Available online at www.sciencedirect.com
SCIENCE DIRECT
Aquatic Botany 84 (2006) 79–84
Aquatic botany
www.elsevier.com/locate/aquabot

Anatomical and nutritional requirements for induction and sustained growth in vitro of *Cymodocea nodosa* (Ucria) Ascherson

Pilar García-Jiménez, Eva P. Navarro, Cristo H. Santana, Ángel Luque, Rafael R. Robaina *

Departamento de Biología, Universidad de Las Palmas de Gran Canaria, 35017 Las Palmas de Gran Canaria, Gran Canaria, The Canary Islands, Spain

Received 13 July 2004; received in revised form 20 June 2005; accepted 6 July 2005

Abstract

In vitro methods of plant micro-propagation are being considered as a possible solution for the decline in seagrass communities registered worldwide. To achieve successful plant micro-propagation, culture conditions are commonly adjusted empirically within almost species-specific conditions, to comply to the following three conditions: (i) culture establishment (ii) shoot production and (iii) rooting and hardening for planting in soil. Cultures of *Cymodocea nodosa* were established from axenic explants of the apical meristem (approx. 0.5 cm) which regenerated new leaf or produced leaf regenerating calli (5% of cultivated explants) in media containing 10^{-6} M of the cytokinin analogue TDZ. Longer ramet explants, not fully axenic, containing internode with leaf and roots were also affected by 10^{-6} M cytokinins and auxin type of regulators, as they promoted leaf extension (in cm), particularly GA. None of the explants progressed further to massive shoot propagation and new plantlet production. Instead, experiments made with ramet explants which simulated potential produced plantlet revealed that there seems to be a strong interaction within leaf, rhizome and root, since the carbon fixed in the leaf was rapidly translocated to the rest of the tissue (50% in the roots in a FW basis). The explants preferred ammonium and dihydrogen inorganic phosphate as a nutrient source, efficiently assimilating the former regardless of whether such were added to the underground or surface tissue. However, underground tissue was required to maintain P status in the cultivated explants.

Keywords: *Cymodocea nodosa*; Explant anatomy; Growth substance; In vitro; Nitrogen; Phosphorus

1. Introduction

The seagrass *Cymodocea nodosa* (Ucria) Ascherson forms dense submerged, ecologically relevant communities as stable and protected habitats, where other vegetation, animals and biota live, spawn and feed. Anthropogenic activities have influenced coastal marine ecosystems to the extent that they are considered to be responsible for the worldwide decline in seagrass communities. Several strategies such as transplantation and micropropagation may help to avoid complete depletion. Transplantation depends on a source (threatened) population and its rates of reproductive or vegetative propagation, which are commonly slow (West et al., 1990; Molenaar et al., 1993; Molenaar and Meinesz, 1992, 1995).

Micropropagation is the propagation of plant species in vitro starting from its cells, tissues or, organs. This can be carried out in a shorter time than conventional vegetative propagation, and produces high biomass from one single plant. In the 1970s Murashige stated that three developmental stages of propagation seems to be mandatory: (i) culture establishment, (ii) shoot production and (iii) rooting and hardening for planting in soil. Using these ground rules, micropropagation of freshwater aquatic plants was successfully carried out concluding in normal and healthy plants (Huang et al., 1994; Agrawal and Mohan Ram, 1995; Kane et al., 1999).

Interesting studies have been produced, aimed at in vitro culture and/or propagation of ecologically relevant marine species, such as *Posidonia oceanica*, *Ruppia maritima* or *C. nodosa*. Whilst it is clear that it is possible to apply the essentials of in vitro methodology to establish explant cultures (i.e. stage I), massive shoot induction, rooting control and plant production (stages II and III) comparable to that of other

* Corresponding author. Tel.: +34 928 452904; fax: +34 928 452922.
E-mail address: rrobaina@dbio.ulpgc.es (R.R. Robaina).

0304-3770/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquabot.2005.07.006

aquatic plants, such as the above mentioned species, have not been achieved (Loquès et al., 1990; Koch and Durako, 1991; Terrados-Muñoz, 1995; Bird et al., 1993, 1996, 1998).

In spite of the relevance of these physiological traits to the success of in vitro propagation of seagrasses, nothing is known about the actual or potential nutritional situation of the explants and plantlets produced in culture. The ecophysiological literature relating to these problems commonly addresses the question in the light of the respective roles of root versus leaf as the organ involved in nutrient absorption (commonly N and P). This is the same in the case of the acquisition of carbon, its fixation and allocation during photosynthesis, although it is clear that seagrasses translocate oxygen and carbon from leaf to non-photosynthetic tissues (Pérez et al., 1994; Terrados and Williams, 1997; Kraemer and Mazzella, 1999; Touchette and Burkholder, 2000a,b). In fact, clone identity experiments revealed that the subsistence of an apical meristem in *C. nodosa* seems to depend on the rhizome (up to 50 cm Terrados et al., 1997).

