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DEVELOPMENT OF A MODEL FOR *IN VITRO* CULTURE OF *Vallisneria americana* Michx.

Desarrollo de un modelo de cultivo *in vitro* para *Vallisneria americana* Michx.

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ABSTRACT. An exploratory experimental design was developed to establish a model for *Vallisneria americana* Michx. following conventional *in vitro* culture procedures for aquatic vegetation. The design included five experiments with stages of presterilization or washing with 0.1 % Tween 20®, sterilization with NaClO and poststerilization of explants (leaf base, rhizome, caulinar apex and seeds), and culture in monophasic or biphasic nutrient media. Leaves and rhizomes were sterilized with 0.3 and 0.6 % NaClO, and contact times of 2.5 and 5 min. Both explants cultured in gellified monophasic White medium with 58.4 mM sacarose and a pH of 7.5 presented on average a 60 % contamination by translucid lipidic exudates, followed by darkening. The presterilization of leaves and rhizomes caused the production of exudates, and the addition of 0.01 % ascorbic acid to the nutrient medium caused its inhibition. Comparing the White medium with the 0.5 MS medium, both with 0.88 x 10^{-3} mM of bencyladenine, asepsia of explants was improved in White and leaf pigmentation was significantly favored in 0.5 MS, although no explant presented regeneration. Presterilization was omitted in the experiments with caulinar apices and seeds, and sterilization was carried out with 0.6 % NaClO for 15 min. The monophasic culture of caulinar apices in liquid 0.5 MS with doses from 0.0 to 8.88 x 10^{-3} mM of bencyladenine regenerated leaves but resulted in excessive contamination and death. In contrast, both the non-aseptic control and the *in vitro* seedlings in biphasic culture were 100 % aseptic in 0.5 MS, White and semi-hard sterile local water. However, the White medium produced lower quality seedlings. The biphasic germination in aqueous medium with local water and gellified support was selected considering its lower cost and germination efficiency.

Key words: Aseptic culture, viable explant, biphasic germination, *Vallisneria americana*, wild celery.

RESUMEN. Con procedimientos convencionales de cultivo *in vitro* efectuados en vegetación acuática se desarrolló un diseño experimental exploratorio para establecer un modelo de cultivo para *Vallisneria americana* Michx. El diseño incluyó cinco experimentos con etapas de preesterilización o lavados de 0.1 % de Tween 20®, esterilización con NaClO y postesterilización de explantes (base de hoja, rizoma, ápice caulinar y semillas), así como su cultivo en medio nutritivo monofásico o bifásico. Las hojas y rizomas fueron esterilizados con 0.3 y 0.6 % de NaClO y tiempos de contacto de 2.5 y 5 min. Ambos explantes cultivados en medio monofásico gelificado de White con 58.4 mM de sacarosa y pH de 7.5 presentaron en promedio 60 % de contaminación por exudados translúcidos lípidicos que después obscurecieron al explante. La preesterilización de la hoja y rizoma causó la producción del exudado y la adición de 0.01 % de ácido ascórbico al medio nutritivo causó su inhibición. Cuando se compararon los medios monofásicos de White y 0.5 MS, ambos con 0.88 x 10^{-3} mM de benciladenina, la asepsia de los explantes fue mejorada en White y la pigmentación foliar fue significativamente favorecida en 0.5 MS, pero ningún explante presentó regeneración. La preesterilización fue omitida en los experimentos con ápices caulinares y semillas y la esterilización se realizó con 0.6 % de NaClO por 15 min. El cultivo monofásico de ápices caulinares en 0.5 MS líquido con dosis desde 0.0 hasta 8.88 x 10^{-3} mM de benciladenina regeneró hojas pero hubo excesiva contaminación y murieron. En cambio, los germinados en el control no aséptico y en el cultivo *in vitro*, ambos en cultivo bifásico, fueron 100 % asépticos en 0.5 MS, White y agua local estéril semidura. Sin embargo, el medio White produjo menor calidad de germinados. La germinación bifásica con medio acuoso de agua local y soporte gelificado fue seleccionada por su menor costo y eficiencia germinativa.

Palabras clave: Cultivo aséptico, explante viable, germinación bifásica, *Vallisneria americana*, cintilla.

INTRODUCTION

Submerged rooted angiosperms are highly productive and are important in providing substrate and services in aquatic ecosystems (Hemminga & Duarte 2000; Hobbs & Harris 2001; Short *et al*. 2006; Best *et al*. 2008). The loss or decrease of this vegetation in coastal areas are related to natural and anthropogenic variations in flood cycles, organic loads, water quality and loss of emergent rooted macrophytes (Mackay *et al*. 2003; Mony *et al*. 2007; Best *et al*. 2008), all of which affect hydrological dynamics and productivity, biodiversity and the abundance of associated flora and fauna and, in consequence, the optimum use of natural resources, including water resources (Hemminga & Duarte 2000).

