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An eco-physiological and biotechnological approach to conservation of the world-wide rare and endangered aquatic liverwort *Riella helicophylla* (Bory et Mont.) Mont.

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Abstract – The rare aquatic liverwort *Riella helicophylla* (Bory et Mont.) Mont., inhabitant of temporary shallow ponds around the Mediterranean basin, is considered threatened throughout its distribution range. In addition, little is known of its biology and ecology or of its role in such an important ecosystem where environmental conditions vary yearly in unpredictable ways. In these variable habitats, due to the seasonal fluctuation of water levels, there is no guarantee of yearly spore input into the spore bank. Spore germination rate and the effects of different culture media in an axenic culture establishment, as well as propagation procedures of *R. helicophylla,* were tested. New insights into the ecology and biology of *R. helicophylla* are given. Spore dormancy is documented, and the protocols for the in vitro culture establishment, propagation and acclimatization of this liverwort are developed. Dry storage at 20 ± 2 °C for about three months broke the dormancy of spores, which subsequently germinated in a high percentage (over 90%). A two phase (solid and liquid) culture media system was developed for the purpose of achieving fully developed gametophytes. The liquid phase contained electrolytes simulating brackish water.

Key words: conservation, ecology, propagation, *Riella,* temporary ponds

Introduction

Temporary Mediterranean ponds are threatened habitats of shallow water bodies that remain flooded for a sufficiently long period of time during winter and spring to allow the development of (semi-) aquatic vegetation and animal communities. These ponds can be of various types, differing in size, shape, depth, altitude, substrates (soil and rock types and combination of these), sources of water, salt concentration in the water, and duration of water reservoir, thus influencing their biological diversity.

Mediterranean temporary ponds comprise a priority habitat according to the Natura 2000 network of the European Union (Natura code 3170, Habitats Directive 92/43/EC) which are scattered primarily in peri-Mediterranean countries with dry and semi-arid climates.

Conservation biologists are aware of the importance of these sites, as they play a significant role in global biogeochemical cycles and biodiversity maintenance (Miracle et al. 2010). Threats to these habitats may vary on different spatial scales and ultimately depend on the conditions at a specific site. The main threats derive from inadequate management of the ponds due to human activities that include agriculture, over-exploitation of aquifers, draining and dredging as well as silting, which, although it constitutes a process in the natural evolution of the ponds, can be accelerated by human activities (Asem et al. 2014).

Despite their high biodiversity levels, not all the organisms in these habitats have received similar attention from a conservation perspective (Della Bella et al. 2005). Conservation efforts have mainly focused on vascular plants, amphibians, crustaceans and other invertebrates such as roti-

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fers (Zacharias et al. 2007). Bryophytes have been subject to very few conservation initiatives, most likely because they represent a very minor proportion of the species diversity in these aquatic habitats. Among bryophytes, a small group of thallose liverworts in the Sphaerocarpales includes the genus *Riella* Mont. This genus includes some 28 species (Söderström et al. 2016), some of which have been described only recently, indicating how poor the knowledge of this genus is (Segarra-Moragues et al. 2012, 2014; Cargill and Milne 2013; Segarra-Moragues and Puche 2014).

Species of *Riella* grow commonly submerged in clean, shallow, fresh or brackish waters of seasonal ponds, more rarely in permanent waters of arid and semiarid regions and have disjunct distributions and scattered populations (Cirujano et al. 1988). Species of *Riella* are rare and/or under-recorded due to their ephemeral habit and fluctuant populations (Segarra-Moragues et al. 2014). The delicate gametophyte is completely intolerant to desiccation and population persistence in drought periods is ensured through their spores which can remain viable through decades in the spore bank (Proctor 1972). Some species of *Riella*, such as *R. helicophylla* (Bory et Mont.) Mont., inhabit brackish water temporary ponds making them, from an ecological perspective, unique amongst bryophytes.

