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ORIGINAL ARTICLE

In vitro propagation and shoot encapsulation as tools for *ex situ* conservation of the aquatic plant *Ludwigia palustris* (L.) Ell.

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Abstract
Ludwigia palustris (L.) Ell. is an aquatic perennial herb present in several regions of Italy, which is one of its native countries. In this research, micropropagation and encapsulation protocols were established from axillary buds of *L. palustris*. Shoots proliferated on half-strength Murashige and Skoog medium without growth regulators. Different culture vessels were tested. Shoots in GROWTEK bioreactor showed the highest fresh and dry weight and total length while the plantlets grown in the RITA bioreactor showed the highest shoot number per explant. Encapsulation of *L. palustris* microcuttings with sodium alginate formed small and whitish beads which were stored for 14 or 28 days at 7° or 25°C. Storage for 14 days at both temperatures gave the best results but prolonged storage at 25°C decreased the shoot viability to 73%. After 4 weeks of recovery, all the plantlets showed the typical features of the species. Even though the latest Italian IUCN Red List does not mention *L. palustris*, conservation measures are proposed at local level because this species locally remains vulnerable mainly due to the loss of adequate habitats.Our protocol could be one of the methods for *ex situ* conservation of *L. palustris* particularly because its seed storage behavior is uncertain.

Keywords: *Bioreactor, culture vessel, encapsulation, marshes, micropropagation, storage*

Introduction

Ludwigia palustris (L.) Ell. belongs to the family *Onagraceae* and is commonly named "water purslane" and "marsh seedbox". It is a little aquatic or amphibian perennial herb. It spreads to form mats on the mud, rooting at nodes in contact with the substrate, or floats in the water where it grows very rapidly up to half a meter long. *Ludwigia* species are morphologically very similar and are difficult to differentiate in the absence of flowers. Some of these are used as ornamental aquarium plants, vegetables, medicines, and aquatic fauna feed. *L. repens*, often ascribed as the synonym of *L. palustris* according to The Plant List (2010), is used for filtration and cleaning of water in canals and lakes (Greenway & Wooley 1999; Öztürk et al. 2004). *L. palustris* makes a perfect plant for aquatic plant hobbyists, and recently, it has been observed that it can offer interesting perspectives for its quality in removing metals, principally mercury, from contaminated waters (Marchand et al. 2010).

The IUCN Red List (Lansdown 2013) reports the native countries for *L. palustris*, including Italy, and the global distribution range of this species, which can be found in western, central, and southern Europe, North Africa, western Asia, and North America. This species is classed as least concern; it is widespread with stable populations throughout most of its European range and does not face any major threats (Bilz et al. 2011; Lansdown 2013). However, it is classed as critically endangered at the national level in Switzerland and Germany, data deficient in Croatia, protected in France, and included in the Red List in Israel (Lansdown 2013).

Its distribution in Italy is mainly located in the northern and central regions (Abbate et al. 2005). Although the latest Italian IUCN Red List (Rossi et al. 2013) does not mention this species, *L. palustris* is locally rare and requires particular protection

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as cited in the Annex A of the Tuscan Regional Law (L.R. n. 56, 2000), and by the Italian Red and Blue List (Pignatti et al. 2001). *L. palustris* was first classified by Linneus in 1753 as *Isnardia palustris* (The International Plant Names Index 2012). The presence of *Isnardia palustris* in Tuscany was documented in the past by Caruel (1860) who described it as abundant in the ditches and marshes near the sea. A manuscript of 1763 (Bernardi et al. 1980) proved the presence of this species in the *Marshes of Fucecchio* (Pistoia, Italy), the largest inland marsh in Italy, today a Regional Nature Reserve. Now conservation measures are proposed at local level because this species locally remains vulnerable mainly due to the loss of adequate habitats (Felicioni & Zarri 2007; Bartolini 2010; Rapetti & Tomei 2010). Worldwide wetlands, such as the Italian ones, are ecosystems strongly influenced by human activities and most of the plants linked to freshwater wetlands are rare, vulnerable, or endangered (Bagella et al. 2013). The situation is so critical that, to avoid the imminent extinction of many aquatic plants, it is necessary to carry out *ex situ* conservation and restoration programs. *In vitro* propagation is one of the methods used for *ex situ* conservation: it is useful in fact to propagate plants whose seeds are recalcitrant, or cannot be dried and stored at low temperatures (Sharrock 2012). These techniques are helpful for recovery programs and for the establishment of "backup collections" of germplasms of endangered species (Pace et al. 2004; Lucchesini et al. 2010; Bhatt et al. 2012), especially for many aquatic plants whose propagation is frequently hindered in the wild (Yapabandara & Ranasinghe 2006; Pence et al. 2007; Rolli et al. 2013). *In vitro* cultures can also be used for *ex situ* storage as slow-growth cultures by maintaining them generally at low temperatures and delaying the subcultures (Negash et al. 2001; Keller et al. 2006). *In vitro* propagation may be combined with the encapsulation technology; alginate encapsulation has two main potential applications: (a) production of encapsulated somatic embryos (synthetic seeds) which maintain the ability to convert to whole plants; (b) production of encapsulated vegetative propagules (capsules) from *in vitro* cultures. In particular, encapsulation of apical shoot buds, nodal segments (microcuttings), and so on offers an efficient and cost-effective system for clonal propagation of plant species and could be used as synthetic seeds for restoration purposes and for the exchange of axenic plant material between laboratories (Reed 2004; Malek 2009; Rai et al. 2009; Engelmann 2011). There are few studies concerning the production of alginate capsules of wetland species to improve their *in vitro* propagation (Rogers 2003; Sarasan et al. 2006; Oh et al. 2010).