The decline in submerged aquatic vegetation has reached 60 % in the last 20 to 30 years in some altered environments (Rybicki & Carter 1986; Rybicki *et al*. 2001). The decrease in the abundance and natural regeneration of seed banks is also considered important (Westcott *et al*. 1997; Ke & Li 2006). Meso and microcosm *in situ* studies have been carried out to evaluate the factors that change submerged vegetation populations (Twilley & Barko 1990; Touchette & Boulkoldher 2002), where the availability of enough organisms and the elimination of epiphytes has been fundamental in obtaining good experimental results. Also, the system of *in vitro* plant culture is useful for evaluations as it excludes the macro and microbiota that is associated with the plants, and it has the capacity to maintain a permanent supply of high quality material for research (Moffler & Durako 1984; Ailstock *et al*. 1991; Li & Gallagher 1996; Kane & Philman 1997). The aseptic reproduction of aquatic plants has been carried out heterotrophically in nutrient media with different phytoregulators. Diverse plant structures such as tissue fragments, organs and seeds cultivated in specific nutrient media have generated seedlings from which *in vitro* clones may be obtained (Kane & Gilman 1991; Kane & Philman 1997; Jenks *et al*. 2000, Kane *et al*. 2002; Zhou *et al*. 2006).

In vitro plants have been used to identify abiotic stress as their greater flexibility favors the design of experiments, lower analyses costs and independence from natural temporal variations (Hall *et al*. 1997; Mohan & Hosetti 1999; McCann *et al*. 2000; Murphy *et al*. 2003). Several ecologically important submerged species such as the seagrasses *Halophila decipiens* (Bird *et al*. 1998), *Posidonia oceanica* (Balestri & Bertini 2003), *Ruppia maritima* (Murphy *et al*. 2003) and *Cymodocea nodosa* (García-Jiménez *et al*. 2006), as well as the freshwater grass *Potamogeton pectinatus* (Ailstock *et al*. 1991; Hall *et al*. 1997; Zhou *et al*. 2006), have been cultivated *in vitro* with this in mind. However, *Vallisneria* ssp. has been studied little (Uma & Mohan-Ram 1972) under this point of view. *In vitro* propagation of this species is required to maintain and re-populate populations that have been reported in drastic reduction and those in ecosystems where it is disappearing (Xiong & Li 2002; Sánchez *et al*. 2007). A permanent supply of plant matter of this macrophyte is needed to experimentally prove the hypotheses generated with respect to the environmental variations and the interactions with other species that are resulting in its declination or disappearance, as well as to sustain programmes to repopulate and restore altered ecosystems where biodiversity has been negatively impacted. Examples of research results include those of Wigand & Stevenson (1994, 1997), Wigand *et al*. (2000), Kurtz *et al*. (2003), Genkai-Kato (2007) and Mony *et al*. (2007).

The Reserva de la Biosfera Pantanos de Centla in one of the greatest wetlands in Mesoamerica, and it harbours the aquatic vascular flora that is representative of this region (Guadarrama & Ortiz-Gil 2000). The dominant species of rooted aquatic vegetation in this wetland, *Vallisneria americana* Michx., has been reported with a wide variety of values of density, biomass, surface area of beds and distribution (Sánchez *et al*. 2007). However, the causes of its spatial-temporal changes and their effects in Pantanos de Centla have not been evaluated, in spite of it harbouring a high biodiversity of associated fauna (Rozas & Minello 2006; Sánchez *et al*. 2007) and providing ecological services similar to those of other species of submerged aquatic vegetation in estuarine ecosystems (Short *et al*. 2006; Genkai-Kato 2007; Best *et al*. 2008). The

in vitro culture model for submerged aquatic vegetation under these conservation conditions is basic for the development of environmental stress bioassays and of restoration programmes for impacted ecosystems, as is the case of the Reserva de la Biosfera Pantanos de Centla. The purpose of this study was to prove the feasibility of cultivating vegetative explants and seeds of *V. americana in vitro*, by reproducing the conventional procedures used for this species and other species of aquatic vegetation. The experiments focused on evaluating the asepsia and viability of explants, and inducing regeneration to micropropagate healthy plants.

MATERIALS AND METHODS

Focal species

Vallisneria americana (Hydrocharitaceae) is a submerged, perennial, dioic and rooted hydrophyte that is distributed from North America to Central America (Korschgen & Green 1988; Ellison 2004). The number and density of vegetation beds in the Reserva de la Biosfera Pantanos de Centla (RBPC) (17° 57' 53"-18° 39' 03" N - 92° 06' 39" -92° 47' 58" W) have recorded a marked seasonal variation and have tended to decline or disappear (Sánchez *et al*. 2007). Populations of this species are monospecific and produce a maximum of flowers and fruits during the low-flood cycle that occurs from May to August (Jiménez 2003). This species presents rosette leaves on a short vertical stem (orthotropic rhizome or caulinar apex) that produces roots and rhizomes with stolons (plagiotropic rhizome). The leaves are strap-like and reach lengths of 50 cm or greater in relation to the local level of flooding (Jiménez 2003). The reproduction of *V. americana* is predominantly vegetative through the stoloniferous rhizome that grows horizontally and develops shoots (ramets). The reproductive ramets fall to the ground and act like turions or dormant buds (Sculthorpe *et al*. 1967).

Sampling

Vallisneria americana plants were collected in Laguna de San Pedrito, in the RBPC (54° 25' 50" N - 20° 30' 63.2" W), during the minimum flooding season of 2004. It has recorded its greatest biomass averages in this lagoon (Jiménez 2003) at a maximum salinity of 4 psu (4450 μS cm^{-2}) and a pH of 7.8 (Sánchez *et al*. 2007). Collection of ramets was done manually and they were washed *in situ* to remove sediment from the roots. Mature fruit was collected the following year. They were recognized by their size, brown color and withering of the pistilate stem. The plant samples were transported in lagoon water at ambient temperature. The *V. americana* seeds were extracted by dissecting the surface of the fruit lengthwise with a scalpel and were preserved, together with their mucilage, in refrigeration (12 $\pm$ 2 °C) for a maximum of three months with periodic changes of water every 15 days.