In this study, we aimed at establishing the protocol for axenic micropropagation of *R. helicophylla*, an endangered species included in Annex II of the European Union Habitats Directive 92/43/EEC. We tested the effect of different culture media in the axenic establishment and propagation of *R. helicophylla* using samples originally collected from a Spanish population, hereafter referred as Spanish genotype, for the purpose of *ex situ* conservation and future research on its biology.

Materials and methods

Plant material

The initial plant materials were collected in spring of 2010 at Marjal del Moro, Sagunto, Valencia Province, Spain by JGS-M and FP. Plants with sporophytes were kept in cultures containing sediments from the original locality until ripe spores were observed. Ripe unopened sporophytes were separated from the plants, air dried and kept in darkness, at room temperature. Sporophytes were collected at different time intervals in order to cover a range of time from the onset of drought conditions (15, 30, 45, 60, 75 and 90 days). Species of *Riella* have been observed to present spore dormancy which prevents immediate germination of the spores (Thompson 1941). They require a drought period, which in natural conditions may correspond to the summer months but that can be extended further in particularly dry years, to break up dormancy. Thus, our sampling of sporophytes covered the minimum drought period necessary to restore the germination capability of the spores.

Sporophyte sterilization assays

For the purpose of axenic culture establishment and to prevent undesired contamination of the cultures, surface sterilization of sporophytes was required. Sodium dichloroisocyanurate (NaDCC) and commercial bleach (NaOCl, 8% of active chlorine) were tested for sporophyte surface sterilization. Sporophytes were sterilized by soaking them for up to 10 minutes using different concentrations (1%, 3%, 5% and 7%) of NaDCC or commercial bleach solutions.

Culture media assays

In this study we used different culture media depending on the experiment. Liquid and solid media were used to germinate the spores whereas plant growth was assessed on solid media or in bi-phasic cultures corresponding to a combination of liquid and solid media. As liquid media we used distilled water or a solution enriched in electrolytes. This electrolyte solution was composed of: $Ca(NO_3)_2 \times 4\ H_2O$ 2876 mg L^{-1}, KH_2PO_4 500 mg L^{-1}, $MgSO_4 \times 7\ H_2O$ 1000 mg L^{-1}, KNO_3 1000 mg L^{-1}, KCl 1000 mg L^{-1}, $KHCO_3$ 400 mg L^{-1}, and Fe-EDTA 130 mg L^{-1}. Three types of solid media, namely MS (Murashige and Skoog 1962), BCD (Sabovljević et al. 2009) and KNOP (Reski and Abel, 1985), were used in plant growth experiments. MS medium contained: NH_4NO_3 1650 mg L^{-1}, KNO_3 1900 mg L^{-1}, $CaCl_2 \times 2\ H_2O$ 440 mg L^{-1}, $MgSO_4 \times 7\ H_2O$ 370 mg L^{-1}, KH_2PO_4 170 mg L^{-1}, Na_2EDTA 37.3 mg L^{-1}, $FeSO_4 \times 7\ H_2O$ 27.8 mg L^{-1}, H_3BO_3 6.2 mg L^{-1}, $MnSO_4 \times 4\ H_2O$ 22.3 mg L^{-1}, $ZnSO_4 \times 4\ H_2O$ 8.6 mg L^{-1}, KI 0.83 mg L^{-1}, $Na_2MoO_4 \times 2\ H_2O$ 0.25 mg L^{-1}, $CoCl_2 \times 6\ H_2O$ 0.025 mg L^{-1}, $CuSO_4 \times 5\ H_2O$ 0.025 mg L^{-1}, agar 8000 mg L^{-1}, and myo-Inositole 100 mg L^{-1}. BCD medium contained: $MgSO_4 \times 7\ H_2O$ 250 mg L^{-1}, KH_2PO_4 250 mg L^{-1}, KNO_3 1010 mg L^{-1}, $FeSO_4 \times 7\ H_2O$ 12.5 mg L^{-1}, and agar 8000 mg L^{-1}. Finally, KNOP medium contained: KH_2PO_4 25000 mg L^{-1}, KCl 25000 mg L^{-1}, $MgSO_4$ 25000 mg L^{-1}, $Ca(NO_3)_2 \times 4\ H_2O$ 100000 mg L^{-1}, $FeSO_4 \times 7\ H_2O$ 250 mg L^{-1}, and 12000 mg L^{-1} of agar. Additionally, cultures on MS medium were assayed on half strength MS or supplemented with sucrose 15000 mg L^{-1}. The pH of the media was adjusted to 5.8 before sterilization of the media at 121 °C for 25 min.

