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Ex situ conservation of rare and threatened Mediterranean Bryophytes*

Abstract

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Four Mediterranean, desiccation-tolerant species were assayed for *in vitro* culture and subsequent cryopreservation using the encapsulation–dehydration method. Two of them are rare species endemic to the Mediterranean area, where they are distributed in several countries or islands (*Entosthodon commutatus* and *Funariella curviseta*), one is an endemic of Fuerteventura, Canary Islands (*Orthotrichum handiense*), and catalogued as Endangered in the Spanish Red list, and one (*Entosthodon hungaricus*) has a wider distribution in central and southern Europe, northern Africa and western Asia. All of them were successfully cultured and cryopreserved with conservation in mind.

Key words: cryopreservation, encapsulation–dehydration, threatened species.

Introduction

The main aim of the *ex situ* conservation is to reduce the risk of extinction of species or populations and, in some cases, for the purpose of restoring them (Bacchetta & al. 2008). Although the conservation of species can be addressed in a much more effective way by the correct management of wild populations and their natural habitats (*in situ*), *ex situ* techniques are considered essential tools for conservation, especially since they have gained international recognition with inclusion in Article 9 of the Convention on Biological Diversity in Goal 8 of the Global Strategy for Plant Conservation (Sarasan & al. 2006).

The plant germplasm necessary for *ex situ* conservation (biological material containing intraspecific genetic variability or genetic materials that can perpetuate a species or a population of an organism, Witt 1985) can be from different structures (spores, tissues or parts of plants) but, particularly from seeds (Bacchetta & al. 2008). In the case of bryophytes, the germplasm usually used is protonema or gametophore fragments obtained from axenic cultures *in vitro*. This is justified by the obvious lack of seeds and, in many cases, spores. Moreover, the reduced size of bryophytes allows a high number of individuals to be grown in a restricted space. In addition, tissue fragments have a very high regenerative potential with no need for elaborate treatments (Lal 1984; Chopra & Kuma 1988) and, furthermore,

* Extended and enriched version of the poster presented by Werner & Ros at the XIV Optima meeting in Palermo, 9-15 Sept. 2013.

many of them are desiccation -tolerant in nature, so that tissues that can survive on little water can be used, which is a prerequisite of cryopreservation protocols (Burch 2003).

The *ex situ* conservation of bryophytes includes the following processes: material collection, propagation and storage, cryopreservation and reintroduction (Ramsay & Rowntree 2004).

The availability of material from threatened species is, by definition, limited and permits must be issued before collection can be made. The collection of plant material should respect the natural populations and avoid potential adverse effects to the population *in situ* due to harvesting (Rowntree 2006; Ros & al. 2011).

In the propagation phase, axenic cultures are mostly used. Bryophytes are relatively easy to culture in comparison with vascular plants and many papers have been published dealing with this subject (see Duckett & al. 2004 for a revision). Axenic cultures have the advantage that vegetal material is kept free of biological and chemical contaminants, and the main problem for obtaining axenic bryophytes is sterilization of the samples. When specimens are available with closed mature capsules the whole process is relatively easy, since fungi and bacteria cannot penetrate the capsules (Sabovljevic & al. 2003; Rowntree 2006). But many species never, or only very rarely, have sporophytes or they are collected in inappropriate seasons for their capsules to be mature. In such cases the samples must be externally sterilized without killing the cells of the gametophores, for which purpose protocols typically use several substances similar to bleach or ethanol (Basile & Basile 1988; Kowalczyk & al. 1997; Sabovljevic & al. 2003; Duckett & al. 2004; Rowntree 2006; Sabovljevic & al. 2009; Ros & al. 2011). Consequently, it is necessary to carry out tests with a relatively high number of plant materials to establish axenic cultures, which implies considerable manual laboratory work. Care must also be taken regarding factors such as temperature, light intensity and day length, whose optimal values have been established in previous works for several species (Rowntree & al. 2011). Additional carbon sources and growth regulators are not strictly necessary in bryophyte axenic cultures because of their autotrophic character (Takami & al. 1988; Hohe & al. 2002; Rowntree 2006).