Handling of plants in aquaria

Plants without damaged leaves were transplanted into glass aquaria (40 L) in a polycarbonate greenhouse. Fifteen ramets were cultivated in the aquaria on a substrate of 10 cm of pre-washed and dried sand and a 30 cm aerated water column. The aqueous medium was renovated every 15 - 20 days throughout an adaptation period of three months. During this time, the greenhouse maintained an average ambient temperature of 29.5 °C and an average relative humidity of 57 % (Thermo-Hygro® Control Company). The aquaria with *V. americana* received a light intensity of 61 to 360 μmol photons m^{-2} s^{-1} (Light Meter® Extech Instruments), the water temperature varied between 25 and 30 °C and the pH varied from 8 to 8.8 (pHmeter 240 Corning®). These parameters were monitored two or three times per week between 11 and 13 h. The *V. americana* ramets produced in the laboratory were placed in transparent polyethylene aquaria with 1 L of White medium (White 1963) at a pH of 7.5. The ratio of water column to sand layer was 3:1 cm. The culture medium was renovated every 15 days to avoid overgrowth of epiphytes. The cultures remained in a laboratory with controlled temperature (25 $\pm$ 5 °C), shade, and natural light and photoperiod. The new ramets were used for the first *in vitro* experiments.

General conditions of *in vitro* culture

The leaf, plagiotropic rhizome and orthotro-

pic rhizome without roots were extracted whole and washed with running water for 10 min. The corresponding explants were dissected with a scalpel and cultivated according to the experimental set.

The *in vitro* culture technique of the explants included the phases of presterilization, sterilization, poststerilization and planting in a culture medium (Torres 1989). Presterilization included washing with Tween 20® for 15 min at 250 rpm. Sterilization or chemical disinfecting was carried out with diluted solutions of Cloralex® (6 % NaClO) at 250 rpm. Poststerilization included three to five rinsings with sterile water to wash and eliminate the NaClO remaining on the explants. The phases of the procedure and the culture media were modified or omitted in regard to the objectives and hypotheses of each experiment, to insure the most adequate conditions of asepsia and viability for the seeds and vegetative structures.

The culture media used in the experiments were White (1963) with 700 μS cm^{-2} and a pH of 6 or 7.5, and Murashige & Skoog (1962) at half ionic strength (0.5 MS) with 3000 μS cm^{-2} and a pH of 6. Both media were used in some experiments. All media were enriched with vitamins and 58.4 or 29.2 mM sucrose.

The monophasic culture was prepared in liquid form or with a gellified support (0.6 % of bacteriological agar) in glass jars (5 cm diameter and 7 cm height). The volume of dosified culture medium was 20 ml. The biphasic cultures were prepared in two receptacles: 1) test tubes with 3 ml of aqueous medium on a support of 15 glass pearls and 2) jars (5 cm diameter and 9 cm height) with 100 ml of aqueous phase on a 20 ml gellified support. The tubes were covered with reinforced aluminum paper and the culture jars with polycarbonate lids (Magenta®). Sterilization of the culture units with nutrient medium was carried out in an autoclave at 121 °C and 104 kPa.

The cultures with explants remained at 25 $\pm$ 5°C under 20 μmol photons m^{-2} s^{-1} for 16 h light:8 h night. Diffuse light equivalent to 1 μmol photons m^{-2} s^{-1} was used to germinate the seeds.

Experiments with leaves and rhizomes

Leaves and plagiotropic rhizomes were obtained from the ramets regenerated under laboratory conditions. The percentage of asepsia in the experiments with leaves and rhizomes was visually diagnosed by observing fungus and bacterial growth in the gellified culture medium. Viability was measured at three levels (0, 50 and 100 %) and was defined with respect to the conservation of color in the leaf and rhizome. The experiment lasted 14 days.

Experiment 1. A random factorial design with three factors and two levels was applied in order to define the effect of the sterilization stage on the asepsia and viability of the leaf and rhizome explants (Zar 1999). The factors included the type of explant (presterilised leaves and rhizomes), the dosis of NaClO (0.3 and 0.6 %) and the time of exposition to the chemical disinfectant (2.5 and 5 min). Each treatment presented five repetitions with a completely random distribution (Underwood 1997). Two 1 cm explants of the same type were placed in each experimental unit. The culture unit was monophasic and was prepared with semi-solid White medium at a pH of 7.5 with 58.4 mM sucrose. The exudates and white halos in the cultures were stained with Gram and the smear was observed with a microscope to identify the origin of the contamination. The analyzed samples were selected considering the type of contaminant, and were obtained from two cultures of leaves and two of rhizomes (10 % of the total).

