritima* seedling grown from sterilized seed. (B) Slices of leaf and rod were finely cut for callus generation. (C) One-month-old calluses derived from rod and leaf slices. (D) Magnification of a callus.

Protoplast Isolation

Protoplasts were isolated from suspension cultures six days after subculture. Fifteen mL of suspension cells were used. After cell sedimentation, the supernatant was removed and replaced by 5 mL of Gamborg B5 fresh medium containing 0.1 g cellulysin, 0.05 g macerase and 0.3 M sorbitol. The digestion was carried out under shaking at 120 rpm at 22°C for 30 min. After incubation, protoplasts were collected by centrifugation at 300 rpm for 3 min and re-suspended in 5 mL of Gamborg B5 fresh medium supplemented with 0.6 M sorbitol or 0.3 M sorbitol.

3. Results and Discussion

The different steps in the development of *Cakile maritima* callus from stem segments are reported in figure 1. Callus appeared on the sides of the discs after two to three weeks. Although produced in light, callus became non-chlorophyllian and pale yellow in color (Fig. 1C, D). Thus *Cakile maritima* loses its power of chlorophyll synthesis during its passage from plant stage to callus stage. To generate cell suspension, approximately 5 g of calluses were transferred in Gamborg medium. These calluses progressively disintegrated to the smallest cell aggregates in the liquid medium. Large quantities of cells could be obtained after two months feeding (Fig. 2A, B). At this stage suspension cells were subcultured every week in fresh Gamborg medium and after an additional 1.5 months the culture became more homogeneous as the cell aggregates became smaller and the cell size increased (Fig. 2C, D). The level of dead cells in the culture also progressively decreased reaching about 10% after four months of culture, in the same range as observed for tobacco or *A. thaliana* suspension cells (Baz *et al*., 2012; Yukihiro *et al*., 2012; Tran *et al*., 2013).

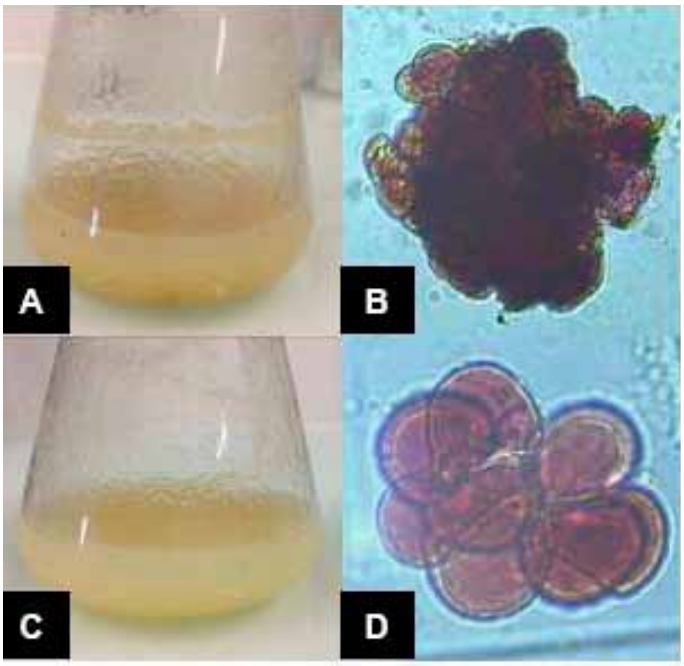


Fig. 2 - Establishment *of Cakile maritima* cell suspensions. (A) Dense suspension cells grown in flask after two months feeding and (B) corresponding cell aggregates magnified 300X. (C) Dense suspension cells grown in flask after 1.5 months of subculture after feeding and (D) corresponding cell aggregates magnified 300X.

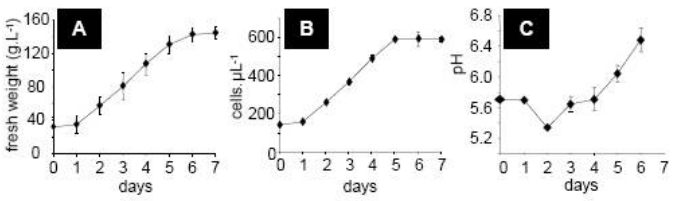


Fig. 3 - Growth pattern of *Cakile maritima* cell suspension determined by fresh weight (A) or cell density measurements (B). Evolution of the medium pH during cell suspension growth. Bars indicate mean ± SD of at least three experiments.

Using four-month-old *C. maritima* culture, growth curves were established by measuring the fresh weight of cells (Fig. 3A) and the cell density (Fig. 3B). Both curves showed a typical sigmoidal shape with a latency period of 24 h, then an exponential phase lasting about four days prior to a plateau phase (Fig. 3A, B) supposed to be due to nutrient depletion. We monitored the medium pH during the culture procedure (Fig. 3C). After a slight acidification at the beginning of the exponential phase, the pH became more alkaline, reaching 6.5 at the end of the culture (Fig. 3C). This alkalization could be involved in the decrease in biomass production as Ling *et al*. (2008) pointed out that pH of 6.7 lowered the growth of suspension culture of *Ficus deltoidea*. However, the pH variations recorded during the culture should remain suitable for nitrate uptake as reported for *Ipomoea* suspension cells (Martin and Rose, 1976).