Once axenic plant material is available it is necessary to ensure that it is stored in viable conditions, maintaining its original genetic characteristics. This is achieved by controlling the storage conditions to suppress or reduce the metabolism of samples and keeping it in optimal culture conditions. In some cases storage consists of keeping the plant material at low temperatures, for example in liquid nitrogen at -196° C, to stop growth while preserving its viability and physiological and genetic characteristics, in a process known as cryopreservation (Bacchetta & al. 2008). Moreover, cryopreservation reduces the need for manpower and the space necessary in culture chambers involved in the maintenance of the cultures (Duckett & al. 2004; Rowntree & Ramsey 2005; Benson 1999; Mallón & al. 2010). There are several protocols that have been successfully followed for the preparation of bryophytes for storage at ultralow temperatures. Since the 80th many protocols describe the conservation of spores, protoplasts and tissue at different temperatures (Takeuchi & al. 1980; Longton 1981; Grimsley & Withiers 1983; Fabre & Dereuddre 1990; Christianson 1998; Pence 1998; Schulte & Reski 2004; Rowntree & Ramsay 2005; Ros & al. 2011), and have been widely developed by Rowntree & Ramsay (2009) and Rowntree & al. (2011). The existence of rehydrins in some desiccation-tolerant bryophytes is linked to the protective function of the cells during desiccation of the vegetative structures (Oliver & al. 2005), and prior treatment with ABA and sucrose may improve post-cryopreservation survival (Pence 1998; Burch & Wilkinson 2002).

The last phase of the *ex situ* conservation process is the reintroduction of plants into their natural habitats. This is a complex and scarcely studied subject in bryophytes and very few attempts have been made (Ramsay & Rowntree 2004). Some papers on this subject have been published by Kooijman & al. (1994), Gunnarsson & Söderström (2007) and Hinde & al. (2010), and reintroduction guidelines have been published by IUCN SSC Reintroduction and Invasive Species Specialist Groups (IUCN/SSC 2013).

The aim of this work was to develop *ex situ* conservation techniques and *in vitro* cultures for four threatened or rare moss species from the Mediterranean basin: *Entosthodon commutatus* Durieu & Mont., *Entosthodon hungaricus* (Boros) Loeske, *Funariella curviseta* (Schwägr.) Sérgio and *Orthotrichum handiense* F. Lara, Garilleti & Mazimpaka species.

Material and Methods

Species selected and material studied

***Entosthodon commutatus* Durieu & Mont.**

(= *E. deserticola* (Trab.) Jelenc, *E. krausei* Besch., *E. saharae* (Trab.) Jelenc, *Funaria fritzei* Geh.)

Distribution: Algeria, Canary Islands, Crete, Greece, Madeira, Morocco, continental Spain (Ros & al. 2013). Mediterranean endemics.

Habitat: saline soils (Brugués & Sérgio 2010), frequently with *Entosthodon hungaricus*.

Threat in the Mediterranean: DD in Spain (Brugués & González-Mancebo 2012)

Locality 1: Spain, Murcia, Cartagena, Boca Rambla, zona de aparcamiento en la carretera a Los Urrutias y El Carmolí, 37º 42' 50.31'' N, 0º 51' 29.42'' W (UTM: 30S XG 8887), saladar aclarado con *Suaeda vera* y *Tamarix sp.*, junto a playa, 0 m s.n.m. (mezclado con *E. hungaricus*), *R.M. Ros & O. Werner s.n.,* 27-03-2010, MUB 34251; *idem* MUB 34252; *idem* 18-04-2011, MUB 40506; *idem* 22-03-2012, MUB 42231; *idem* MUB 42232; *idem* MUB 42233.

For cryopreservation experiments: samples MUB 40506 and MUB 42231.

***Entosthodon hungaricus* (Boros) Loeske**

(= *Funaria hungarica* Boros, *Physcomitrium longicolle* Trab., *Entosthodon longicollis* (Trab.) Ros & M.J. Cano *nom. illeg., E. maroccanus* (Meyl.) Hébr. & Lo Giudice)

Distribution: central European steppes with an Aral-Caspian distribution (Gams 1934) and Mediterranean area. According to Pisarenko & al. (2001), the central European countries where the species is known are: Austria, Germany, Hungary, Romania and Ukraine; the Asian countries are Kazakhstan Altai, Lower Volga area, Asian Russia, and Kirgizia, although these authors consider this last report doubtful. In the Mediterranean area the species is known from Algeria, Canary Islands, Crete, Greece, Israel, Malta, Morocco, Serbian Republic, Sicilia, and continental Spain (Ros & al. 2013).