Experiment 2. The lipidic contamination generated over the explants in experiment 1 was evaluated in the presterilization stage, and a culture medium with a random incomplete factorial arrangement with three factors and two levels (Zar 1999). A hypothesis was established to demonstrate that the asepsia and viability of the leaves and rhizomes were affected by the presence of exudates that were generated by the use of Tween in the aseptic procedure of *V. americana*, and that an anti-oxidant added to the culture medium might reduce this effect. The factors included the type of explant (leaf and rhizome), the presterilization with Tween 20® (0.0 and 0.1 %) and the addition of an anti-oxidant

(0.0 and 0.01 %) to the culture medium. The design included only five treatments with a completely random distribution (Underwood 1997). The anti-oxidant was ascorbic acid added in solid form to the sterile nutrient medium before gellification. From this experiment onwards, the sterilizing condition for these explants was fixed at 0.3 % NaClO for 2.5 min, and the general conditions of the explant cultures (size of explants, experimental unit, repetitions and culture medium) were similar to those of the first experiment.

Experiment 3. The viability and asepsia of the leaves and rhizomes were determined with respect to the composition of the culture medium. The White nutrient medium and the 0.5 MS with 0.88 x 10^{-3} mM of bencyladenine (BA) and 58.4 mM sucrose were compared in this experiment. BA was used to induce organogenesis (Ailstock *et al*. 1991). One 1.5 cm explant was placed in each receptacle. The other general culture conditions were not modified. Viability was determined through leaf regeneration.

Experiments with the caulinar apex

Caulinar apices were provided by the greenhouse plants, as the laboratory plants cultivated in the White medium died after approximately three months due to conditions that are analyzed in the discussion. Asepsia percentages were calculated visually as in the previous experiments. The percentage of viability was quantified through leaf regeneration, and the level of viability was quantified through the number of leaves produced.

Experiment 4. The asepsia and viability of caulinar apices were evaluated with a random unifactorial design (Zar 1999) that included doses of 0.0, 2.22 x 10^{-3}, 4.44 x 10^{-3} and 8.88 x 10^{-3} mM of BA in the culture medium. Sterilization was carried out in two phases to reduce the contamination of the explants. The apices were disinfected with 0.3 % NaClO for 1 min, washed in sterile water and disinfected a second time with 0.6 % for 10 min. The culture medium was 0.5 MS prepared with 29.2 mM sucrose in liquid form. A 1.5 cm explant was placed in each receptacle. The treatments prepared with five experimental units remained static for 14 days.

Seed experiments and evaluations with germination kinetics

The aseptic procedure followed in the experiments with seeds was reduced to the stages of sterilization and poststerilization with five washings. Sterilization was carried out with 0.6 % NaClO for 10 min.

Experiment 5. Aseptic seeds were germinated under the biphasic condition in test tubes with a completely random unifactorial design in order to obtain qualitatively healthy *in vitro* plants (Zar 1999). The studied factor was the aqueous phase, and its three levels were 0.5 MS, White and local potable water as culture media. The physicochemical characteristics of the local water (Table 1) indicated it was semi-hard (Anonymous 2000). The culture media were prepared with 29.2 mM sucrose and were adjusted to a pH of 6. Each treatment included five repetitions of the experimental unit distributed completely randomly (Underwood 1997). From 7 to 10 aseptically treated seeds were planted in each experimental unit. The seeds in the control group did not receive aseptic treatment and were cultivated in local sterile water without sucrose.

The effect of the treatments on the asepsia of the seeds and the quality of the regenerated material in the different aqueous treatments were recorded after 30 days. The criterium used to qualify the quality of the seedlings was the development of the whole plant (with coleoptile, at least one green leaf, and root growth).

Experiment 6. The germinative efficiency of *V. americana* in biphasic culture with gellified support was evaluated with local water with unchanged pH (Table 1). This aqueous medium was chosen as it provides low-cost plants of good quality. The design included culture time as a factor. Six repetitions of the experimental unit were carried out, each with 10 seeds that were distributed completely randomly (Underwood 1997). The seedlings were visually counted, considering the emergence of the coleoptile as the start of germination as the emergence of the radicle in this culture system is difficult to see. Germination was quantified on different days

Table 1. Physical and chemical parameters of local water in the experiments.
Tabla 1. Parámetros físicos y químicos de agua local en los experimentos.

Parameter	Measure
pH	6.95 units (APHA 1992, Method 4500)
N-NO_3	0.360 mg L^{-1} (APHA 1992, Method 4500)
N-NO_2	0.005 mg L^{-1} (APHA 1992, Method 4500)
Hardness	201.6 mg L^{-1} (APHA 1992, Method 2340)
Alcalinity	100 mg L^{-1} (APHA 1992, Method 2320)
Conductivity	310 μS cm^{-2} (APHA 1992, Method 2510)

during one month. Germination kinetics were obtained through the germination percentages.

Statistical analyses

Kolmogorov-Smirnov & Lilliefords tests were used to determine the normality of the data, and the Cochran C test was used for the heterogeneity of variances (Underwood 1997). Variability of data was estimated with an ANDEVA of principal effects (experiments 2 and 3), and with a one-way analysis (experiment 1, 4) in the experiments with vegetative explants (Zar 1999). Post-hoc comparisons were analyzed with a Fisher LSD test. Contamination with fungus and dry white colonies (experiment 1), asepsia (experiments 2 and 3), viability (experiments 3 and 4) and the level of pigmentation (experiment 3) measured in the corresponding experiments did not present a normal distribution due to the abundance of cero values. In consequence, these variables were analyzed with the Kruskall Wallis (H) test, except for the level of pigmentation for which the Mann-Whitney (U) test was used. Statistical significance was established at $p < 0.05$. All statistical tests were carried out with general lineal models in the Statistica software (Anonymous 2004).