L. palustris can be propagated by sprigs but not easily by seeds (USDA, NRCS 2013). In fact, Thompson et al. (1997) reported that seeds of *L. palustris* are short-lived under ambient conditions and can persist in the soil for less than 1 year. Although further experimental data are necessary to certainly define the storage behavior of the taxon, it can be reliably classified recalcitrant or intermediate (SID 2008). Therefore, *in vitro* techniques might be very useful for this species but there is only one report concerning an *in vitro* micropropagation protocol for this genus starting from apical and axillary shoots (Öztürk et al. 2004).

The purposes of the present research were (a) to determine a rapid, simple, and efficient micropropagation hormone-free protocol from axillary buds of *L. palustris* and (b) to verify the possibility of encapsulating vegetative micro-shoots of *L. palustris* and then assessing the subsequent shoot survival, their recovery, and finally their proliferation and rooting abilities.

Materials and methods

Micropropagation

Some *L. palustris* cuttings were collected from the wild habitat named *Paduletta di Ramone* located in the *Fucecchio Marshes* (Provincial Nature Reserve of Pistoia, Italy) and transferred into a growth chamber at the Department of Agriculture, Food, and Environment, University of Pisa, Italy. Cuttings were cultured in pots filled with wet sand. This allowed to increase the availability of healthy plant material to begin the *in vitro* culture (Stage 0). After the cuttings had rooted and new stems had developed, some apical and nodal segments were removed. Leaves were eliminated to obtain stem explants. These were preliminarily washed first with distilled water containing – two to three drops of Tween 20 for about 30 min and continuous stirring, then with sterile water. Under laminar flow cabinet, the explants were subsequently submerged for 10 min in 15% NaOCl (8% of chlorine active) and then they were rinsed three times with sterile water and were excised to 1-cm long segments containing – one to two buds. Each segment was individually cultured in polycarbonate vials with 5 ml of a solid culture medium for 4 weeks. The substrate, named LUD medium, consisted of half-strength MS salts and vitamins (Murashige & Skoog 1962), 30 $g\,l^{-1}$ sucrose, 300 $mg\,l^{-1}$ reduced glutathione (GSH) as the anti-oxidant agent, 500 $mg\,l^{-1}$ 2-(*N*-morpholino)ethanesulfonic acid (MES) to stabilize the pH, adjusted to 5.6, and 2 $g\,l^{-1}$ Gelrite® (Duchefa Biochemie B.V., Haarlem, The Netherlands). The medium was autoclaved (20 min at 121°C) for