Habitat: saline soils.

Threat in the Mediterranean: NT in Spain (Brugués & González-Mancebo 2012).

Locality 1: Spain, Murcia, Las Torres de Cotillas, Urbanización Los Romeros, 38º 00' 49.50'' N, 1º 14' 41.86'' W (UTM: 30S XH5408), suelo descubierto en jardín, junto a un estanque artificial, 120 m s.n.m., *R.M. Ros s.n.,* 06-04-2010, MUB 34249.

Locality 2: Spain, Murcia, Cartagena, Boca Rambla, zona de aparcamiento en la carretera a Los Urrutias y El Carmolí, 37º 42' 50.31'' N, 0º 51' 29.42'' W (UTM: 30S XG 8887), saladar aclarado con *Suaeda vera* y *Tamarix sp.*, junto a playa, 0 m s.n.m. (mezclado con *E. commutatus*), *R.M. Ros & O. Werner s.n.,* 27-03-2010, MUB 34250; *idem* 18-04-2011 MUB 40507; *idem* MUB 40508; *idem* MUB 40509; *idem* MUB 40510; *idem* 22-03-2012, MUB 42229; *idem* MUB 42230.

Locality 3: Murcia, San Javier, Los Narejos, 37º 45' 55.35'' N, 0º 50' 52.15'' W (UTM: 30S XG 9082), suelo arcilloso con *Suaeda vera* y otras nitrófilas, 13 m s.n.m., *R.M. Ros & O. Werner,* 27-03-2010, MUB 34253.

Locality 4: Spain, Murcia, Alhama de Murcia, polígono industrial de Alhama de Murcia, 37º 49' 19.0'' N, 1º 24' 11.5'' W, saladar de *Atriplex*, *Suaeda*, *Limonium* y *Tamarix*, suelo con afloramientos de sal protegido bajo arbustos, 150 m s.n.m. *O. Werner & R.M. Ros s.n.*, 19-04-2011, MUB 38591; *idem* MUB 40505.

Locality 5: Spain, Murcia, Cartagena, carretera Cartagena-Los Alcázares, km 21a, 37º 42' 28.8'' N, 0º 51' 44.2'' W (UTM: 30S XG 8876), saladar aclarado con *Suaeda vera* y *Tamarix sp.*, junto a playa, 0 m s.n.m., *R.M. Ros & O. Werner s.n.,* 22-03-2012, MUB 42218; *idem* MUB 42219; *idem* MUB 42220; *idem* MUB 42221.

For cryopreservation experiments: samples MUB 38591 and MUB 40505.

***Funariella curviseta* (Schwägr.) Sérgio**

Distribution : Algeria, Balearic Islands, Canary Islands, Corsica, Crete, Croatia, Cyprus, France, Greece, Israel, Italy, Lebanon, Lybia, Malta, Montenegro, Morocco, continental Spain, Portugal, Sardinia, Serbian Republic, Tunisia, Turkey (Ros & al. 2013). Mediterranean endemics.

Habitat: stony slopes, protosoils in openings among bushes and rocks of acidic or basic nature.

Threat in the Mediterranean: EN in Italy (Cortini Pedrotti & Aleffi 1992).

Locality 1: Spain, Murcia, Cartagena, Los Nietos, Cabezo Mingote, UTM: 30S 694394; 4168152, fisura de roca caliza, 50 m s.n.m., *R. M. Ros & I. Aledo,* 27-04-2010, MUB 34244; idem *R.M. Ros & O. Werner s.n.,* 22-03-2012, MUB 42236; idem 42237; idem 42238;

Locality 2: Spain, Murcia, Cartagena, Cabezo La Fuente, Los Belones, 37º 36' 28,5'' N, 0º 46' 36,3'' W (UTM: 30S XG9664), hendidura de roca caliza, 107 m s.n.m., *R.M. Ros & O. Werner s.n.,* 22-03-2012, MUB 42240; *idem* 42241.