RESULTS

Asepsia and viability of leaves and rhizomes

All the cultures of *V. americana* leaves treated with NaClO presented contamination and were not viable. In contrast, 60 % of the rhizomes were aseptic and survived with 0.3 % of NaClO 2.5 min. The two explants darkened in all the culture units. Contamination by fungus in leaves and rhizomes accounted for 30 and 35 %. Another 5 % presented dry white halo forming bacteria. The other 65 % of the leaves and 60 % of the rhizomes in the contaminated units presented mucilaginous translucid masses or exudates over the tissues. The microbiological analysis of the cultures with exudates and without mixed contamination showed only micelles. The dry white colonies were Gram negative.The explant, the concentration of chlorine and the time of exposition had no significant effect on the microbial contaminants that developed ($p > 0.05$, respectively).

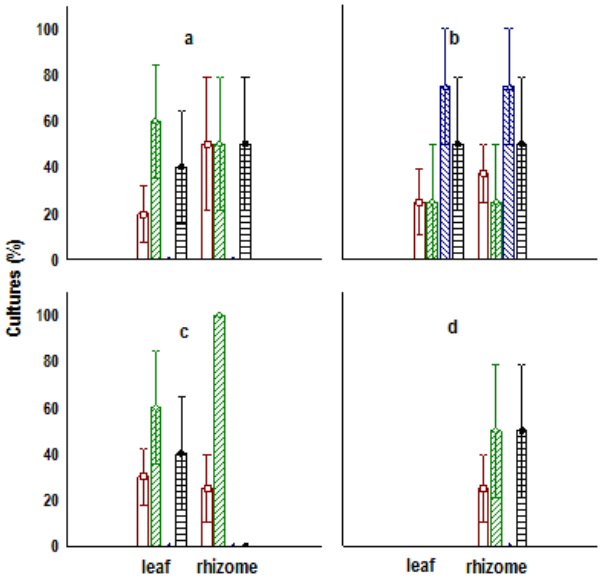

Figure 1. *Vallisneria americana*. Viability, asepsia, contamination with exudate and white halo, in leaf and rhizome cultures as a result of presterilization with Tween and of the addition of the antioxidant ascorbic acid in the White culture medium at a pH of 7.5. a) without Tween and without antioxidant, b) with Tween and without antioxidant, c) without Tween and with antioxidant, and d) with Tween and with antioxidant. Vertical bars = mean ± standard error. Viability ▫, asepsia ▨, exudate ▧, white halo ▣.
Figure 1. *Vallisneria americana*. Viabilidad, asepsia y contaminación por exudado y halo blanco en los cultivos de hojas y rizomas por efecto de la preesterización con Tween y la adición de antioxidante ácido ascórbico en el medio nutritivo de White a pH de 7.5. a) sin Tween y sin antioxidante; b) con Tween y sin antioxidante; c) sin Tween y con antioxidante; d) con Tween y con antioxidante. Barras verticales = promedio ± error estándar. Viabilidad ▫, asepsia ▨, exudado ▧, halo blanco ▣.

The contamination by exudates disappeared in the leaf and rhizome cultures that did not receive treatment with Tween ($F = 16.29$, $p < 0.05$)

(Figure 1). No difference for this result was observed in relation to the type of explant ($F = 0.63$, $p > 0.05$). Ascorbic acid significantly inhibited the formation of exudates ($F = 5.18$, $p < 0.05$). The independent variables explant, Tween and ascorbic acid did not significantly affect asepsia (explant: $H = 0.11$, Tween $H = 3.11$, ascorbic acid: $H = 2.25$; $p > 0.05$), viability (explant: $F = 0.9$; Tween: $F = 0.18$; ascorbic acid: $F = 0.43$; $p > 0.05$) or contamination by dry white colonies (explant: $F = 0.13$, Tween: $F = 0.65$, ascorbic acid: $F = 0.42$; $p > 0.05$). This experimental set did not present fungal contamination.

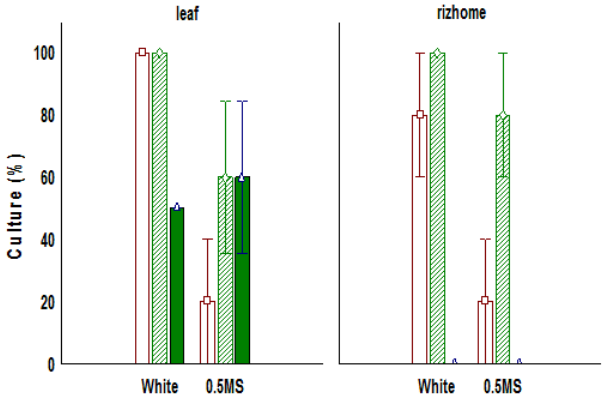

Figure 2. *Vallisneria americana*. Cultures of aseptic and viable leaves and rhizomes, and pigmentation level in leaves, in White medium and in 0.5 MS with 0.88 mM of bencyladenine (BA). Vertical bars = mean ± standard error. Asepsia ▫, viability ▨, pigmentation leaves ▲.
Figure 2. *Vallisneria americana*. Cultivos asépticos y viables de hojas y rizomas, y nivel de pigmentación en las hojas, en medio White y 0.5 MS con 0.88 mM de benciladenina (BA). Barras verticales = promedio ± error estándar. Asepsia ▫, viabilidad ▨, pigmentación de hojas ▲.