sterilization. All cultures were incubated at 25 ± 1°C under cool white fluorescent light (70 μmol s^{-1} m^{-2} photon flux density) with a 16-h photoperiod. Subsequently, when the shoot culture was established, the best *in vitro* condition was tested using four different vessels. Two of them contained the solid medium: Plant Culture Containers vented PCCV25 (Ø 70 mm × h 90 mm, volume 141.75 ml; TQPL Co., New Milton, UK) with 9 explants/vessel; Microbox Eco2 (Duchefa, Micropoli, Italy; 80 mm × 125 mm × 65 mm, volume 650 ml) with 15 explants/vessel. The other two vessels contained the liquid medium: GROWTEK™ (Ø 100 mm × *h* 150 mm, volume 1178 ml; Scienceware®, Bel-Art Products, Wayne, NJ, USA) with 25 explants/vessel; RITA® temporary immersion system (Ø 130 mm × *h* 150 mm, volume 980 ml, Vitropic, Saint-Mathieu-de-Tréviers, France) with 25 explants/vessel. Shoot growth rate and multiplication parameters (fresh and dry weight and mean shoot length, number of new shoots and their length) were recorded at the end of the cultivation period (fourth week). Three vessels per each vessel type were used and the results were analyzed using one-way analysis of variance (ANOVA), and the mean values were separated by Tukey's test ($p < 0.05$).

To assess the acclimatization process, well-developed plantlets (5 plantlets/vessel) were transferred in polycarbonate boxes (107 mm × 107 mm × 96 mm) (Sigma-Aldrich®, Milano, Italy) equipped with ventilation filters formed by an autoclavable polypropylene membrane (Ø 40 mm, Ø pores 0.3 mm) on the cover. The boxes contained 100 ml of aquarium sand and 50 ml of a nutritive solution that consisted of MS/3 mineral salts at pH 5.6. The cultures were maintained in the growth chamber in the same conditions described above. After 7 days, the vessel caps were raised slightly to favor air circulation, maintaining at the same time a high humidity inside the vessels. After another 4-day period, the caps were removed permanently. A week later, the plants were transferred to a greenhouse under natural light and temperature conditions during spring time. At this stage, the plants with the aquarium sand were placed in a floating system consisted of a nonwoven porous sheet suspended in polyethylene boxes (1100 ml) containing the same nutritive solution used in the stage described above. To ensure an oxygen content of about 6.0 mg l^{-1}, the boxes were kept constantly aerated with an air pump.

Shoot encapsulation

The plant material for the experiment was obtained from 4 weeks *in vitro* proliferated *L. palustris* shoots on LUD medium. At the end of the subculture period, the *in vitro* proliferated shoots were separated into nodal portions (3–4 mm long), without leaves with two axillary buds. Before encapsulation, the explants were cut again 1 or 2 mm each side of a node. To provide nutrients to the explants during the storage period, a calcium-free LUD medium was prepared. For encapsulation, sodium alginate solutions were used at concentrations of 25 g l^{-1} and 30 g l^{-1}. The alginate-coated uninodal explants were dropped with a pipette in a complexing solution containing LUD medium added with $CaCl_2$ (1.1%, w/v). The pH of all media and solutions used in the experiment was adjusted to 5.6, and the media and solutions were autoclaved at 121°C for 20 min. For polymerization of sodium alginate, the capsules were held in the complexing solution for about 30 min under continuous stirring. After polymerization, the hardened alginate capsules were gently rinsed twice, for 15 min each time, with sterile water and next were placed in Petri dishes (Ø 50 mm) with a sterile filter paper inside to eliminate water excess.

The encapsulated nodal explants were stored in Petri dishes (Ø 50 mm, ≥ 20 capsules/dish) with 5–6 ml of the LUD liquid medium without sucrose, to maintain the relative humidity inside the dishes during the storage period. One dish represented an experimental unit and five dishes were maintained for each storage period. The dishes were stored in the following conditions:

(A). 14 days storage at cold temperature of 7 ± 1°C;
(B). 14 days storage at room temperature of 25 ± 1°C;
(C). 28 days storage at cold temperature of 7 ± 1°C;
(D). 28 days storage at room temperature of 25 ± 1°C;
0 Control (0 days storage) (sowing of capsules immediately after encapsulation).

The aseptic capsules were maintained in the dark. At the end of the storage periods, capsules were sown in microboxes Eco2 containing the LUD propagation medium (21 capsules per vessel, 4 vessels per each treatment). Then, the cultures were maintained in the growth room in the same conditions of the micropropagation stages. After 4 weeks, the values of the following growth parameters were recorded: viability (green appearance, lack of necrosis), sprouting (shoots at least 5 mm long), shoots per capsule (number of shoots produced), length of the shoots produced, potential multiplication rate (number of nodes of proliferated shoot per capsule), fresh weight, dry weight, and rooting percentage. The experimental design was completely randomized. Data were subjected to two-way ANOVA, with means separated using Bonferroni multiple range test

within each temperature tested. The percentage conversions into angular values were performed before statistic analysis.