For cryoconservation experiments: samples MUB 42236 and MUB 42240.

***Orthotrichum handiense* F. Lara, Garilleti & Mazimpaka**

Distribution : Fuerteventura, Canary Islands (González-Mancebo & al. 2008; Patiño & al. 2013). Canarian endemics.

Habitat: epiphyte on *Asteriscus sericeus* and occasionally on *Kleinia neriifolia* and basaltic rocks at elevations ranging from 650 m to c. 800 m (González-Mancebo & al. 2009).

Threat: EN in Spain (González-Mancebo & al. 2008, 2012).

Localidad 1: Spain, Fuerteventura, Pico de Ingenieros, UTM: 28R 0563731; 3109049, Matorral dominado por *Asteriscus sericeus,* 790 m s.n.m., *J. Leal & J. Patiño s.n.,* 11-2008, MUB 44615.

Localidad 2: Spain, Fuerteventura, Pico de Ingenieros, UTM: 28R 0564166; 3109204, matorral dominado por *Asteriscus sericeus*, 729 m s.n.m., *J. Leal & J. Patiño s.n.,* 11-2008, MUB 44616.

Locality 3: Spain, Fuerteventura, Pico de la Zarza, UTM: 28R 0563614; 3108388, matorral dominado por *Asteriscus sericeus* (por fuera de la zona vallada), 680 m s.n.m., *J. Leal & J. Patiño s.n.*, 11-2008, MUB 44617.

Locality 4: Spain, Fuerteventura, Pico de la Zarza, UTM: 28R 0563321; 3108640, matorral dominado por *Asteriscus sericeus* (dentro de la zona vallada), 805 m s.n.m., *J. Leal & J. Patiño s.n.,* 11-2008, MUB 44618.

Localidad 5: Spain, Fuerteventura, Pico de la Palma, UTM: 28R 0562783; 3108459, matorral dominado por *Asteriscus sericeus*, , 730 m s.n.m., *J. Leal & J.M. González-Mancebo s.n.*, 11-2008, MUB 44619.

In vitro cultures

Spores of the four species from the collections mentioned above were sown without previous sterilization of the capsules under aseptic conditions on Knop-agar plates (dehydrated 2% Agar Agar in strips (SERVA Kobe I, research grade) with Knop salts: 0.025% KH_2PO_4, 0.025% KCl, 0.05% $MgSO_4$ * 7 H_2O, 0.1% $Ca(NO_3)_2$ * 4 H_2O, and 0.0007% FeEDTA) covered with cellophane sheets (Bopp & al. 1964). The medium was sterilized by autoclaving for about 30 min and leaving at room temperature to cool to ~65 ºC prior to addition to Petri dishes (6 cm diameter).

Spores contained in the closed ripe capsules were transferred to the Petri dishes under sterile conditions and maintained in an incubator at 20 ± 3 ºC a 16 h light period (3000 ± 500 lux) and 15 ± 2ºC during the 8 h dark period.

Cultures were maintained on this medium for several weeks prior to these experiments, with subcultures made every 3-4 weeks

Cryopreservation

The dehydration–encapsulation technique and pre-treatment with 10 µM Abscissic Acid (ABA), modified from Burch and Wilkinson (2002), without the addition of sucrose was used. Protonemata with or without gametophores were transferred to a fresh culture medium similar to that described above but supplemented with 10^{-5} M ABA for a period of 6 d under the above mentioned temperature and photoperiod. Small portions of protonema or gametophores (approximately 2-3 mm long) were encapsulated in alginate beads at 3% and solidified in a calcium chloride solution for 3 min (Burch & Wilkinson 2002). Then, the capsules were transferred to an empty

Petri dish and placed in a culture chamber for dehydratation. After 24-48 h, the dry capsules were placed in Eppendorf tubes, which were immersed in liquid N_2 for about 6 h, after which they were removed and placed in a rack at room temperature until the capsules were completely thawed (approximately 10 min). The capsules were placed in Petri dishes containing culture medium and maintained in the growth chamber under the above temperature and photoperiod regime. Bryophyte regeneration was monitored every week for 5 weeks.