The leaf and rhizome cultures turned out to be more aseptic when cultivated in the White medium than in 0.5 MS ($H = 9.4$; $p < 0.05$), independently of the type of explant ($H = 1.92$; $p > 0.05$) (Figure 2). The culture media did not generate significant differences in the viability percentage of the explants ($H = 3.35$; $p > 0.05$) or in regard to the structure that was cultivated ($H = 0.37$; $p > 0.05$). However, in the leaf cultures, a green pigmentation was favored when the 0.5 MS medium was used ($U = 2.5$; $p < 0.05$). The addition of BA to both culture media did not have a regenerative effect on any explant.

Asepsia and regeneration in caulinar apices

No significant differences were recorded for the viability of the caulinar apices in the treatments with BA ($H = 0.76$; $p > 0.05$). All cultures turned turbid and 80 % were contaminated with fungus. The average total viability of the apices was 50 % and foliar regeneration was recorded for all BA concentrations. The cultures that were supplemented with 0.88×10^{-3} mM of BA presented a greater foliar regeneration ($F = 5.20$, $p < 0.05$) than the other three treatments (Figure 3). However, all the explants began to rot after 14 days and finally died due to the high contamination.

In vitro germination

The *in vitro* germination of *V. americana* in different aqueous media and in the control was established with no sign of contamination. Seedlings with a high visual quality were obtained in 0.5 MS and in local water (Figure 4). In contrast, seedlings in the White medium presented an arrested development after the emergence of the coleoptile (Figure 4). The control seedlings were of good quality except for a low foliar pigmentation. The maximum germination percentage of *V. americana* in biphasic culture with gellified support and local water was 97 % (Figure 5). Asepsia and seedling quality were comparable to the biphasic culture with glass pearls, as no microbial growth was recorded and whole plants developed with pigmented leaves.

DISCUSSION

The *in vitro* establishment of *V. americana* through vegetative explants presented complications due to excessive contamination, as has been recorded for other aquatic plants (Balestri *et al.* 1998; Jenks *et al.* 2000; García-Jiménez *et al.* 2006). The leaf and rhizome explants were contaminated mainly by exudates, and the caulinar apices by fungal and bacterial growth. The formation of exudates was a peculiar characteristic, and was apparently the main cause of the darkening of leaves and rhizomes. Ho-

wever, no bacterial cells were detected when the exudates of both explants were stained with Gram and observed microscopically. In contrast, the micellar or mucilaginous appearance presented evidence of the production of lipidic substances and opened the hypothesis of a masked asepsia with a probable effect on explant viability. Potentially allelopathic essential oils with a strong activity against algae and fungus have been identified in the leaves of *Vallisneria spiralis* (Qiming *et al*. 2006). Also, allelochemical auto-toxicity has been observed *in vitro* in aquatic plants (Ervin & Wetzel 1999).

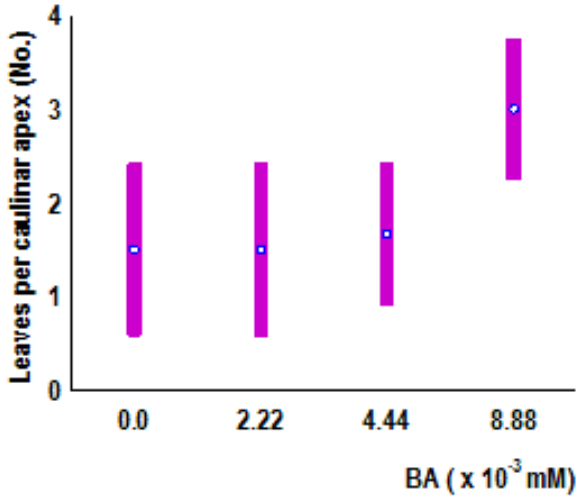

Figure 3. *Vallisneria americana*. Average of regenerated leaves in caulinar apices in response to bencyladenine level. The average (n = 5) includes aseptic and contaminated explants (ANOVA; p < 0.05). Vertical bars = mean ± standard error.
Figure 3. *Vallisneria americana*. Promedio de hojas regeneradas en ápices caulinares en respuesta al nivel de benciladenina. El promedio (n = 5) incluye explantes asépticos y contaminados (ANOVA p < 0.05). Barras verticales = promedio ± error estándar.

The disappearance of the exudates in the later cultures of *V. americana* leaves and rhizomes that were not presterilized with Tween during the aseptic procedure indicated: 1) that extracellular lipidic products caused the mortality of the explants, and 2) the need to omit the use of this surfactant with *V. americana*. The low aseptic efficiency and viability of explants obtained in this study coincided with data recorded for other macrophytes (Balestri *et al*. 1998; Jenks *et al*. 2000; García-Jiménez *et al*. 2006). The low efficiency obtained in experiment 1 is explained by the use of surfactants in the presterilization stage, as the presence of mucilaginous substances in aquatic macrophytes has been associated with excessive mechanical cleaning which modifies the quality and composition of excreted organic substances (Godmaire & Nalewajko 1986). The use of Tween is commonly recorded in presterilization and sterilization procedures. However its exclusion has not been explained (McCann *et al*. 2000).