For the axenic culture establishment experiments, spore germination was tested on the three aforementioned solid media: KNOP, MS (standard composition and half strength), and BCD. The different media were prepared following the protocol described above and replicates supplemented with sucrose or plant growth regulators were also tested. The unopened sporophytes were surface sterilized, and then opened in an air flow chamber to release the spores on sterile solid-phase media described above and kept at 25 ± 2 °C under long day conditions (16 h light / 8 h darkness).

Spore germination assays

In order to test whether spore germination was affected by the time from which the sporophytes were subjected to drought conditions, they were collected at the aforementioned six time intervals and then kept dried up to 10 days prior to experimentation. Spore (total content of three spore capsules) germination was recorded seven days after incubation in tubes by quantification of germlings using a hemocytometer slide. Water or electrolyte solution containing gibberellins (0.03 mM, 0.1 mM, 1 mM and 10 mM) (GA_3

and GA_7) were tested in order to investigate the influence of these phytohormones on spore germination, since dormancy of spores has been reported in *Riella* (Thompson 1941).

Axenic culture establishment assays

As plants of *Riella* inhabit shallow water ponds and remain attached to sediments by their rhizoids, bi-phasic axenic culture media were established and tested. Spores were firstly sown on KNOP, MS or BCD solid sterile medium under long day conditions. Once the spore germinated, the green structure appeared in less than 10 days after sowing. Thin solid basal media containing plants were subsequently covered with liquid solutions of sterilized distilled water or electrolytes (liquid phase). Bi-phasic cultures were grown at 18 ± 2 °C under long day conditions, at 47 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by cool-white fluorescent tubes. Cultures were grown for six weeks under these conditions. Four replicates of each culture were established in order to account for the variation in total biomass.

Quantification of vegetative growth

It was not possible to estimate the vegetative growth of *R. helicophylla* through the multiplication index (that represents the number of newly grown shoots which derived from one starting shoot) because of its thallose habit. To overcome this, estimation of the vegetative growth was based on quantification of total biomass of individual gametophores. For this purpose individual gametophores were kept fully hydrated, to avoid variations in water content and then weighed on a Sartorius analytical balance (weight estimated to the nearest 0.0001 g).

Results

Spores collected in the field and immediately spread on solid medium of any type did not germinate. The exposure to light or dark during the three weeks after spread on media did not affect germination. Our results showed an effect of the sporophyte (i.e. spores) drought duration time on the germination ability of *R. helicophylla* spores. Germination proportions were negligible for spores that had remained dry less than two months (Fig. 1). Spore germination increased after 75 days since drying out and reached maximum values at 90 days since drought (Fig. 1). Although germination percentages were higher for spores germinated on the electrolyte solution than on distilled water, the differences between the two treatments were not significant (Fig. 1).

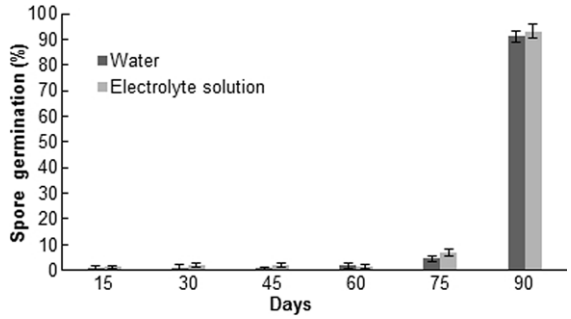

Fig. 1. Spore germination rate of *Riella helicophylla* expressed in percentage (± standard deviations) after dry storage at room temperature (in days) prior to test for seven days in liquids.