For *Entosthodon commutatus*, *E. hungaricus* and *Funariella curviseta* a total of (90-)100(-153) alginate beads from each of its two geographical locations were prepared. In the case of *Orthotrichum handiense* plants from only one locality were used and 50 alginate beads were made due to the scarcity of this rare and threatened species.

Results

In vitro cultures

In *Entosthodon commutatus*, *E. hungaricus* and *Funariella curviseta* spore germination started 3-7 d after sowing the spores, although in some samples of *Entosthodon commutatus* they needed longer (about 15 d). In *Orthotrichum handiense* germination took place 15-20 days after inoculation. The development of the protonema was successful in all cases. The time required for the formation of the first gametophores ranged from 20-25 d after spore germination in the case of *Funariella curviseta*, to 40-50 d in *Entosthodon hungaricus*, 30-40 in *Entosthodon commutatus*, and 60-80 d in *Orthotrichum handiense* (Table 1, Fig. 1)

Since the *in vitro* development of *Entosthodon commutatus* and *Funariella curviseta* is very similar to that described by Sabovljević & al. (2012) for the taxonomically closely related *E. hungaricus*, only the *in vitro* development of *Orthotrichum handiense* is described below.

When spores germinate they give rise to chloronema-type unipolar or bipolar protonema filaments (with transversal cell walls and abundant chloroplasts) that ramify transversally (Fig. 2, A-E). These creeping filaments turn brown and become caulonema as they develop oblique cell walls, but they also contain numerous chloroplasts, which are difficult to see under the brown cell walls. They are very long and give rise to numerous perpendicular or oblique erect ramifications pale brown or orange in color with perpendicular cell walls (secondary chloronema) (Fig. 2, F). The gametophores grow sparsely from the secondary chloronema, so they are not very abundant (Fig. 2, G, H). Very frequently old cultures develop new plants on caulonema filaments, which can easily break off and become independent plants (Fig. 2, I). Approximately two year old cultures were observed to develop abundant axillary perigonia.

Table 1. Results of *in vitro* cultures and cryopreservation assays in the four studied species. The survival rate after 5 weeks is given (protonema fragments or gametophores looked green and healthy and contained actively dividing cells), along with the percentage of material considered to be in very good condition (it developed new protonema filaments and (very frequently) also gametophores from the alginate beads). * In *Orthotrichum handiense* only one cryopreservation assay was made due to the scarcity of such a rare and threatened species.

Species	***In vitro* cultures**		**Cryopreservation assays**	
	Start of germination after sowing (days)	**Appearance of the first gametophores (days)**	**Survival rate (% ± S.D.) 5 weeks after treatment**	**Material in very good condition (%) 5 weeks after treatment**
Entosthodon commutatus	3-7(15)	30-40	93.6 ± 6.36%	17-30%
Entosthodon hungaricus	3-7	40-50	54.5 ± 27.58%	6-20%
Funariella curviseta	3-7	20-25	69.5 ± 21%	7-47%
Orthotrichum handiense	15-20	60-80	90%*	75%

Cryopreservation

The survival rate, considered as the percentage of regeneration of frozen beads, was acceptable (>54 ± 27.58%) for all four species (Table 1). The condition of frozen material was considered bad when all the cells within a replicate died. Regeneration was considered good when the protonema fragments or gametophores within the beads looked green and healthy and contained actively dividing cells (Fig. 3-A, B). Finally, it was considered very good when they developed new protonema filaments and (very frequently) gametophores (Fig. 3-C-F).

The species that showed the best results was *Entosthodon commutatus*, where the two populations used had a survival rate of 89% and 98%. In *Funariella curviseta* the survival rate was slightly lower, ranging from 84% in one population to 55% in the other; *Entosthodon hungaricus* had a survival rate of 74% in one population but only 35% in the other. And in the case of *Orthotrichum handiense*, the only population used in the assay (due to the scarcity of wild material) had a survival rate of 90%.

