Journal of Coastal Research	17	1	188–194	West Palm Beach, Florida	Winter 2001

Isolation of Leaf Protoplasts from *Pancratium maritimum* L. and Two Other Dune Plants: Possible Applications

Elena Balestri†, Gualtiero Luccarini‡ and Francesco Cinelli§

†Centro Interuniversitario di Biologia Marina ed Ecologia Applicata
P.le Mascagni 1
57126 Livorno, Italy

‡Istituto di Mutagenesi e Differenziamento CNR
Via Svezia 10
56100 Pisa, Italy

§Dipartimento di Scienze dell'Uomo e dell'Ambiente
Via Volta 6
56100 Pisa, Italy

ABSTRACT

BALESTRI, E.; LUCCARINI, G., and CINELLI, F., 2001. Isolation of leaf protoplasts from *Pancratium maritimum* L. and two other dune plants: possible applications. *Journal of Coastal Research*, 17(1), 188–194. West Palm Beach (Florida), ISSN 0749-0208.

Protoplast techniques can be applied for studying physiological and biochemical processes in plant cells. In order to utilize these techniques, a method for isolation of viable protoplasts from dune plants was established. Using a combination of cellulase R-10 Onozuka, hemicellulase and pectinase protoplasts were isolated from younger leaves of *Pancratium maritimum*. Field-grown plants harvested in autumn or winter yielded approximately six times as many protoplasts per g of fresh tissue as did plants collected in spring ($3.6–5.3 \times 10^6$ versus 9×10^5 protoplasts per g of fresh tissue). No protoplasts were released from plants harvested in summer. The production of protoplasts from cultivated plants reached high yields of protoplasts (3.6×10^6) independent to the season. The viability of these protoplasts was 89.2% (±1.1). Within 2–3 days of liquid culture 88.6% (±3.6) of the protoplasts were able to regenerate cell-walls. First divisions were detected after 5–7 days of culture. By using the same procedure, yields of 2.31×10^6 and 1.28×10^4 protoplasts were obtained from field-collected leaves of *Cakile maritima* and *Ammophila arenaria*, respectively. Viability of protoplasts were 87.5% (±4.3) and 75.1% (±2.4), respectively. The potential applications of this method are discussed.

ADDITIONAL INDEX WORDS: *Dune plants, protoplast techniques, ecophysiology.*

INTRODUCTION

Coastal dune plants perform critical functions in dune initiation, growth and stabilization (PLUIS and DE WINDER, 1990; HESP, 1991; MAUN, 1997). Because of their features, dune plants have long been regarded as a model for the study of plant adaptations and responses to environmental stresses (i.e. salinity, low nutrient and water availability, sand instability). Until now, research on this topic has been at in situ and whole-plant level of investigation (ISHIKAWA *et al.*, 1990; ISHIKAWA *et al.*, 1996; BLITS and GALLAGHER, 1991; MAUN, 1997; VALVERDE *et al.*, 1997). Understanding the ecophysiological mechanisms at cellular and molecular levels requires additional research. To date, the scarce availability of systems to culture dune plants (COOK *et al.*, 1989; KANE *et al.*, 1993) has precluded this approach.

Protoplasts isolated from terrestrial plants and, more recently, marine plants have provided a powerful experimental system for studying physiological and biochemical processes in plant cells (ROSEN and TAL, 1981; LYNCH *et al.*, 1987; RAINS, 1989; ELZENGA *et al.*, 1991; FOWKE and WANG, 1992; GARRIL and TYERMAN, 1994; PATRICK, 1997; WANG *et al.*, 1997; ZHANG *et al.*, 1997; SHABALA *et al.*, 1998; NEWMAN and RAVEN, 1999). The knowledge of ion transport mechanisms at the plasma membrane associated with ionic and osmotic stress has greatly advanced by the use isolated protoplasts in patch-clump and electrorotation techniques. Protoplast technology has also been applied to genetic manipulation for selection of specific physiological traits (*i.e.* environmental stress tolerance, disease resistance) and plant improvement (POTRYKUS and SHILLITO, 1988; ROEST and GILISSEN, 1989; PUITE 1992; PASZKOWISKI *et al.*, 1992; KOOP *et al.*, 1992). To date, there have been no reports on protoplast isolation from dune plants. Theoretically there are no reasons why such technique cannot be extended to this group of plants.

Therefore, we demonstrate here the possibility to isolate viable and cell-wall regenerating protoplasts from the dune plant *Pancratium maritimum* L. (sand-lily), and we assess the influence of the donor plant source (field-collected versus cultivated plants) and season on protoplast yield. We also investigate whether such method is suitable for producing protoplasts from two other dune plants, *Ammophila arenaria* (L.) Link. and *Cakile maritima* Scop. These plants were chosen for the investigation because of their contrasting morphology and ubiquitous distribution in coastal dune regions in Italy. *Pancratium maritimum* (Amaryllidaceae) is a perennial bulbous plants which has severely treathened along the Mediterranean coasts (BELLAN-SANTINI *et al.*, 1994). Little is known on the ecology of this pioneer plant (KILINÇ and YÜKS-

99036 received 5 May 1999; accepted in revision 3 January 2000.