Results and discussion

Micropropagation

Before the sterilization of *L. palustris* explants, a standard procedure, named Stage 0 (Debergh & Maene 1984), was carried out, which consisted in maintaining the mother plant in a protected environment for several weeks. This stage allowed to increase the availability of *L. palustris* explants to begin the *in vitro* culture. The percentage of the detected contamination was 22.6 ± 7.8 but the uncontaminated explants on LUD medium appeared robust and deep green colored with a mean shoot number and length of 2 ± 0.4 and 1.5 ± 0.15 cm, respectively. They showed elongated internodes and primary roots and, sometimes, secondary ones; in addition, there was no callus formation at shoot bases (Figure 1). LUD medium contained half of the salts present in the MS formulation. A medium formulation was used with a reduced salt concentration on the basis of the attitude of aquatic plants to grow in oligotrophic environments. The use of growth regulators in preliminary experiments (Mensuali-Sodi et al. 2011) did not improve the multiplication rate and the quality of *L. palustris* plantlets (data not shown). In this work, LUD medium did not contain growth regulators: generally the use of culture media with reduced or no growth regulators is adopted to avoid somaclonal variation. In the case of *Ludwigia palustris*, which will be reintroduced in the wild, this procedure is strongly recommended. Moreover, as reported by Öztürk et al. (2004) on *L. repens*, the use of high levels of growth regulators during the induction and multiplication phases caused a pronounced inhibition or suppression of the elongation and growth of the shoots after 2–3 weeks in culture, hindering also the rhizogenesis during the next micropropagation phase.

The subsequent experiment had the aim to define the most suitable micro-environment for the multiplication of *L. palustris*, using different types of vessels filled with the solid or liquid medium (Figures 2–4). Figure 5 shows the average values of the growth parameters measured after one culture cycle (4 weeks). The percentage of rooting is not shown because in all experiments 100% of the explants rooted, forming a root system consisting of primary and secondary roots. Also, the formation of aerial roots from the stem nodes was observed, as it happens *in vivo*. As regards the growth parameters, shoots obtained from the liquid cultures in the vessels GROWTEK showed the highest fresh and dry weight and total length of plants in comparison with all the others. In addition, the plantlets growing in the RITA system showed the highest shoot number per explant. No clear effects were observed in the length of the newly formed shoots. These results might suggest that the plants were affected by the different micro-environments: the cultures in GROWTEK vessel developed mainly the principal shoots being constantly in contact with the substrate; the cultures in the RITA system, not being constantly in contact with the substrate, demonstrated to be under a sort of water stress as shown by the fresh and

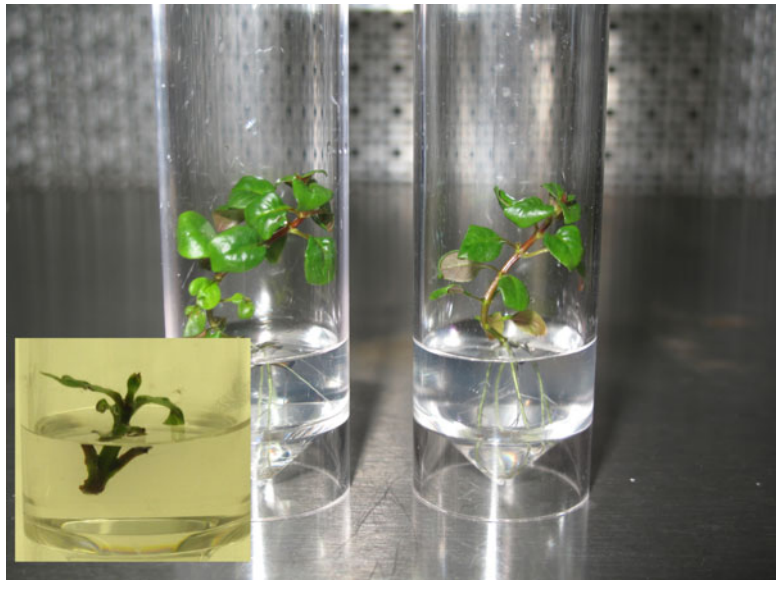

Figure 1. *L. palustris* on agarized LUD medium (modified half-strength MS salts and vitamins) during the induction phase (box) and at the end of the micropropagation process.