Histological analysis on six-day-old suspension cells revealed distinct morphological features of the cultured cells (Fig. 4). Although the cell aggregate size diminished during culture establishment, a few large aggregates (>20 cells) remained. However the largest part of the culture consisted of compact small groups of less than 20 cells, 60% corresponding to groups of three to 10 cells, single cells representing about 10% of the population (Fig. 4A). Most of the cells were rounded in shape (Fig. 4B, left and center); less than 2% were elongated (Fig. 4B, right). Interestingly, as observed during the establishment of the culture (Fig. 2B, D), the size of the cells seemed to be dependent on the size of the groups, the isolated cells being the largest (Fig. 4C).

It is known that protoplasts can be used for transient expression, trafficking assay or ion homeostasis analysis, notably in studies on plant resistance to salinity (Laohavisit *et al*., 2012; Haro *et al*., 2013; Morgan *et al*., 2013; Mottaleb *et al*., 2013; Son *et al*., 2013). Thus, we evaluated the usefulness of the cell cultures for the isolation of protoplasts. Protoplasts were isolated from six-day-old suspension cultures. Based on the different cell sizes measured (Fig. 4B, C) we observed protoplasts of different sizes. Most of these protoplasts maintained in 0.6 M sorbitol ap-

peared plasmolyzed and numerous protoplasts seemed to be shrunken (Fig. 5A). We then used 0.3 M sorbitol and obtained rounded protoplasts with large vacuoled evidenced by neutral red staining (Fig. 5B).

Finally, *Cakile maritima* being a halophyte, we checked the salinity resistance of the suspension cells. Suspension cells were subcultured in Gamborg medium complemented with 100, 400 or 800 mM NaCl. Sigmoidal growth curves were obtained and no significant differences were observed between the control and the suspension cells growing in presence of 100 mM NaCl when 400 mM and 800 mM strongly decreased the growth of the suspension cells (Fig. 6A). These data are in accordance with what was described for seedlings, *C. maritima* even requiring the presence of a moderate salt concentration (50-100 mM NaCl) to maintain a significant growth activity and plant development (Debez *et al*., 2004, 2008). Although strongly reduced at 400 mM or 800 mM NaCl, the growth of the suspension cells was also in accordance with previous data indicating that *C. maritima* can survive up to 800 mM

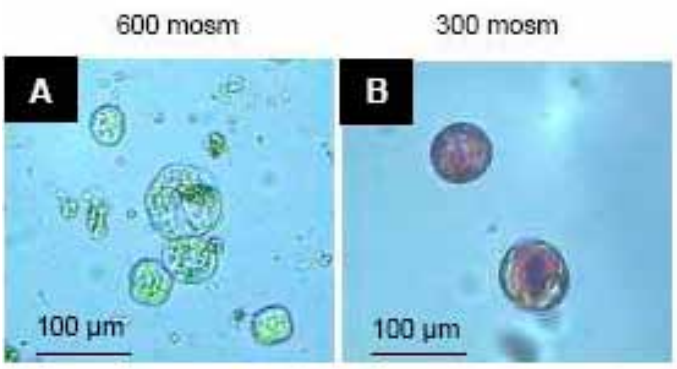


Fig. 5 - Protoplasts derived from six-day-old *Cakile maritima* cell suspension maintained in 0.6 M sorbitol (A) or 0.3 M sorbitol (B).