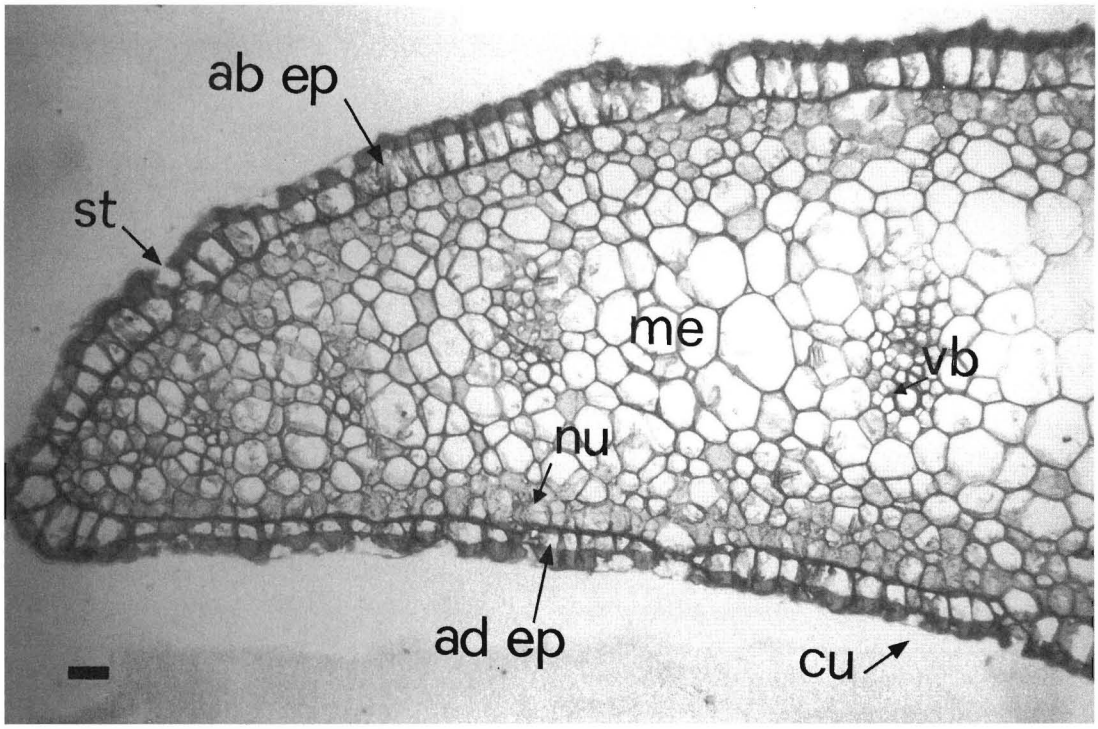

Figure 1. Transversal section of the basal part of a young leaf of *Pancratium maritimum* after embedding and staining, showing the cuticle (cu), the epidermis both adaxial and abaxial (ad ep and ab ep) with stomata (st), mesophyll (me), vascular bundles (vb) and nuclei (nu). Bar = 30 μm, ×125.

EL, 1994; MEDRANO *et al.*, 1999). This species has a great potential economic value for flower production (PROTICH, 1987) and for sources of several compounds (TATO *et al.*, 1988; IDE *et al.*, 1996, 1998). *Cakile maritima* (Cruciferae) is a strandline annual herbaceous species which has been used to study responses to environmental factors (i.e. nutrient availability and salinity; ROZEMA *et al.*, 1982; PAKEMAN and LEE, 1991*a*; PAKEMAN and LEE, 1991*b*). *Ammophila arenaria* (Graminaceae) is the most widely used species for planting purposes in Mediterranean (HUISKES, 1979; VAN DER PUTTEN and KLOOSTERMAN, 1991). Possible applications of this method are discussed.

MATERIALS AND METHODS

Pancratium maritumum

The basal parts of the younger leaves (2–10 cm long) of *Pancratium maritimum* were used as tissue sources for protoplast isolation. Leaves were excised both from plants growing on a dune area at Rosignano Solvay (Livorno, Italy) and plants growing into pots (14 cm diameter, 20 cm height) filled with fertilized sand, under conditions giving "soft" growth (18 °C, a 16 hr photoperiod provided by cool-fluorescent lights (Philips) at 30 μmol photons $s^{-1} \cdot m^{-2}$). The latter plants were allowed to adapt to the cultivation system for at least three weeks prior to experiments.

Portions of leaves were washed with sterile distilled water, rinsed with 70% (v/v) ethanol, and surface-sterilized with 3% (v/v) sodium hypochlorite for 20–30 min with gentle shaking. They were then rinsed three times in sterile distilled water. The cuticle was removed and the leaf portions were cut transversally into approximately 2–3 mm^2 sections and weighed. To test tissue axenicity, pieces of sterilized leaves were incubated in the standard media Plate count agar and Marine agar (Difco) at 22 °C for 1–2 weeks. Approximately 8 g (fresh tissue) of cut leaves were incubated in 15 ml filter-sterilized (0.22-μm membrane filter, Sartorius) enzyme mixture prepared by dissolving 1% (w/v) of cellulase (Onozuka R10; Yakult Honsha, Japan), 1% (w/v) pectinase (Boerozym; Sigma Chemical Co., St Louis, Mo., USA), 0.5% (w/v) hemicellulase (Sigma) in 50% filtered seawater (17‰), at pH 5.8. Digestion was performed for 3–10 h at 22–24 °C in the dark. After the enzymatic treatment, the enzyme mixture was pipetted off and replaced with 10 ml of washing solution (W) consisting of 10 mM $CaCl_2$ and 30 g/l sucrose dissolved in 50% filtered seawater, at pH 7.8. Digested tissues were maintained in the W solution for 1 h at room temperature with a rotary shaker (30 rpm) to improve protoplast release. The protoplast mixture was filtered through a 100-μm nylon net and the filtered protoplast suspension was centrifuged at 100 *g* for 8 min. The protoplast pellet was purified by centrifugation at 150 *g* for 10 min with gradients of Ficoll 400 (30% w/v, 12% w/v, 0). The cell debris formed a green pellet at the bottom of the tube, leaving band of purified protoplasts at the interface between Ficoll 12% and the W solution. Protoplasts were retrieved from the boundary and resuspended in 2 ml of a fresh W solution. The number of protoplasts was measured using