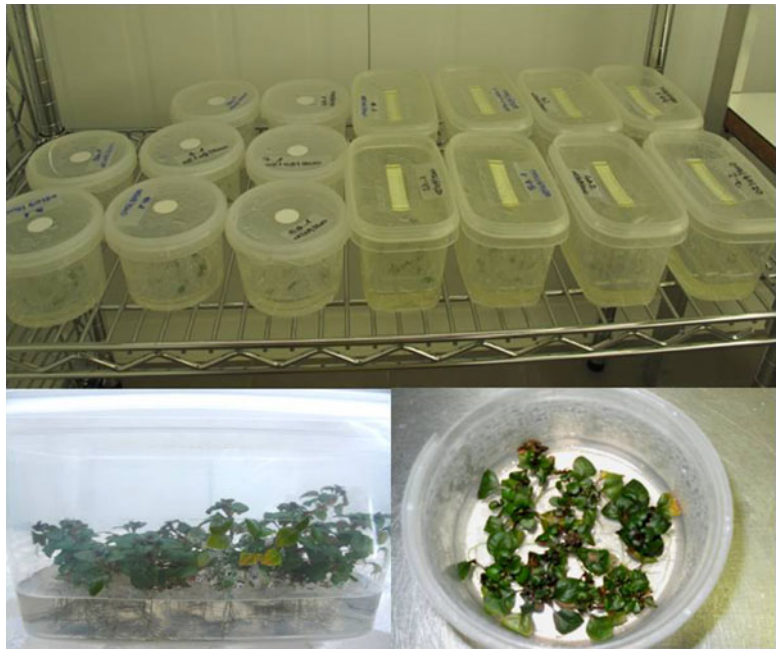

Figure 2. *L. palustris* growing on agarized LUD medium into Plant Culture Containers vented (PCCV25) and Microbox Eco2 during the multiplication phase.

dry weight values (Figure 5). Summarizing, taking into account the overall plantlets features and the analysis of all the growth parameters, the GROW-TEK vessels were the more suitable *in vitro* culture system for the species *L. palustris*. This bioreactor permits a constant feeding supply in comparison with the conventional gelled media or with a temporary nutrient supply (Dey 2005). Moreover, comparing the type of culture on solid and liquid substrates, it is evident that the scale-up of *L. palustris* propagation, being this species a hydrophyte, is better established in a semi-submerged liquid system.

Shoot encapsulation

This experiment had the aim of verifying the effect of different storage conditions of encapsulated explants on their regrowth and proliferation. A preliminary experiment tested two sodium alginate concentrations in the matrix, 25 and 30 $g l^{-1}$. Sodium alginate and calcium chloride play an important role in gel matrix formation and gel physical dehydration and bead hardiness depends upon optimal ion exchange of Na^{+} and Ca^{2+} (Singh et al. 2006a). The alginate-encapsulated nodal segments of *L. palustris* formed small and whitish beads with an average diameter of 3–5 mm. The capsules obtained using 30 $g l^{-1}$ sodium alginate were firm, clear, and isodiametric (Figure 6(A)) whereas a lower concentration of sodium alginate (25 $g l^{-1}$) not only prolonged the polymerization time, but also resulted in fragile and irregularly shaped beads which were difficult to handle. Similar observations on the effect of the sodium alginate concentration were also made in other species (Naik & Chand 2006; Singh et al. 2006a, 2006b, 2009; Rai et al. 2008; Hegazi 2011).

The composition of the gel matrix was based on previous studies demonstrating that gelling matrix

Figure 3. *L. palustris* growing on liquid LUD medium into GROWTEK™ bioreactors during the multiplication phase.

