Axenic cell culture of the seagrass *Cymodocea nodosa* from cotyledonary tissue

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INTRODUCTION

Plant Tissue Culture, also called "micropropagation", is the propagation of plants from different tissues (or explants) in a shorter time than conventional propagation, making use of the ability that many plant cells have to regenerate a whole plant (totipotency) [1, 2, 3, 4].There are two alternative mechanisms by which an explant can regenerate an entire plant, namely organogenesis and somatic embryogenesis [5]. Since the last decades, the number of higher terrestrial plants species from which these techniques have been successfully applied has continually increased [6, 7]. However, few attempts have been carried out in marine plants.

Previous seagrasses authors have focused their studies on i) vegetative propagation of rhizome fragments as explants in *Ruppia maritima* [8, 9, 10], *Halophila engelmannii* [11], *Cymodocea nodosa* [12] and *Posidonia oceanica* [13, 14]; ii) culture of meristems in *Heterozostera tasmanica* [15], *C. nodosa* [16] or *P. oceanica* [17]; and iii) culture of germinated seeds on aseptic conditions, in *Thalassia testudinum* [18, 19], *H. ovalis* [20], *P. coriacea* [20], *P. oceanica* [21, 22], and *H. decipiens* [23]. All these studies determine the most adequate culture medium for each species (seawater, nutrients, vitamins, carbon sources, etc...), often supplemented with different plant growth regulators and the necessary conditions for the culture maintenance, such as light and temperature [16, 20]. On the other hand, several studies have previously established protocols for cell or protoplast isolation in the species *Zostera marina* [24], *Z. muelleri* [25], *P. oceanica* [26, 27, 28], and *C. nodosa* [27], using shoots collected from natural meadows as original vegetal source, but further cell growth was never accomplished. Due to the absence of somatic embryogenesis or organogenetic studies in seagrasses we wonder:

IS THE SUCCESSFUL APPLICATION OF TISSUE CULTURE TECHNIQUES POSSIBLE IN SEAGRASSES?

PRELIMINARY APPROACHES OF *C. NODOSA* TISSUE CULTURE.

MATERIAL AND METHODS

Explants source:
Cotyledons from fifteen-day-old seedlings (Fig. 1A) germinated in axenic conditions [29] were cut in 5 or 1 mm (Thin Cell Layer) fragments.

Sterilization procedures:
Seedlings were immersed for 10 min in a 10% sodium hypochlorite solution with a drop of Tween 80, washed 3 times in distilled autoclaved water and incubated 24-48 h in a 10% antibiotic solution [16].
Cotyledon's fragments were immersed for 10 min in a 2% KBr solution and washed 3 times in distilled autoclaved water.

Culture Media
Two different culture media were tested:
PES medium [30] modified by García Jiménez et al., (2006) [16].
MS medium [31], with or without NaCl (3.20 g l^{-1}).
Both media were supplemented with 8g l^{-1}agar, 1:10 (v/v) antibiotic solution, 100 mg l^{-1} L-glutamine, 30 g l^{-1} sucrose, 1 g l^{-1}activate charcoal, and the pH adjusted to 7.

Culture conditions
Experimental conditions of the culture were 22 ± 2ºC, 16:8 photoperiod and 30 µmoles of photons m^{-2} s^{-1}.

Treatments applied
PGR´S. Cultures media were supplemented with different plant growth regulators and concentrations: NAA, 2,4-D, KIN, TDZ, BAP, PICL, IBA, Spm and Put, from 10^{-3}M to 10^{-9}M, added alone or in combination.
Anti- Browning. The explants were cut immersed in antioxidant solution (100 mg l^{-1} ascorbic acid and 100 mg l^{-1} citric acid). Culture media were also supplemented with the same antioxidant solution [32, 33].