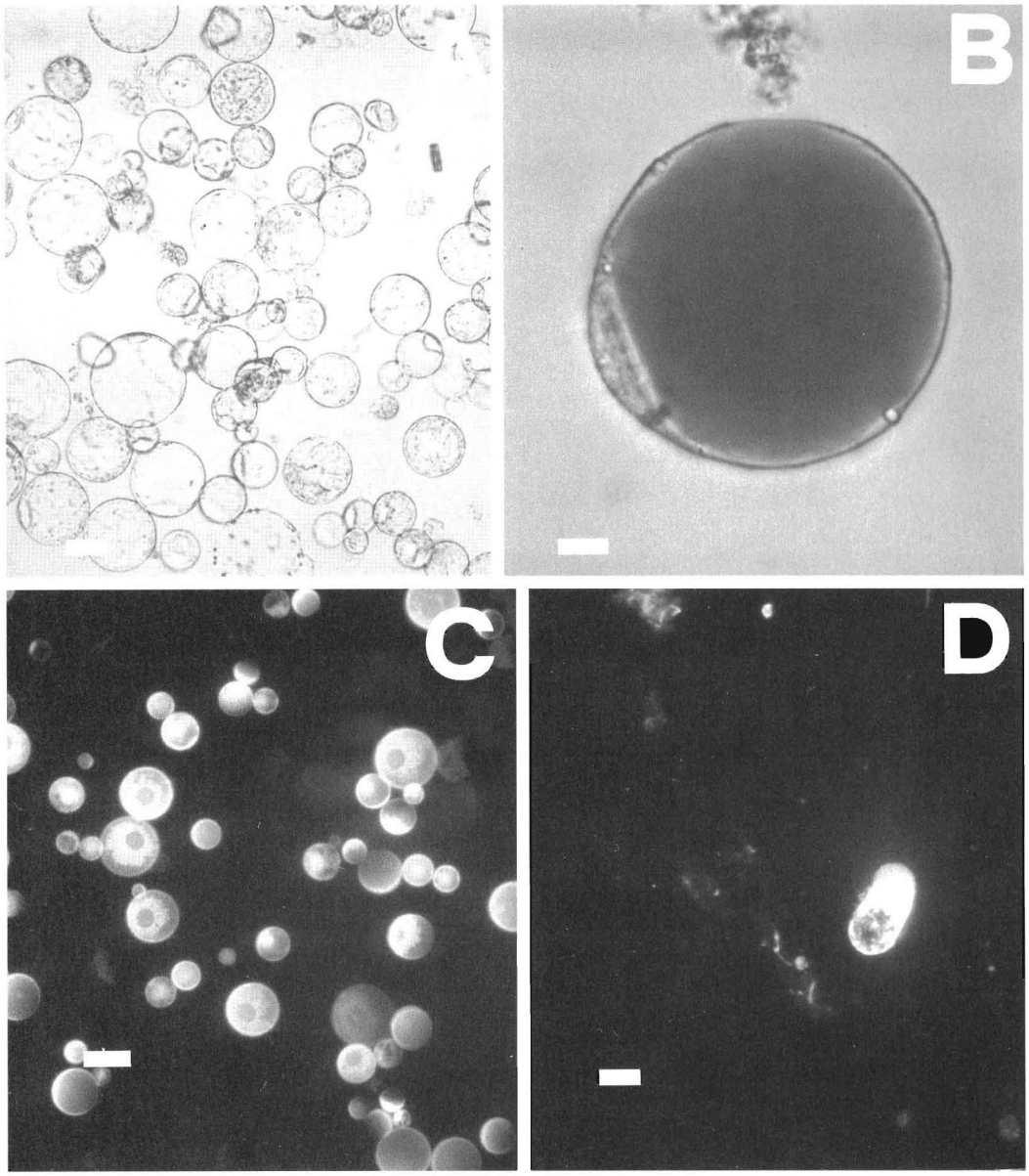

Figure 2A–D. Mesophyll protoplasts isolated from young leaves of *Pancratium maritimum*. (A) Freshly isolated protoplasts. Two type of protoplasts are shown: larger highly vacuolated and smaller densely cytoplasmatic. Bar = 30 μm, ×125. (B) Protoplast stained with neutral red showing a large vacuole. Bar = 10 μm, ×500. (C) Yellow-green fluorescence in 24 h-old protoplasts after staining with FDA. Bar = 50 μm, ×78.8. (D) Fluorescence in a 5 day-old protoplast undergoing first cell division after staining with calcofluor white. Bar = 30 μm, ×125.