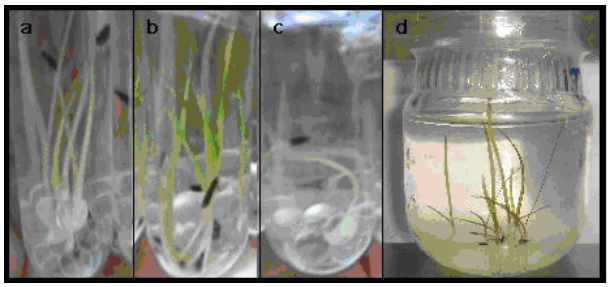

Figure 4. *Vallisneria americana*. Seedlings in biphasic cultures: a) 0.5 MS: glass pearls, b) local water: glass pearls, c) White: glass pearlsand d) local water: agar.
Figure 4. *Vallisneria americana*. Germinados en cultivos bifásicos: a) 0.5 MS: perlas de vidrio, b) agua local: perlas de vidrio, c) White: perlas de vidrio y d) agua local: agar.

The addition of an antioxidant reduced the exudate on the leaves and roots cultivated in experiment 2. Antioxidant agents, such as ascorbic acid, modify the redox potential in the culture medium, as they donate electrons that inhibit the oxidation of labile substrates (George 1996). Ascorbic acid and other antioxidants inhibited senescence of *V. spiralis* leaves by increasing chlorophyll and protein content that retarded the decrease of Hill activity (Sasadhar & Choudhuri 1987). However, the exclusion of presterilization with Tween in the leaf and rhizome explants (experiment 2) proved that it is not necessary to add an antioxidant if the surfactant Tween is not included in the procedure. This result made it possible to modify the procedure of the culture in gellified nutrient media (experiment 3), as both explants became established with aseptic efficiency and without darkening (experiment 4).

The leaves and rhizomes that were cultivated heterotrophically in monophasic nutrient media with BA (experiment 3) showed no signs of regeneration,

although the viability of the leaves increased in the MS medium, together with an increase in pigmentation. In contrast, the caulinar apex showed their

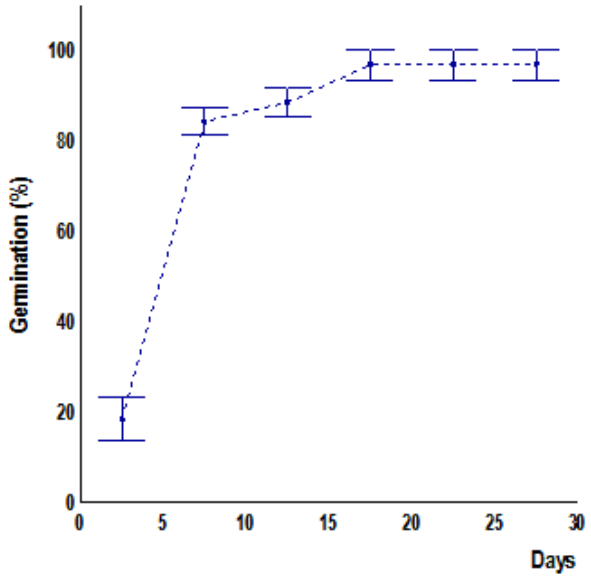

Figure 5. *Vallisneria americana*. Germination kinetics in biphasic culture (in aqueous medium and in gellified support with 29.2 mM sucrose). Vertical bars = mean ± standard error.
Figure 5. *Vallisneria americana*. Cinética de germinación en cultivo bifásico (en medio acuoso y soporte gelificado con 29.2 mM de sacarosa). Barras verticales = promedio ± error estándar.

potential to form new leaves in spite of the turbidity and the mass of fungus that developed. The foliar organogenesis in the apex of the plagiotropic rhizome of *Cymodocea nodosa* was favored only by TDZ (*N*-phenyl-*N*'-1,2,3-thidiazol-5-ylurea) through the formation of a callus. However, when the structure was a ramet, the regeneration and foliar extension of a leaf occurred with the phytoregulators GA (giberellic acid), BA, KIN (kinetin) and TDZ (García-Jiménez *et al*. 2006). It was experimentally demonstrated that the use of the whole ramet of *C. nodosa* was a necessary requirement for carbon fixation, its translocation to the rest of the tissue and the later stimulus in the process of foliar development (García-Jiménez *et al*. 2006). Similarly to *C. nodosa*, the caulinar apices of *V. americana* cultivated in experiment 4 were the most adequate structures to regenerate leaves *in vitro*, although the cultures were not completely aseptic. In agreement with results published by Bird *et al*. (1998) and García-Jiménez *et al*. (2006), it is probable that the *in vitro* morphogenesis of the leaves of some submerged species present a greater genetic (division and cellular expansion) and environmental (light, temperature and nutrients) control. Foliar regeneration in the caulinar apices of *V. americana* was favored with 8.88 x 10^{-3} mM of BA. Similar doses have been used to regenerate and multiply other submerged aquatic species (Bird *et al*. 1998; Ailstock *et al*. 1991; García-Jiménez *et al*. 2006).

The development of *V. americana* cultures via vegetative explants was considered a priority in this study as this leads to the propagation of more genetically homogeneous plants and to a lower experimental error in the bioassays, considering that the genetic plasticity of aquatic plants has been cited as a problem for their use in bioassays (Mohan & Hossetti 1999). Also, the sexual route to provide submerged plants for bioassays is limited as a result of the availability of pollen and seeds (Sullivan & Titus 1996; Ke & Li 2006). The establishment of fruit in natural populations of *V. americana* is also limited by the availability of pollen and this, in turn, is affected by water depth, exposure to air and waves, and surface current speed (Sullivan & Titus 1996). The acquisition of a sufficient amount of *V. natans* seeds has restricted its success as an alternative method for the restoration of Chinese lakes (Ke & Li 2006), although submerged aquatic vegetation in general boasts a relatively successful reproductive process (Hemminga & Duarte 2000; Ke & Li 2006). The species *Ceratophyllum demersum* and *Halophila decipiens*, both cultivated *in vitro*, have been recorded with low germination frequencies (Wyman & Francko 1986; Bird *et al*. 1998).