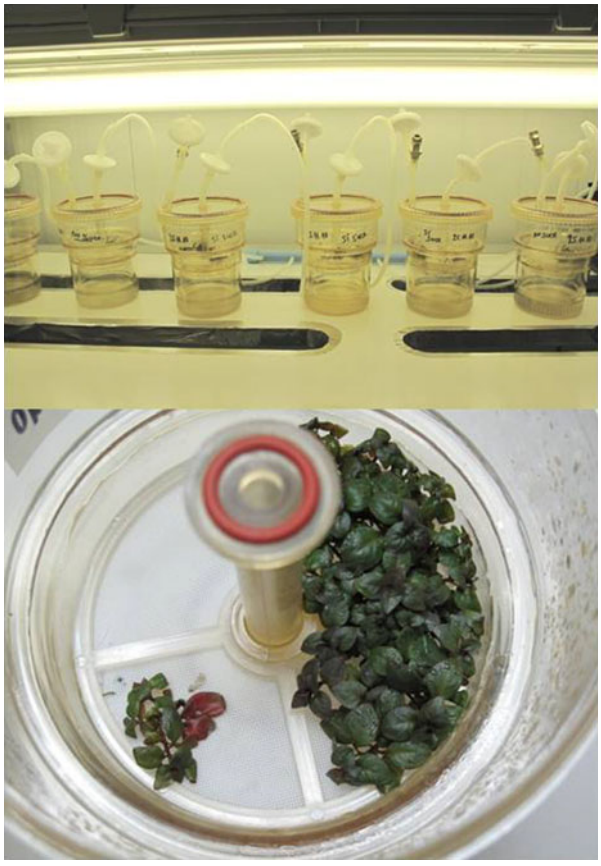

Figure 4. *L. palustris* growing on liquid LUD medium into RITA® temporary immersion system during the multiplication phase.

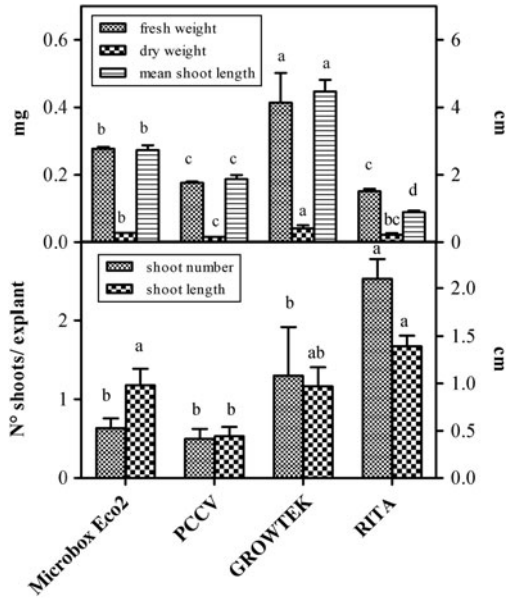

Figure 5. Mean values ± SE of the shoot growth (fresh and dry weight and mean shoot length) and multiplication parameters (number of new shoot and their length) recorded at the end of the cultivation period (fourth week) on *L. palustris* shoots cultured in different types of vessel: microbox Eco2, PCCV, Growtek bioreactor and RITA systems. Differences between means were analyzed using one-way ANOVA separated by Tukey's test ($p < 0.05$). Different letters indicate statistically different values ($p \leq 0.05$).

supplemented with nutrient ingredients (nutrient medium salts, sugars, and growth regulators) served as an "artificial endosperm" which provided nutrients to the encapsulated propagules during their recovery and directly affected the efficiency and practical applicability of the technique (Sarkar & Naik 1997; Singh et al. 2006a, 2009, 2010; Tsvetkov et al. 2006; Micheli et al. 2007; Kumar et al. 2010; Verma et al. 2010; Hegazi 2011). In the present work, an artificial endosperm consisting of LUD medium with 3% of sucrose was chosen. The storage in plastic Petri dishes, along with a small amount of nutritive solution without sucrose, was useful to maintain high humidity and resulted to be an essential strategy for the retention of viability of the encapsulated microcuttings (Micheli et al. 2007; Rai et al. 2008). The present work confirmed that the use of minimal growing medium lacking sucrose was fundamental to prevent the shoot emergence from alginate beads during the storage period. After this phase, the beads were cultured on the agarized LUD medium to evaluate their regrowth (Figure 6(B)–(D)). After 1 week of culture on this medium, the encapsulated nodal explants exhibited the shoot development whereas the roots were visible after 3–4 weeks. Similar observations were reported by Verma et al. (2010) on *Solanum nigrum* and by Ray and Bhattacharya (2010) on *Eclipta alba*. Two-way ANOVA analysis was carried out considering the time and temperature effects on several parameters recorded 4 weeks after transferring the beads on the LUD medium for recovery (Table I). As regards the shoot viability percentage, there was a significant interaction between the storage period and temperature: only when the beads were conserved at 25°C, the storage of 28 days decreased the shoot viability. No interactions were observed in all the other parameters recorded: the longer storage period gave extremely significant negative effects both on the number of new shoots formed from the nodal explants and on their sprouting percentage, whereas their length and potential multiplication rate (expressed as the number of the shoot nodes per each spherule) were similar in all the samples measured.