a standard hemocytometer. Protoplasts were cultured at a density of 1.5×10^5 protoplasts per ml in Petri dishes containing 3 g/l sucrose, 10 mM $CaCl_2$, and 0.2 M sorbitol dissolved in ½ Murashighe and Skoog (1962) basal medium (MS), at pH 7.8. Cultures were maintained at 20 °C under a 12 h photoperiod provided by cool-fluorescent lights at 30 μmol photons $s^{-1} \cdot m^{-2}$). Every week the osmotic pressure of the culture medium was decreased by dilution (v/v) with fresh MS medium. The percentages of viable protoplasts were determined just after isolation and after, 15 days of culture. Two methods were used to assess viability: 8% (w/v) fluorescein diacetate staining (FDA; Sigma; POWER and DAVID, 1990) followed by observation with a UV fluorescence microscope, and 0.1% (w/v) neutral red which specifically stains metabolically active vacuoles. The absence of cell walls in freshly isolated protoplasts as well as cell wall regeneration in cultured protoplasts was detected using 0.1% fluorescent brightener 28 (Calcofluor white; Sigma) staining, followed by observation with UV fluorescence (POWER and DAVID, 1990). The percentages of cell-wall regenerated and divided protoplasts were determined by taking samples of 0.3 ml at 24 h intervals after isolation. Samples of protoplasts were also

stored in the W solution at 4 °C to monitor the baseline decrease of viability.

In order to certify the anatomical origin of the isolated protoplasts, samples of younger leaves of *P. maritimum* were fixed in ethanol : acetic acid 3 : 1 (v/v) for hystological study. Permanent material was prepared by paraffin embedding technique (LIPUCCI *et al.*, 1987). The sections (12 μm thick) were cut with a microtome and stained by safranine-fast green staining technique, and observed with a light microscopy. To evaluate the influence of season and growth rate on the yield of protoplasts experiments were conducted monthly from November 1997 to June 1998. Concomitantly, leaf growth rate (cm·d $^{-1}$) and leaf production (leaves·d $^{-1}$) of plants were estimated by using a non-destructive leaf marking method (ZIEMAN, 1974).

Cakile maritima and *Ammophila arenaria*

Cakile maritima and *Ammophila arenaria* were collected at the site from October to December 1998. The basal part of the youngest leaves was excised and used immediately for protoplast production. Isolation of protoplasts was carried out using the same conditions to those described for *Pancratium maritimum*, except for the peeling procedure. Five grams (fresh weight) of cut leaves were incubated in 10 ml of enzyme solution. After the centrifugation, cell debris formed a green pellet at the bottom of the tube, leaving purified protoplasts at the surface of the W solution. Protoplasts were collected and suspended in 2 ml of fresh washing medium and counted. The percentages of viable protoplasts were monitored after 24 h from isolation by the FDA staining.

RESULTS

Pancratium maritimum

The *Pancratium maritimum* leaf is composed of (from outside to inside): a thick cuticle, a monolayer epidermis with stomata in both ad- and abaxial surface and a relatively undifferentiated mesophyll consisting of nearly isodiametric parenchyma cells, in which several parallel vascular bundles are embedded (Figure 1). Freshly isolated protoplasts of *P. maritimum* were spherical in shape and lacked the cell wall, as confirmed by Calcofluor white staining. Two main types of protoplasts were isolated (Figure 2A): large ones (70–100 μm) with a central vacuole as shown by neutral red staining (Figure 2B) were derived from the inner parenchyma cells, and smaller (20–40 μm) with dense cytoplasm corresponded to the sub-epidermal parenchyma cells.

The yield of protoplasts obtained from field-grown plants varied depending on the season (Figure 3). The highest yield ($5.3 \times 10^6 \pm 0.2$ SE protoplasts per g fresh wt tissue) was obtained from leaves excised in winter (from January to March 1998), when plants showed higher leaf growth rates (0.97 ± 0.38 cm d $^{-1}$) and production (0.06 ± 0.05 leaves d $^{-1}$). Protoplast release usually started 30 min after enzymatic incubation and peaked within 3 h. A yield of 0.9×10^6 (± 0.09) protoplasts per g fresh wt tissue was obtained after 8–10 h of incubation from leaves excised in spring (from April to May 1998), when plants showed lower leaf growth rates (0.014 ± 0.02 cm d $^{-1}$) and no leaf production. No significant production of protoplasts was obtained from June to October, during the reproductive phase. By using cultivated plants which showed a mean leaf growth rate of 0.25 (± 0.15) cm d $^{-1}$ and leaf production of 0.05 (± 0.01) leaves d $^{-1}$, high yields of protoplasts ($3.6 \times 10^6 \pm 0.5$ per g fresh wt tissue) was obtained independently to the season. Thus, cultivated plants were preferred as source of protoplasts in the preliminary culture experiment.

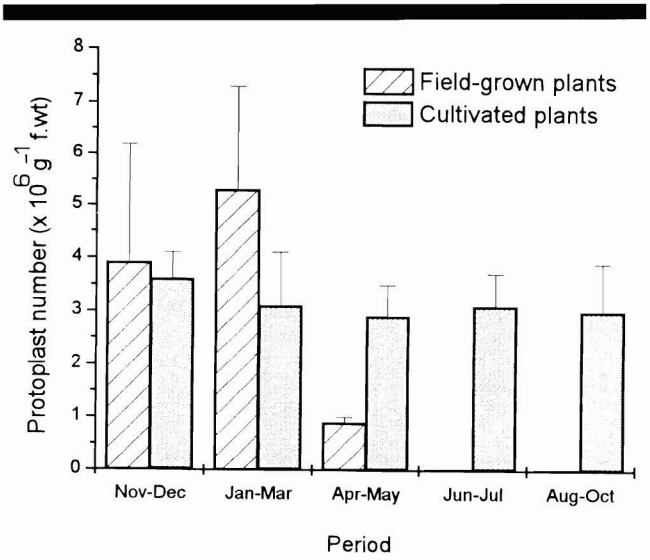

Figure 3. Seasonal variation of protoplast yields from field-grown and cultivated plants of *Pancratium maritimum*. The data are means of 3 replicates ± standard error.