RESULTS

There were no differences between PGR's or antibrowning treatments. The explants stay in an aseptic condition during few days (even better in free NaCl medium), but do not produce any callus-like structure or somatic embryos. The unique response was the formation of a swelling structure (Fig. 1B), where a new leaf is produced (Fig. 1C). This response occurs in explants that previously have a shoot apical meristem, as the swelling's histology shows (Fig. 1D). Finally, the induction of rizhogenetic responses could not be successfully achieved.

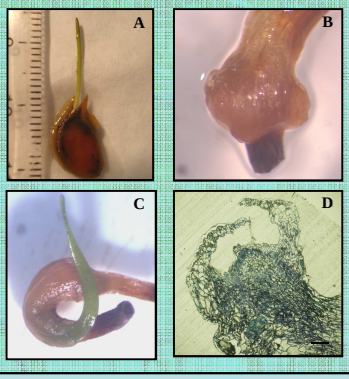

Fig 1. A. *Cymodocea nodosa* fifteen-day seedling used as material source for tissue culture. B. Swelling structure (5-8 mm) produce from cotyledonary explants. C. Regenerating leaf (5-8 mm) from swelling structures. D. Swelling histology (Bar = 0.1 cm)

DEVELOPED PROTOCOL TO PRODUCE AXENIC CELL CULTURES FROM *C. NODOSA* COTYLEDONARY TISSUE

MATERIALS AND METHODS

Explants source:
Fifteen-day-old seedlings germinated in axenic conditions [29] (Fig. 3A).

Sterilization procedure
The exterior cover of seedlings (2 per digestion) were extracted for sterilisation and washed in double distilled water. Cotyledons were cut in 1 mm fragments and immersed in 70% ethanol for 1 min. Next, these fragments were sterilised for 30 min in a 2% sodium hypochlorite solution, and finally washed 3 times in distilled autoclaved water.

Digestion procedure
To improve cell release, fragments were incubated for 5 min in a sterilised hyperosmotic medium to induce slight cell plasmolysis (plasmolysis medium, Table 1).
The fragments (ca 100 mg fresh weight) were digested in 2 ml plasmolysis medium containing hydrolytic enzymes (1% w:v Cellulase Sigma Co. USA 6.9 Units mg^{-1} and 1% w:v hemicellulase Sigma Co. USA 1.5 Units mg^{-1}), which was sterilised via filtration (0.2 µm pore membrane filters; Millipore). Digestion was performed in a Stuart S130H hybridization-shaker oven for 18h at 5 rpm, and 36ºC in the dark.

Table 1. Composition of the plasmolysis medium

Component	Concentration (g L^{-1})
KH_2PO_4	0.0272
$CaCl_2$ x $2H_2O$	1.480
$CuSO_4$ x $5H_2O$	0.025
KNO_3	0.101
KI	0.16
$MgSO_4$ x $2H_2O$	0.246
Citric acid	0.2
Glycine	0.1
MES buffer	1
Mannitol	130
pH	5.8

Culture Media
The released cells were sieved through a 60µm sterile metallic net (Sigma Co. USA) and centrifuged at 1000g for 10 min. The resulting pellet, consisting of free cells, was gently resuspended in a MS medium [31] containing 10 mg l^{-1} malic acid and citric acid as antioxidants [32, 33] at pH 5.8, but devoid of seawater or any NaCl addition. The cell pellet was twice purified by centrifugation in order to remove the manitol, and resuspended in the same MS medium containing 2,4-D and BAP at 10^{-4} M respectively. The medium was autoclaved and the plant growth regulators were filter sterilised from a previously filtered stock solution (0.2 µm pore membrane filters; Millipore, Germany) and added to the medium. To avoid cell death, 2,4-D and BAP had to be removed after one week of culture.

Culture conditions
Experimental conditions of the culture were 28ºC and darkness during the first 5 weeks, and then switched to light conditions (30 µmoles of photons m^{-2} s^{-1}, 16:8 photoperiod). The experiments lasted three months and were performed twice with the same results.