Low temperature and high humidity were essential conditions for retention of viability of the encapsulated shoot tips (Hegazi 2011). However in some species, a low-temperature storage (4°C) prolonged up to 30 days caused a drastical loss of viability (Singh et al. 2006a, 2006b; Rai et al. 2008) probably due to low respiration rates of the encapsulated plant tissues (Kavyashree et al. 2006). On the basis of these reports, half of the beads were maintained at a temperature of 7°C. This procedure

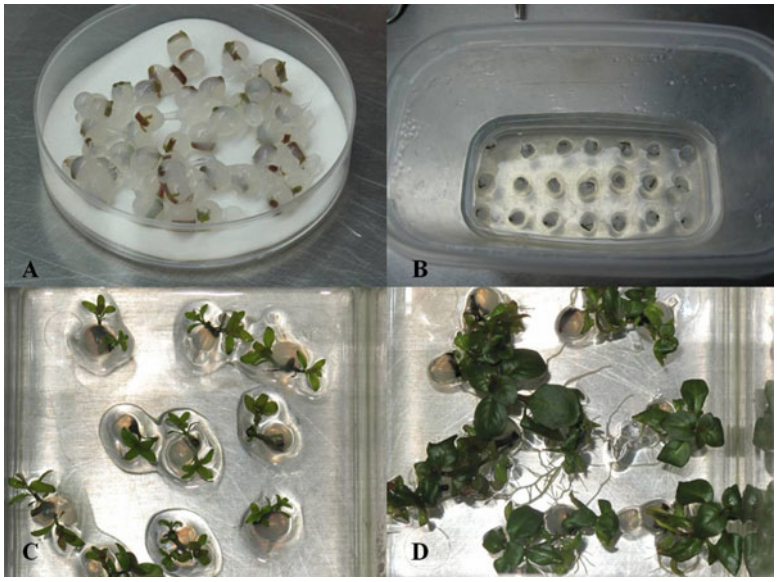

Figure 6. Alginate-coated *L. palustris* uninodal explants during the desiccation process (A), after their sowing on LUD medium (B), during the shoot sprouting (C), and after the complete recovery of the plantlets (D).

permitted to maintain the maximum shoot viability during the 4 weeks of culture. The other alginate spheres were stored at room temperature (25°C), but a slight decline in the shoot viability after the 14th day was observed. Probably, this temperature combined with the presence of sucrose into the alginate beads speeded the metabolic processes of the microcuttings causing progressive tissue deterioration. Slightly lower temperatures did not compromise the encapsulated tissue; in fact, it was reported that spherules of olive microcuttings maintained at 18°C (Micheli et al. 2007) showed high viability percentages when cultured for their recovery and propagation.

As regards the fresh weight accumulation, shoots coming from the 28-day period at 7°C reduced the water retention in comparison with the shoots maintained at the same temperature for 14 days. No differences were observed on dry matter accumulation between the treatments. After the storage periods, *L. palustris* shoots at the second week in culture showed curled light green leaves. Successively, at the 4th week of culture, all the plantlets recovered the typical features achieving a more intense color with developed leaves and showed analogous potential multiplication rate (Table I). In addition, after sowing, a recurrent outgrowth of both axillary shoots from each node was observed (Figure 6(C)). This phenomenon was previously observed on synthetic seeds of *Olea europea* L. (Micheli et al. 2007). This could be due to the alginate matrix which, covering the whole body

Table I. Average values of the growth parameters of *L. palustris* shoot after 4 weeks recovery from synthetic capsules stored at 25 and 7°C for 0, 14, and 28 days.