After isolation, viability of protoplasts assessed by the FDA staining (Figure 2C) was 89.2% (±1.1). The protoplasts became nonspherical within 24–72 h in culture, when regeneration of cell wall began, as shown by Calcofluor white staining. The percentage of cell wall regenerating protoplasts was 88.6% (±3.6). Within 5–7 days in culture, in 0.15% of the regenerated protoplasts initial stages of cell division were observed (Figure 2D). Most cells (86.7% ± 9.2) remained viable after 15 days in culture, but further development was not observed. Approximately 90% of the protoplasts stored at 4 °C in the W solution were found still viable after 40 days. 80–100% of the surface sterilized leaves were axenic after one week in the standard media.

Cakile maritima and *Ammophila arenaria*

After 3 h of enzyme incubation, a yield of 2.31×10^5 (±0.4 SE) protoplasts per g fresh weight tissue was isolated from leaves of *Cakile maritima*. Protoplast release begun approximately after 30 min of enzyme treatment and peaked within 1 h of incubation in the W medium. Freshly isolated protoplasts were 20–50 μm in diameter and lacked the cell wall, as confirmed by Calcofluor white staining. Protoplasts showed a large vacuole with the cytoplasm and the chloroplasts packed in the peripheral region (Figure 4A). Estimate of protoplast viability was 87.5% (±4.3) at 24 h after isola-

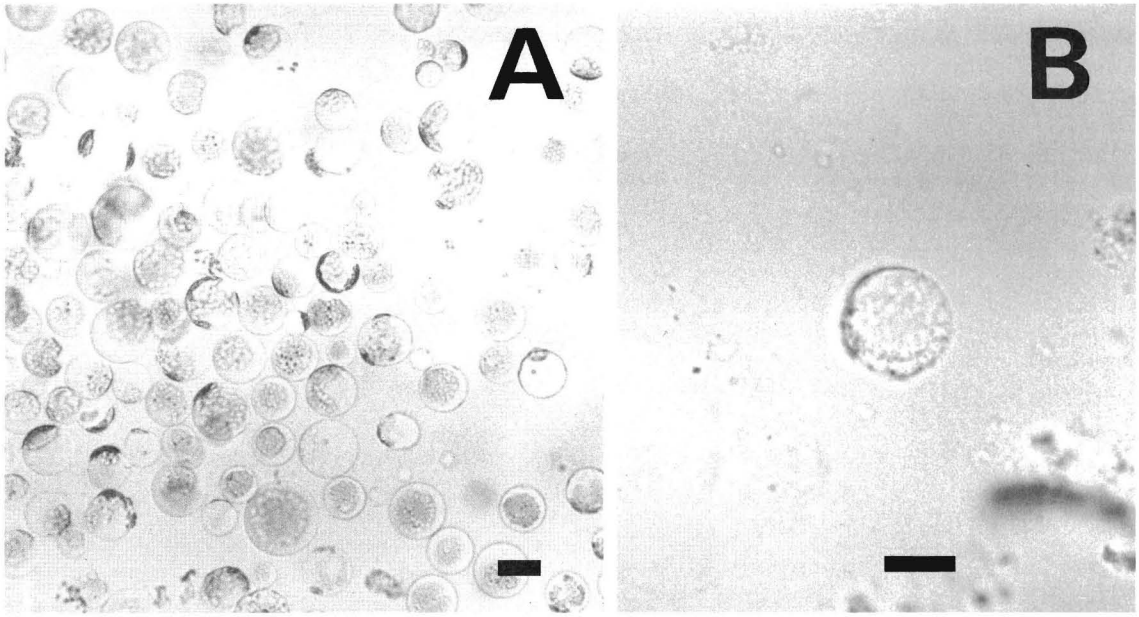

Figure 4A–B. Freshly isolated protoplasts. (A) *Cakile maritima*. Bar = 30 μm, ×125. (B) *Ammophila arenaria*. Bar = 8 μm, ×500.

tion. Isolation of protoplasts from *Ammophila arenaria* was obtained only from the basal part of the youngest leaf (approximately 2–3 mm long). The highest yield of protoplasts obtained from this tissue was 1.28×10^4 (±0.1) per g fresh weight after 10–12 h of enzymatic incubation. Protoplasts were 7–20 μm in diameter and showed a dense cytoplasm (Figure 4B). The viability of these protoplasts was 75.1% (±2.4) at 24 h after isolation.