Gibberellins, which are widely known to break seed dormancy, did not bring the expected results in breaking dormancy in spores that had been dried out for up to ten days (Fig. 2). The treatments of spores with 0.03, 0.1, 1 and 10 mM GA_3 and GA_7 added to basal media did not break the germination inhibition either in dark or in light condition at 18 ºC. The imitation of the natural dry and hot period (Fig. 1) breaks spore dormancy.

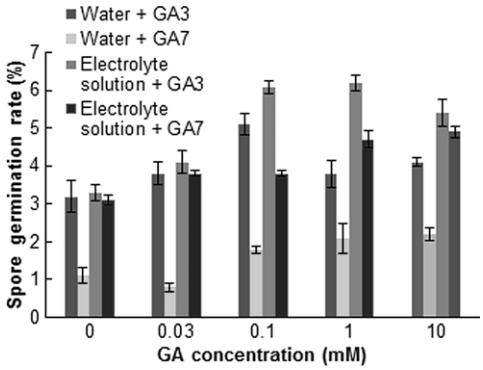

Fig. 2. Test of breaking dormancy with different concentrations of selected gibberellins (GA) dissolved in water or electrolyte solution. The difference in control (0 mM GA) clearly indicates the presence of some variation within spores from the same liverwort population.

The dormancy noted caused the problems with in vitro establishment of *R. helicophylla.* Once, we have found the way to break dormancy in spores we precede to axenic in vitro culture establishment. The most effective way to get viable axenic spores was treatment with 3% NaDCC for 7 minutes. The percentage of uncontaminated media was over 95%.

The spores (released from dormancy) were germinated in a high percentage in all tested solid media under long day conditions. However, the gametophytes did not equally develop (Fig. 3), and instead a callus-like tissue was formed that afterwards started developing some buds (Fig. 4a).

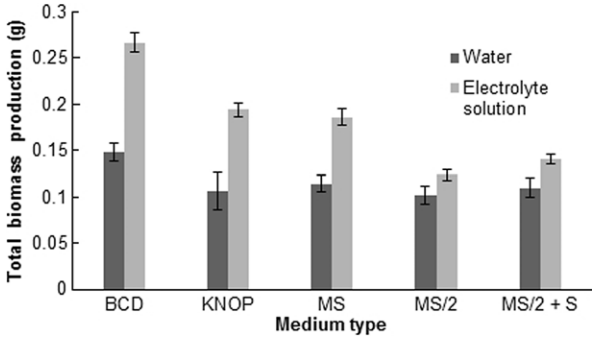

Fig. 3. Biomass production of *Riella helicophylla* gametophytes on different solid media covered with water or electrolyte solution. MS – Murashige and Skoog medium; MS/2 – half strength Murashige and Skoog medium; MS/2 + S – half strength Murashige and Skoog medium enriched with 15 g L^{-1} of sucrose.

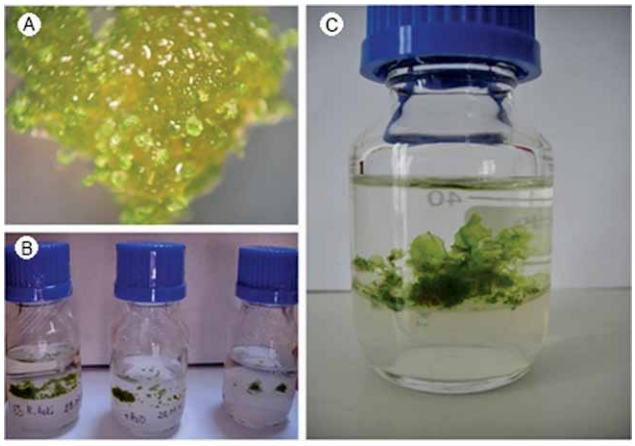

Fig. 4. A) Development of a callus-like tissue and buds from spores germinated on solid BCD medium, B) test of various solid media types covered with water on the development of *Riella helicophylla* gametophytes, C) fully developed gametophytes in biphasic axenic cultures on solid BCD medium covered with electrolytes.