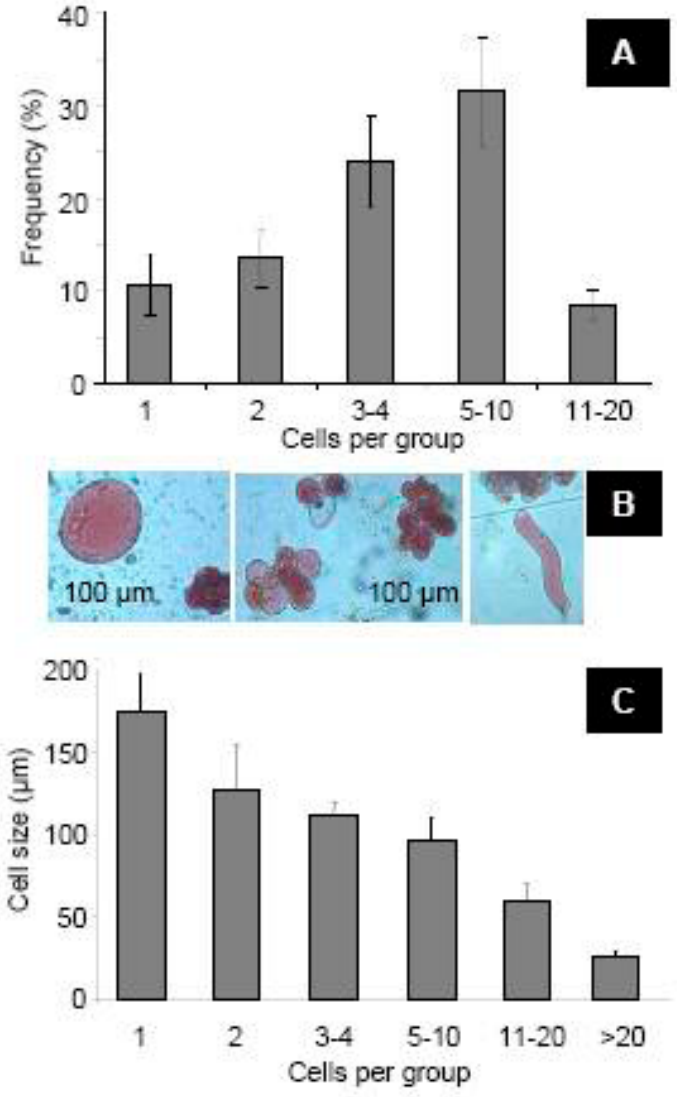


Fig. 4 Morphology of six-day-old *Cakile maritima* suspension cells. (A) Frequency of cell groups in the culture. (B) Different morphologies of cells. (C) Sizes of the cells according to the size of the cell groups. At least 250 cells were analyzed; bars indicate mean ± SD.

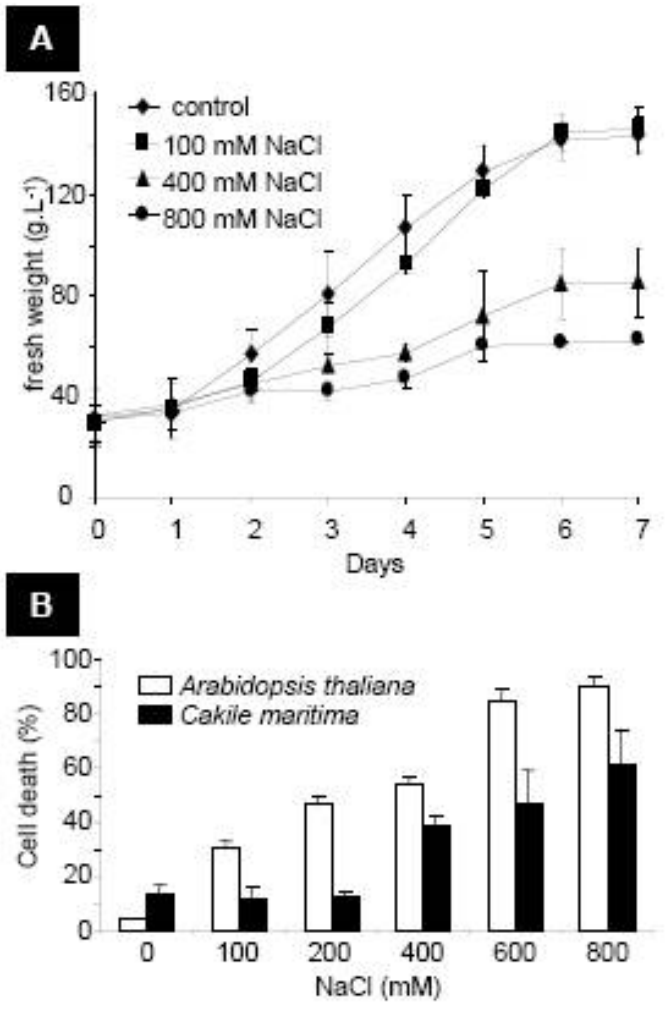


Fig. 6 - Evaluation of *Cakile maritima* cell suspension resistance to salinity. (A) Growth pattern of cell suspensions grown in presence of 100, 400 or 800 mM NaCl determined by fresh weight measurement (B). Comparison of cell death extents of *C. maritima* and *Arabidopsis thaliana* cell suspensions treated 6 h with NaCl concentrations ranging from 100 to 800 mM. Bars indicate mean ± SD of three experiments.

NaCl (Ellouzi *et al*., 2013) and successfully reproduces till 500 mM NaCl salinity even if the biomass was reduced (Debez *et al*., 2004). We further compared the extent of cell death induced 6 h after the addition of various NaCl concentrations on *C. maritima* and *A. thaliana* suspension cells. Cell death began to increase from 100 mM NaCl to almost 100% at 600 mM for *A. thaliana* (Fig. 6B). For *C. maritima*, the increase in cell death was significant only with 400 mM NaCl and reached only 60% at 800 mM, remaining largely inferior when compared to *A. thaliana* (Fig. 6B). It is worth noting that 40% of surviving cells probably go on dividing, which explains the growth of the culture, although reduced, at 800 mM NaCl (Fig. 6A). As a whole, these data demonstrate that *C. maritima* suspension cells preserve their ability to resist salinity.

4. Conclusions

The present study reports the development of *C. maritima* cell suspension cultures which maintained their salt resistance, offering greater understanding about adaptation to saline environments, as well as traits associated with biofuel production.

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