DISCUSSION

In the present study we report a method for isolating mesophyll protoplasts from three important dune plants of the Mediterranean coasts which show different morphological adaptive features. The enzyme mixture used in this study is a combination of recipes used for protoplast isolation from other terrestrial plants. *Pancratium maritimum* appeared a very suitable species for protoplast isolation. The yield of protoplasts obtained from *P. maritimum* (10^6 protoplasts per gram of fresh tissue) was comparable to the other protocols used for important bulbous ornamental crops of Liliaceae (Mii *et al.*, 1994). It is well recognized that in higher plants the yield and division of protoplasts are affected by various factors including the kind of source materials, the developmental state and environmental conditions of donor plants (Potrykus and Shillito, 1988; Roest and Gilissen, 1993; Krautwing and Lörz, 1995; Kikkert, 1997). These factors are particularly important for successful isolation of protoplasts from wild species. Indeed, cultures of marine algae and angiosperms and dune plants have frequently hampered by the seasonal availability, marked variability in growth characteristics and difficulty in removing micro-organisms (Björk *et al.*, 1990; Walland *et al.*, 1990; Loquès *et al.*, 1990; Koch and Durako, 1991; Kane *et al.*, 1993; Mollet, 1995). In the present study, we showed that the season and growth rate of plants are of critical importance for the release of protoplasts from *P. maritimum*. Plants with high growth rates (in winter) yielded six times as many protoplasts (per g fw weight tissue) as did plants with lower growth rates (in late spring). The decline in leaf growth rate and production can be explained by the changes in resource allocation patterns occurred in plants during the reproductive phase (Kiling and Yüksel, 1995). We demonstrated, however, that by controlling the growth conditions of the plants it is possible to obtain yields of protoplasts comparable to those obtained under the best conditions from field-growing plants and, in this case protoplasts production is independent of constrains of seasonality. Thus cultivated plants may provide a better source of tissue for protoplast isolation. In liquid culture protoplasts of *P. maritimum* have proved to be capable of cell wall regeneration and initial divisions. Further studies need to identify the conditions for plant regeneration (*i.e.* growth regulators and osmoticum). Protoplasts were also obtained from *Cakile maritima* and *Ammophila arenaria* by using the same procedure adopted for *P. maritimum*. The optimal conditions for protoplast isolation from *A. arenaria,* however, should be investigated more intensively.

The method presented here may offer a novel experimental system for the characterization of the physiological and biochemical mechanisms which regulate dune plant responses to stress at cellular and molecular level, contributing to advances in ecophysiological research. For example, the role of ion transport regulation in response to salinity and drought, two environmental factors which frequently may affect plant performance, could be elucidate by applying electrophysiological techniques to isolated protoplasts of dune plants. The ability to obtain largely vacuolated protoplasts from *P. mar-*

itimum which are able to rapidly regenerate cell-wall and undergo cell division, make this plant also particularly interesting for the use of vacuoles (LEIGH, 1983; NEWELL *et al.*, 1998; WILLMER *et al.*, 1999) for studying the role of compartmentation of osmotica in stressed cells. On the other hand, protoplasts originating from the epidermal cells of *C. maritima* may allow the study the photosynthetic characteristics and responses to environmental factors. Moreover, comparison of species with different susceptibility to environmental factors (i.e. salt-resistance) such as *C. maritima*, *A. arenaria* and *P. maritimum* (ROEZEMA *et al.*, 1982; SYKES and WILSON, 1989) could be useful to relate such differences to genetic regulation at the molecular level. To date, the knowledge of the physiology and metabolism of species which grow in arid and saline environment is scarce. These approaches, therefore, could to contribute to clarify how these plants can cope with its stressful environment. Owing to increasing salinisation and degradation of soils this topic is an important subject of further research (FLOWERS *et al.*, 1986; AUSTIN, 1989; YEO and FLOWERS, 1989). Finally, when the optimal culture conditions for regenerating plants will be identify protoplasts could provide a complementary system for selection and production of the planting material required for large-scale restoration programs.

ACKNOWLEDGMENTS

We are grateful to Prof. A. Pardossi (Dipartimento di Biologia delle Piante Agrarie, Pisa University) for its contribution to the study. The authors wish also to thank Prof V. Nuti Ronchi for valuable suggestions and Claudia Nelli for laboratory assistance.

LITERATURE CITED

AUSTIN, R.B., 1989. Prospect for improving crop production in stressful environments. *In:* HAMLYN, G.J.; FLOWERS, T.J. and JONES, M.B. (eds.), *Plant under stress*. Cambridge University Press, pp. 235–249.

BELLAN-SANTINI, D.J.; LACAZE, C., and POIZAT, C. 1994. *Les biocénoses Marines et Littorales de Méditerranée, Synthèse, Menaces et Perspectives*. Collection Patrimoines Naturels—Sèrie Patrimoines écologique. Muséum National d'Histoire Naturelle, Paris, 19, 147–159.

BLITS, K.C. and GALLAGHER, J.L., 1991. Morphological and physiological responses to increased salinity in marsh and dune ecotypes of *Sporobolus virginicus* (L.) Kunth. *Oecologia*, 87, 330–335.

BJÖRK, M.; EKMAN, P.; WALLIN, A., and PEDERSÉN, M., 1990. Effects of growth rate and other factors on protoplast yield from four species of *Gracilaria* (Rhodophyta). *Botanica Marina*, 333, 433–439.

COOK, D.A.; DECKER, D.M., and GALLAGHER, J.L., 1989. Regeneration of *Kosteletzkya virginica* (L.) Presl. (Seashore Mallow) from callus cultures. *Plant Cell Tissue Organ Culture*, 17, 11–119.

ELZENGA, J.T.M.; KELLER, C.P., and VAN VOLKENBURGH, E., 1991. Patch clamping protoplasts from vascular plants. *Plant Physiology*, 97, 1573–1575.