Culture monitoring
Culture development was photographed weekly using an Olympus CK40 optic inverted photomicroscopy. Cell growth was measured by counting the number of cells in random microscopic fields (magnification, 20x) at day 0 and 7 days after initiation of cultures.
Complementary histological studies of the cotyledon were made to locate the potential source of active growing cells. Specimens of 3-mms along the proximal to distal axe of the cotyledon were fixed and processed as described in García-Jiménez et al., (1998) [34].

RESULTS

The digestion of the cotyledonary explants liberated cells (20-60µm) from the tissue which were able to divide in the medium. The initial cell number increased from 15±2 to 48±7 (mean number of cells per microscopic field) after 7 days in culture in MS medium + 2,4-D + BAP (Fig. 2A-B). Cultures in MS free hormone medium progressed further to develop cell masses.
Over the 3 month culture period, the cells kept dividing and spontaneously joined to form cell aggregates (Fig. 2C) and structures resembling pre-embriogenic stages, such as the globular stage (Fig. 2D).

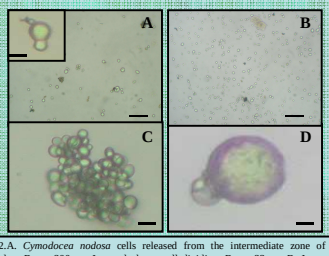

Fig. 2.A. *Cymodocea nodosa* cells released from the intermediate zone of the cotyledon. Bar = 300µm. Inserted photo: cell dividing. Bar = 33µm. B. Increasing number of cells after one week in culture. Bar = 300µm. C. Cell aggregates. Bar = 50µm. D. Globular cell in culture resembling pre-embriogenic stages. Bar = 20µm.

The histological study from seedling tissue showed the presence of a shoot apical meristeme on the intermediate area of the cotyledon, formed by cells with a high morphogenetic potential (Fig. 3B). However, the apical zone of the cotyledon was constituted by elongated and vacuolated cells unfit to induce morphogenetic responses (Fig. 3C).

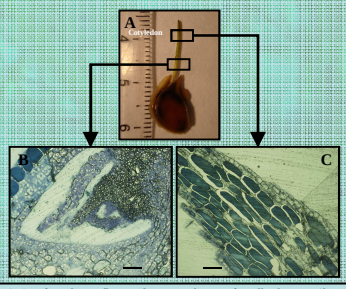

Fig. 3. A. Fifteen-day seedling used as material source for cell culture. B. Shoot Apical Meristem located in the intermediate zone of the cotyledon containing active growing cells. Bar = 200µm. C. Apical zone of the cotyledon containing vacuolated cells unfit to induce morphogenetic responses. Bar = 400µm.

DISCUSSION

Due to the weak response of *Cymodocea nodosa* cotyledonary explants cultured *in vitro*, we consider the culture of cells as the most adequate way to produce somatic embryos. We have achieved aseptic culture, cell division, and cell growth throughout 3 months of study, by multiplying by 300% the initial number of cells after 7 days in culture (Fig. 2A-B). During the experimental time established, cells divided and joined, forming cell aggregates with likely embryogenic potential (Fig. 2C). There were two key factors in the establishment of these cultures: one was the use of cotyledonary explants from seedlings germinated in aseptic conditions, as compared to plant material sourced from natural meadows; the other was the removal of NaCl from the MS culture medium, as contaminants associated with the explants not appear due to their apparent halophytic nature. We have generated a basic tool for the future of *C. nodosa* tissue culture, since it allows the establishment and maintenance of cell cultures to test any regeneration protocol, and enables the possibility to induce the development of whole plants by embryogenesis or organogenesis. In fact, we have noticed globular somatic embryo-like cells in our cultures (Fig. 2D). Thus, the technique proposed in this study should be complemented with the development of morphogenetic ways that allow the regeneration of the whole plant.

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