Temperature (°C)	Storage (days)	Viability[1] (%)	Sprouting[2] (%)	Shoots/bead[3] (n)	Shoot length (cm)	PMR[4] (n)	FW (g)[5]	DW (g)[6]	Rooting (%)
–	0	97.5 a	90.0 a	1.34 a	10.69 a	5.24 a	0.22 ab	0.013 a	70.0 a
25	14	97.5 a	97.5 a	1.22 ab	11.78 a	4.53 a	0.20 ab	0.011 a	100 a
25	28	73.0 b	50.0 b	0.62 b	8.83 a	4.74 a	0.15 ab	0.008 a	88.0 a
–	0	97.5 a	90.0 a	1.34 a	10.69 a	5.24 a	0.22 ab	0.013 a	70.0 a
7	14	100 a	100 a	1.76 a	11.20 a	5.54 a	0.23 a	0.013 a	100 a
7	28	97.5 a	47.5 b	0.67 b	7.69 a	4.05 a	0.14 b	0.008 a	80.0 a
Storage (A)		***	***	***	NS	NS	**	NS	NS
Temperature (B)		***	NS	NS	NS	NS	NS	NS	NS
Interaction (A × B)		***	NS	NS	NS	NS	NS	NS	NS

Notes: Data were subjected to two-way ANOVA, with means separated using Bonferroni multiple range test within each temperature tested. It was performed by the percentage conversions into angular values before statistic analysis. Different letters indicate statistically different values (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). [1] Viability: green appearance, lack of necrosis; [2] sprouting: shoots from the bead at least 5 mm long; [3] shoots/bead: number of shoots produced; [4] pmr (potential multiplication rate), number of nodes of proliferated shoot per capsule. [5] FW, fresh weight; [6] DW, dry weight.

Figure 7. Acclimatization phase of *L. palustris* plantlets: (A) cultivation in boxes in the growth chamber; (B) cultivation in the floating system in the greenhouse.

of the microcuttings, provided nutrients to both axillary buds without full prevalence of one bud on the other.

In vitro *rooting, acclimatization, and utilization of micropropagated plants*

L. palustris showed a high *in vitro* rooting potential. During the multiplication phase, the explants (100%) developed adventitious roots. As regards the explants restored from the encapsulation process, all the shoots demonstrated to retain a high rooting potential as observed during the micropropagation phase. Öztürk et al. (2004), using growth regulators during the multiplication phase of *L. repens*, were forced to perform a hormone-free subculture before the rooting phase. On the contrary, in this work, the use of a hormone-free multiplication medium allowed to avoid loss of time in manipulating and subculturing the plantlets. Thanks to this feature, it has not been necessary to establish a separate rooting phase (phase III), allowing to proceed directly with the acclimatization phase (phase IV). The plantlets easily acclimatized as long as the weaning procedures with the programmed times described above were followed carefully (Figure 7). Some plantlets derived both from the GROWTEK scale-up system and the restored encapsulated shoots were acclimatized and grown under greenhouse conditions in hydroponic systems at the Department of Agriculture, Food, and Environment (University of Pisa, Italy); others were sent to collaborators at the Center CRDP Padule di Fucecchio (Pistoia, Italy) where the cultures were acclimatized and maintained in large washtubs. At this Center, *L. palustris* plantlets were monitored for 2 years under the same environmental conditions of their natural habitat with the aim to carry out possible outplantings.

Conclusions

Plants such as *L. palustris* linked to freshwater wetlands are a component of wildlife and most of them are rare, vulnerable, or endangered, *ex situ* conservation practices is therefore essential for wetlands genetic diversity safeguard.

Among the various *ex situ* conservation methods, seed banking is the most convenient. This involves desiccation of seeds to low moisture contents and storage at low temperatures. However, there are a large number of species which produce recalcitrant seeds that quickly lose viability and do not survive desiccation, hence conventional seed-storage strategies are not possible. Plant tissue culture can be a benefit for the *ex situ* conservation of recalcitrant species assuming that technical and cost issues are solved (Pence 2010). As regards *L. palustris*, our results demonstrated that requirements are both satisfied: this study reports, for the first time, the micropropagation of this aquatic species defining a rapid (no long subcultures, easy handling of the explants, and rapid acclimatization process) and cheap (hormone-free media and scale-up in bioreactors) propagation protocol. Moreover, this work reports the possibility to encapsulate *L. palustris* microcuttings, to easily store the alginate beads using normal household refrigerators or at room temperature and to obtain a successful plant recovery from the encapsulated nodal segments following storage. The methods described in this work could be potentially used to preserve the germplasm of this species over a short period. This could also facilitate the transport and the exchange of encapsulated nodal segments among laboratories, thereby avoiding shoot desiccation and maintaining their viability. Generally, capsules consisting of *in vitro* propagules have many advantages for propagation making the storage and the production scale-up easier. Further investigations might be addressed to evaluate the tolerance of *L. palustris* to prolonged storage periods for possible long-term germplasm conservation.

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