FLOWERS, T.J.; HAJIBAGHERI, M.A. and YEO, A.R., 1896. Ion relations of plants under drought and salinity. *Australian Journal of Plant Physiology*, 13, 75–91.

FOWKE, L.C. and WANG, H., 1992. Protoplasts as tools in cell biology. *Physiologia Plantarum*, 85, 391–395.

GARRIL, A.; TYERMAN, S.D., and M.A. FINDLAY, 1994. Ion channels in the plasma membrane of protoplasts from the halophyitic angiosperm *Zostera muelleri*. *Journal of Membrane Biology*, 42, 381–393.

HESP, P.A., 1991. Ecological processes and plant adaptations on coastal dunes. *Journal Arid Environment*, 21, 165–191.

HUISKES, A.H.L., 1979. Biological flora of the British Isles. *Ammophila arenaria* (L.) Link. (*Psamma arenaria* (L.) Roem. *et* Schult.; *Calamagostris arenaria* (L.) Roth). *Journal of Ecology*, 67, 363–382.

IDE, S.; SENER, B.; TEMIZER, H., and KONUKOL, S., 1996. The crystal and molecular structure of two drug compounds: tazettine and corydaldine. *Crystal Research Technology*, 31, 617–624.

IDE, S.; SENER, B., and OZTURK, S., 1998. The crystal and molecular structure of (−) Crinine (C16H17NO3). *Journal of Chemical Crystallography*, 28(7), 577–579.

ISHIKAWA, S.; OIKAWA, T., and FURUKAWA, A., 1990. Photosynthetic characteristics and water use efficiency of three coastal dune plants. *Ecological Research*, 5, 377–391.

ISHIKAWA, S.; FURUKAWA, A., and OIKAWA, T., 1990. Photosynthetic responses to drought conditions in three coastal dune plants in relation to their zonal distribution. *Australian Journal of Botany*, 44, 381–391.

KANE, M.E.; BIRD, K.T., and LEE, T.M., 1993. In vitro propagation of *Ipomoea pes-caprae* (Railroad-Vine). *Journal Coastal Research*, 9, 356–362.

KIKKERT, J.R., 1997. Protoplast isolation and culture. *In:* CHUN, N.B.; KIM, Y.W.; AHUJA, M.S., and KLOPFENSTEIN, M.R. (eds). *Micropropagation, genetic engineering, and molecular biology of Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. pp. 326.

KILINÇ, M. and YÜKUSEL, S., 1995. A morphological, anatomical and ecological study on *Pancratium maritimum* L. (Amaryllidaceae). *Turkish Journal of Botany*, 19, 309–320.

KOCH, E.W. and DURAKO, M.J., 1991. In vitro studies of the submerged angiosperm *Ruppia maritima*: auxin and cytokinin effects on plant growth and development. *Marine Biology*, 110, 1–6.

KOOP, H.U.; EIGEL, L., and SPÖRLEIN, B., 1992. Protoplasts in organelle research: transfer and transformation of plastids. *Physiologia Plantarum*, 85, 339–344.

KRAUTWIG, B. and LÖRZ, H., 1995. Cereal protoplasts. *Plant Science*, 111, 1–10.

LEIGH, R.A. and TOMOS, A.D., 1983. An attempt to use isolated vacuoles to determine the distribution of sodium and potassium in cells of storage roots of red beet (*Beta vulgaris*). *Planta*, 159, 769–775.

LIPUCCI DI PAOLA, M.; FOSSI, D.; TOGNONI, F., and NUTI RONCHI, V., 1987. Adventitious bud induction *in vitro* from juvenile leaves of *Cupressus arizonica* Green. *Plant Cell Tissue Organ Culture*, 10, 3–10.

LOQUES, F.; CAYE, G., and MEINESZ, A., 1990. Axenic culture of selected tissue of *Posidonia oceanica*. *Aquatic Botany*, 37, 171–188.

LYNCH, J.; CRAMER, G.R., and LAUCHLI, A., 1987. Salinity reduces membrane-associated calcium in corn root protoplasts. *Plant Physiology*, 83, 390–394.

MAUN, M.A., 1997. Adaptations of plants to burial in coastal sand dunes. *Canadian Journal of Botany*, 76, 713–738.

MEDRANO, M.; GUITAN, P., and GUITAN, G., 1999. Breeding system and temporal variation in fecundity of *Pancratium maritium* L., (Amaryllidacea). Reproductive ecology of *P. maritimum*. *Flora*, 194, 13–19.

NEWMAN, J.R. and J.A. RAVEN, 1999. CO2 is the main inorganic C species entering photosyntetically active leaf protoplasts of the freshwater macrophyte *Ranunculus penicillatus* ssp. *pseudofluitans*. *Plant Cell and Environment*, 22, 1019–1026.

NEWELL, M.; LEIGH, R.A., and HALL, J.L., 1998. Vacuole development in cultured evacuolated oat of mesophyll protoplasts. *Journal of Experimental Botany*, 49, 817–827.

MII, M.; YUZAWA, Y.; SUETOMI, H.; MOTEGI, T., and GODO, T., 1994. Fertile plant regeneration from protoplasts of a seed-propagated cultivar of *Lilium* × *formolongi* by utilizing meristematic nodular cell clumps. *Plant Science*, 100, 221–226.

MOLLET, J.C.; VERDUS, M.C.; KLING, R., and MORVAN, H. 1995. Im-

proved protoplast yield and cell wall regeneration in *Gracilaria verrucosa* (Huds.). Papenfuss (Gracilariales, Rhodophyta). *Journal of Experimental Botany*, 283, 239–247.