In order to direct the *in vitro* culture of *V. americana* towards the development of ecophysiological or ecotoxicological experiments, it is fundamental to reproduce the natural submerged life conditions of the species. The *in vitro* culture of mature seeds was developed in a biphasic system with natural water or with a culture medium and glass pearl support considering the above statement. The seedlings in the biphasic culture with natural water and 0.5 MS were of good quality and there were no

rhizospheric bacteria or microalgae. The efficiency of the system with respect to germination percentage was demonstrated when this was replaced by an agar layer to obtain the aseptic biphasic germination of *V. americana* in local water, and this was comparable to *V. natans* cultivated in non- aseptic, aerobic and light environment without the effect of the depth in boreal areas (Ke & Li 2006). The rooting of seedlings is also considered important in the growth of aquatic grasses (Bird *et al*. 1998).

The recommendation made by Uma & Mohan-Ram (1972) of using the White medium to cultivate *V. spiralis* seeds could not be reproduced in this study for the leaf and rhizome explants, nor for the seedlings or the stock of laboratory plants of *V. americana*. The White nutrient medium was prepared at a pH of 7.5 in this study, as this is the pH value recorded in Pantanos de Centla (Sánchez *et al*. 2007). The natural pH value is a basic factor in the procedure to cultivate *V. americana*. However, inorganic and organic components in culture media may modify the pH and affect the *in vitro* growth and propagation of plants (Owen *et al*. 1991). The White medium, compared with other better known media, is the formula with the lowest buffer capacity (Owen *et al*. 1991). According to Titus & Hoover (1993), the reproductive processes of *V. americana* in greenhouse conditions were affected at a pH of 5 as, under these conditions of acidity, the plants produced from seed did not flower, the rhizomes presented a lower biomass, they were different from those in the field, and they developed as very small plants. Nutrient enrichment has also represented an obstacle to micropropagate submerged marine vegetation (Balestri *et al*. 1998; Touchette & Boulkoldher 2002). Seedlings of the aquatic grass *Halophila decipiens* were found to be not viable in nitrate concentrations above 1.7 mM (Bird *et al*. 1998).

The *in vitro* germination in the biphasic system with an aqueous phase of local water or 0.5 MS produced healthy and rooted plants that are considered ideal for biological assays. In accordance with the results, the sterility of the seeds had a positive effect on germination, except when they were cultivated in the White medium. The hardness and alcalinity of the culture media may modify the speciation of ions, which favors the germination process or increases toxic effects (Owen *et al*. 1991).

Seeds were selected as candidates to obtain *in vitro* plants of *V. americana*, as the prevailing contamination in the three vegetative structures prevented their use as culture material. The genetic variability particular to the seeds is important in ecophysiological bioassays (Bird *et al*. 1998, Balestri & Bertini 2003). However, the *in vitro* production of plants from seeds has the inconvenience of depending on a natural seasonal and unpredictable supply, as has been frequently recorded for submerged aquatic vegetation (Zhou *et al*. 2006). The inter-annual availability of seeds of the *V. americana* populations distributed in Pantanos de Centla is very variable and unpredictable.

The choice of a culture medium is important for *in vitro* regeneration or multiplication (Kane *et al*. 2002). The MS medium at half ionic strength has been frequently used, and other aquatic plants have been micropropagated successfully (Jenks *et al*. 2000; Kane *et al*. 2002). In the case of *V. americana*, this nutrient medium did not affect the asepsia and viability of the seedlings, and it improved only the viability in the case of leaves and caulinar apices. Both results represent new possibilities for the *in vitro* clonation of *V. americana* in the MS medium, as an opportunity is opened to develop systems with fewer sources of contamination, extracting vegetative explants from aseptic seedlings.

Notwithstanding that the biphasic culture of *V. americana* with seeds in the MS medium was feasible (experiment 5), the model of *in vitro* plants with local water is even more advisable as its lower cost and simplicity present greater advantages for the development of ecophysiological and ecotoxicological tests with *V. americana*. The procedure of culture in water may also be reproduced and standardized if hard reconstituted local water is used in the development of tests under controlled conditions. This *in vitro* model of *V. americana* makes it possible to develop experimental studies to evaluate the factors that negatively affect its populations in Pantanos de Centla and other ecosystems. At present, the biphasic system with reconstituted water has been useful in starting *in vitro* tests with *V.*

americana to determine tolerance to different ionic nitrogenated environments (Ruiz-Carrera, unpublished data).

The proposal to use alternative methods that include procedures with more natural *in vitro* cultures, with less stages and chemical substances, and with lower costs generates possibilities to prove hypotheses that will provide information to support the operation of programmes of wetland management where aquatic vegetation is declining or disappearing. This procedure offers this option for the *V. americana* populations in Pantanos de Centla or in any other ecosystem within its wide distribution from North America to Central America (Korschgen *et al.* 1988; Ellison 2004).

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