Fully developed gametophytes were obtained from biphasic axenic in vitro cultures that imitated the shallow water ecological conditions in the wild. When all the tested solid media were covered with distilled water, fully developed gametophytes were formed (Fig. 3). But when they were covered with the electrolyte solution, the total biomass production was significantly increased on all the media tested.

Discussion

The results clearly show the existence of spore dormancy in the tested *Riella helicophylla* population. This is rather expected since this species inhabits temporary ponds which can be dried out after few months and stay without water for couple of years. Dormancy is a response to the harsh environment during the drought period and in *R. helicophylla* is a prime example of genotype-environment interaction. By the dormancy present in *R. helicophylla*, it can be inferred that the drought period is anticipated geneticaly. It can, however, be broken by signals from the environment. As usually stated, dormancy is complex phenomenon, and induction, maintenance and breaking of dormancy in vascular plants is quite unknown and not consistent across all species. Even less is known for non-tracheophytes, such as bryophytes. This evidence opens many other questions such as defining external signals in processes such as induction, establishment and breaking of spore dormancy in the interesting aquatic liverwort *R. helicophylla.*

It was shown that photomorphogenesis in bryophytes is positively influenced by light, as one of the most important environmental factors that influences bryophyte development (Nakazato et al. 1999). Nevertheless, bi-phase culture with electrolytes influenced full development of the gametophytes. Having in mind that these plants grow in brackish environments and that the uptake of minerals takes place on the whole through the thallose gametophyte, fully developed liverwort in such conditions is rather expected than in a bi-phase culture with water where ions are dissolved from the solid medium (Figs. 4b, 4c). It has been previously reported that gibberellins have an influence on the process of morphogenesis of selected moss species (Sabovljević et al. 2010). However, the exposures of spores to selected concentrations of two widely used gibberellins did not cause germination. Therefore, we decided to test out simulation of natural conditions, with the aim of achieving the spore germination ability. We chose to keep the spores in dry conditions at 20 ºC, since in natural condition spores survive a long dry summer period. The germination dormancy was not broken after 15, 30, 45, 60 and 75 days. However, spores germinated after 90 days in dry and relatively hot conditions, up to 91%. Thus, dryness and warmth, here combined, break the spore dormancy in the tested *R. helicophylla* population.

The germinated spores showed slightly better growth in BCD medium. The other tested media did not show a statistically significant difference in basal media preferential (ANOVA P value 0.914). Accordingly, the other basal media tested did not have any significant effect on spore germination. Solid, but soft, media were good for spore germination in contrast to the completely liquid media tested.

Two phase system (solid and liquid) culture media were developed for the purpose of achieving fully developed gametophytes. Spores were able to germinate on a solid rather than in purely liquid medium, and they developed some kind of callus tissue that developed into green plants, gametophores, after being transferred to the two phase culture. Two types of liquid phases: (1) distilled water and (2) water containing electrolytes simulating brackish water (electrolyte solution), were tested over BCD medium, in which callus mass had good growth. The best plant morphogenesis was achieved on BCD medium covered with electrolyte-enriched liquid phase.

Experiments on sporophyte development induction (i.e. spore production) by growing female and male plants and acclimation to xenic condition are ongoing. The aim is to develop biotechnical model, which can be widely used for reintroduction and release of the *Riella* plants to potential natural and semi-natural aquatic habitats.

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