PAKEMAN, R.J. and LEE, J.A., 1991*a*. The ecology of the strandline annuals *Cakile maritima* and *Salsola kali*. II. The role of nitrogen in controlling plant performance. *Journal of Ecology*, 79, 155–165.

PAKEMAN, R.J. AND LEE, J.A., 1991*b*. The ecology of the strandline annuals *Cakile maritima* and *Salsola kali*. I. Environmental factors afecting plant performance. *Journal of Ecology*, 79, 143–153.

PASZKOWSKI, J.; PETERHANS, A.; SCHÜPMANN, H.; BASSE, C.; LABEL, E.G., and MASSAN, J., 1992. Protoplasts as tools for plant genome modification. *Physiologia Plantarum*, 85, 35–356.

PLUIS, J.L.A. and DE WINDER, B., 1990. Natural stabilization. *Catena*, 18, 195–208.

POTRYKUS, I. and SHILLITO, R.D., 1988. Protoplasts: Isolation, culture, plant regeneration. *In:* WEISS-BACH, A. and WEISSBACK, H. (eds.), *Methods for Plant Molecolar Biology*. Academic Press Inc, San Diego, California, pp. 355–383.

POWER, J.B. and DAVID, M.R., 1990. Protoplasts of higher and lower plants: isolation, culture and fusion. *In:* POLLARD, J.W. and WALKER, J.M. (eds), *Methods in Molecular Biology: Plant Cell and Tisue Culture*. Human Press, Clifton, New Jersey, pp. 237–260.

PROTICH, N., 1987. Studies on six bulb plant species of the Bulgarian flora promising for flower production. *Rasteniev'd nauki*, 24, 59–63.

PUITE, K.J., 1992. Progress in plant protoplast research. *Physiologia Plantarum*, 85, 403–410.

RAINS, D.W., 1989. Plant tissue and protoplast culture: applications to stress physiology and biochemistry. *In:* HAMLYN, G.J.; FLOWERS, T.J., and JONES, M.B. (eds.), *Plant under stress*. Cambridge University Press, pp. 181.

ROEST, S. and GILISSEN, L.J.W., 1993. Regeneration from protoplasts—a supplementary literature review. *Acta Botanica Neerland*, 42, 1–23.

ROSEN, A. and TAL, M., 1981. Salt tolerance in the wild relatives of the cultivated tomato: responses of naked protoplasts isolated from leaves of *Lycopersicon esculentum* and *L. peruvianum* to NaCl and proline. *Zeitschrift für Pflanzenphysiologie*, 102, 91–94.

ROZEMA, J.F.; BIJL, F.; DUEK, T., and WESSELMAN, H., 1982. Salt-spray stimulated growth in strand-line species. *Physiologia Plantarum*, 56, 204–210.

SHABALA, S.; NEWMAN, I.; WHITTINGTON, J., and JUSWONO, U., 1998. Protoplast ion fluxes: their measurement and variation with time, position and osmoticum. *Planta*, 204, 146–152.

SYKES, M.T. and J.B., 1989. The effect of salinity on the growth of some New Zealand sand dune species. *Acta Botanica Neerland*, 38(2), 173–182.

TATO, M.P.V.; CASTEDO, L., and RIGUERA, R., 1988. New alkaloids from *Pancratium maritimum* L. *Heterocycles*, 27, 2833–2838.

VAN DER PUTTEN, W.H. and KLOOSTERMAN, E.H., 1991. Large-scale establishment of *Ammophila arenaria* and quantitative assessment by remote sensing. *Journal of Coastal Research*, 7, 1181–1194.

VALVERDE, T.; PISANTY, I., and PINCON, E., 1997. Growth response of six tropical dune plant species to different nutrient regimes. *Journal of Coastal Research*, 13, 497–505.

WALLAND, J.R.; DIKSON, L.G., and WATSON, B.A., 1990. Protoplast isolation and regeneration in the marine red alga *Porphyra nereocystis*. *Planta*, 181, 522–528.

WANG, J.; SUKHORUKOV, L.; DJZENOVA, C.S.; ZIMMERMANN, U.; MÜLLER, T., and FUHR, G., 1997. Electrorotational spectra of protoplasts generated from the giant marine alga *Valonia utricularis*. *Protoplasma*, 196, 123–134.

WILLMER, C.M.; PADMASREE K., and RAGHAVENDRA, A.S., 1999. A novel method of measuring volume changes of mesophyll cell protoplasts and the effects of mercuric chloride on their osmotically-induced swelling. *Journal of Experimental Botany*, 50, 401–406.

YEO, A.R. and FLOWERS, T.J., 1989. Selection for physiological characters—examples from breeding for salt tolerance. *In:* HAMLYN, G.J.; FLOWERS, T.J., and JONES, M.B. (eds.), *Plant under stress*. Cambridge University Press, pp. 217–234.

ZHANG, W.H.; WALKER N.A.; TYERMAN, S.D., and PATRICK, J.W., 1997. Mechanisms of solute efflux from seed coats: whole-cell K^+ currents in transfer cell protoplasts derived from coats of developing seeds or *vicia raba* L. *Journal of Experimental Botany*, 313, 1565–1572.

ZIEMAN, J.C., 1974. Methods for the study of the growth and production of the turtle grass, *Thalassia testudinum* König. *Aquaculture*, 4, 139–143.