In this work, explants of *C. nodosa*, containing only the apical meristem, or a ramet, containing the internode, leaf, and associated root (i.e. simulating plantlet), were cultivated under different nutritional conditions, which included plant growth regulators, and nitrogen and phosphorus sources to determine: (i) the effect of growth regulators, (ii) the preferred chemical form of the nutrients and (iii) the relative importance of the underground and overground parts of the explant in N, P and C assimilation. This was an attempt to clarify whether growth regulators affect growth, to optimise the type and form of addition to the culture vessel of the limitant nutrients, and to evaluate to what extent both parts are necessary to avoid poor nutritional status during the propagation of the regenerated plantlet during stages II and III of propagation.

2. Materials and methods

2.1. Plant material

Samples of *C. nodosa* (Ucria) Ascherson were collected at Castillo del Romeral (on the southeast coast of Gran Canaria, the Canary Islands, 27°48′00″N; 15°25′40″W) from shore pools of 1–2 m depth. To avoid any damage to the populations, the experiments were regularly carried out with material collected in the Winter and Spring of the years from 2001 to 2003. Within 2 h after collection, the youngest and cleanest plant material was selected at the laboratory, where explants of approx. 3 cm consisting of internode rhizome, leaf, and associated root were excised from the rhizome (henceforth ramets, Fig. 1). Leaves from ramets were further cut transversely in order to analyse the effect of regulators on new leaf tissue generation (meaning both the growth (cm) of the young leaf and new leaf tissue produced by those that were cut off). Explants were surface sterilized previous to cultivation, by dipping in distilled water and immersion in 1% sodium hypochloride in autoclaved seawater for 5 min. After rinsing three times in sterile seawater, explants were incubated for 48 h with a filter-sterilized antibiotic mixture containing rifampicin, penicillin, nystatin, ampicillin (150 mg l^{-1} each) and germanium dioxide (5 mg l^{-1}). This method produced not axenic cultures of such a centimeter long explants, but together with sterilization through autoclaved seawater, nutrient solutions, sand and the culture vessel, ensured they were clean enough to avoid contaminant overgrowth and interference during the experimental time (up to 15 days). Axenic explants were obtained from cylindrical fragments (0.5 ± 0.1 cm) excised from the meristematic apex of the plagiotropic rhizome (henceforth, apical explants).

2.2. Culture conditions

To check the effects of plant growth regulator (PGRs) auxins, indole acetic acid (IAA), indole butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D); cytokinins, kinetin (KIN), benzyladenine (BA), *N*-phenyl-*N*′-1,2,3-thidiazol-5-ylurea (thidiazuron or TDZ), and gibberellic acid (GA) were added individually as filter-sterilised stock solutions at 10^{-6} M final concentration ramets were cultured in 30 ml Provasoli's medium (PES, Provasoli, 1968) in tubes, supplemented with the respective growth regulator. Axenic apical explants were cultivated in solidified (agar 0.8%) PES medium (prepared with diluted seawater to adjust the osmolarity to that of the seawater; Robaina et al., 1990) in Petri dishes, supplemented with 10^{-6} M of the same regulators and sucrose 60 g l^{-1}. Preliminary experiments carried out with most of the regulators tested did not reveal the existence of a clear dose response, thus effective concentration was directly adjusted to 10^{-6} M as a standard hormonal concentration ramets and apical explants were kept for 15 days in their respective media. Experiments were repeated three times with five replicates of each regulator (i.e. 15 ramets and 15 apical explants per growth regulator).

Enriched seawater Provasoli medium in Magenta®-G7 (Sigma Co.) culture vessels was used as a culture medium in the experiments with nutrients. The vessels were filled with 40 ml autoclaved sand and 200 ml of liquid culture medium as shown in Fig. 1. Enrichment of the medium was achieved by adding nutrients from sterilized stock solutions to the seawater. Nutrient enriched sand was obtained by incubating autoclaved sand during 4 days in the appropriate nutrient solution. To check nutritional preferences for different chemical forms of N and P, a simplified factorial experimental design of Box–Behnken was used (Tox and Behnken, 1969) for three factors (nitrate, glutamic, ammonium, for N, and inorganic KH_2PO_4 and organic glyceraldehyde-3-phosphate for P) with three concentration levels based on regular PES enrichment of sand and water (0, 0.05, 0.1 mM, and 0, 0.5, 1 mM phosphorus and nitrogen, respectively) and three central points. A semiquantitative response variable (GH) was used to measure greenness and youthful vigor of leaves during the experimental time (15 days). One ramet per vessel (Fig. 1) was used together with five replicates per treatment (GH = 1 no evident change or even degeneration symptoms, GH = 2 healthy as pigmented, and GH = 3 healthy and regenerating new leaves. Fig. 1C and D). The entire experimental design was performed twice with five