Discussion

All the species used were seen to grow very well and easily in *in vitro* conditions using a standard culture medium commonly used for bryophytes, despite their strict ecological requirements in the wild: terricolous on saline soils in the case of *E. commutatus* and *E. hungaricus*, casmocomophytic in the case of *Funariella curviseta* and epiphytic or saxi-

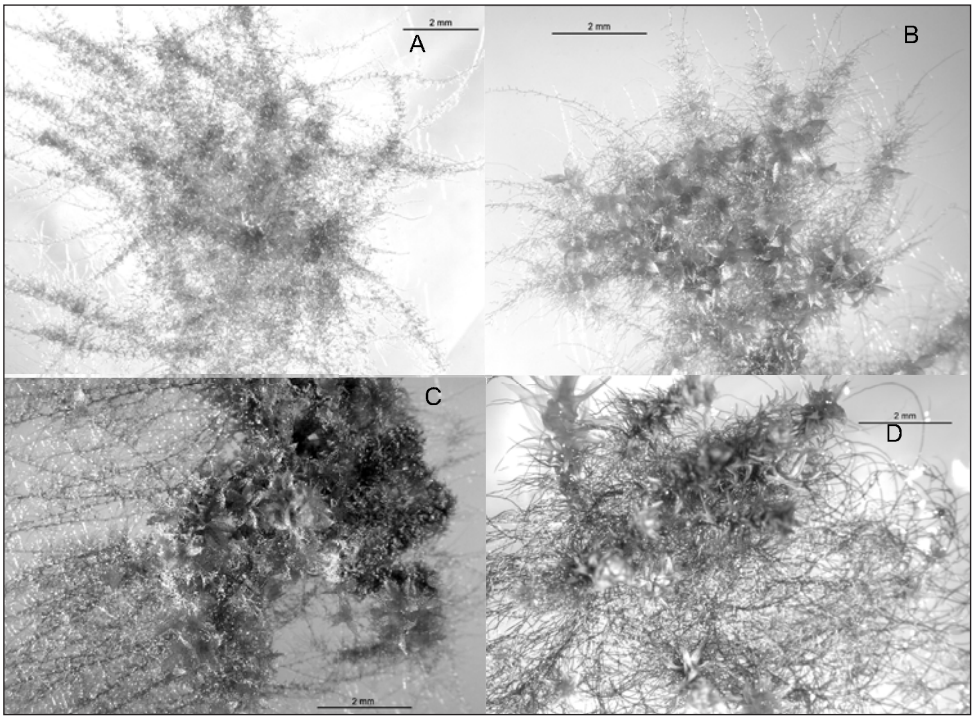

Fig. 1. Plants obtained axenically in *in vitro* cultures after protonema have developed gametophores: **A.** *Entosthodon commutatus*; **B.** *Entosthodon hungaricus*; **C.** *Funariella curviseta*; **D.** *Orthotrichum handiense*.

colous in the case of *Orthotrichum handiense*, this last in a very reduced area estimated at 45 ha (González-Mancebo & al. 2009) on Fuerteventura Island. One possible interpretation of these results is that in conditions other than those in which they were formed other factors limit their growth, e.g. competition with other species, low dispersal capacity, etc.

Of the four species, only *Entosthodon hungaricus* had previously been tested for axenic *in vitro* cultures by Sabovljević & al. (2012). They succeeded in establishing cultures after sterilizing spores with 3% NaOCl for 90 seconds followed by a double rinse of sporophytes with distilled water. In our case, the sporophytes were not sterilized, and we simply opened ripe capsules and directly sowed spores on the agar. This makes the *in vitro* process easier as we also obtained axenic *in vitro* material without problems. The development of the protonema and the formation of gametophores described by Sabovljević & al. (2012) were, as expected, similar to that observed in our experiments.

The *in vitro* development process in *Entosthodon commutatus* and *Funariella curviseta* was very similar to that described for *E. hungaricus*, as they belong to the same family, developing numerous gametophores from both the primary and secondary protonema formed from a single spore. In the case of *Orthotrichum handiense*, the development of protonema and gametophores was slower than in the Funariaceae studied, but many gametophores also grew from a single spore.

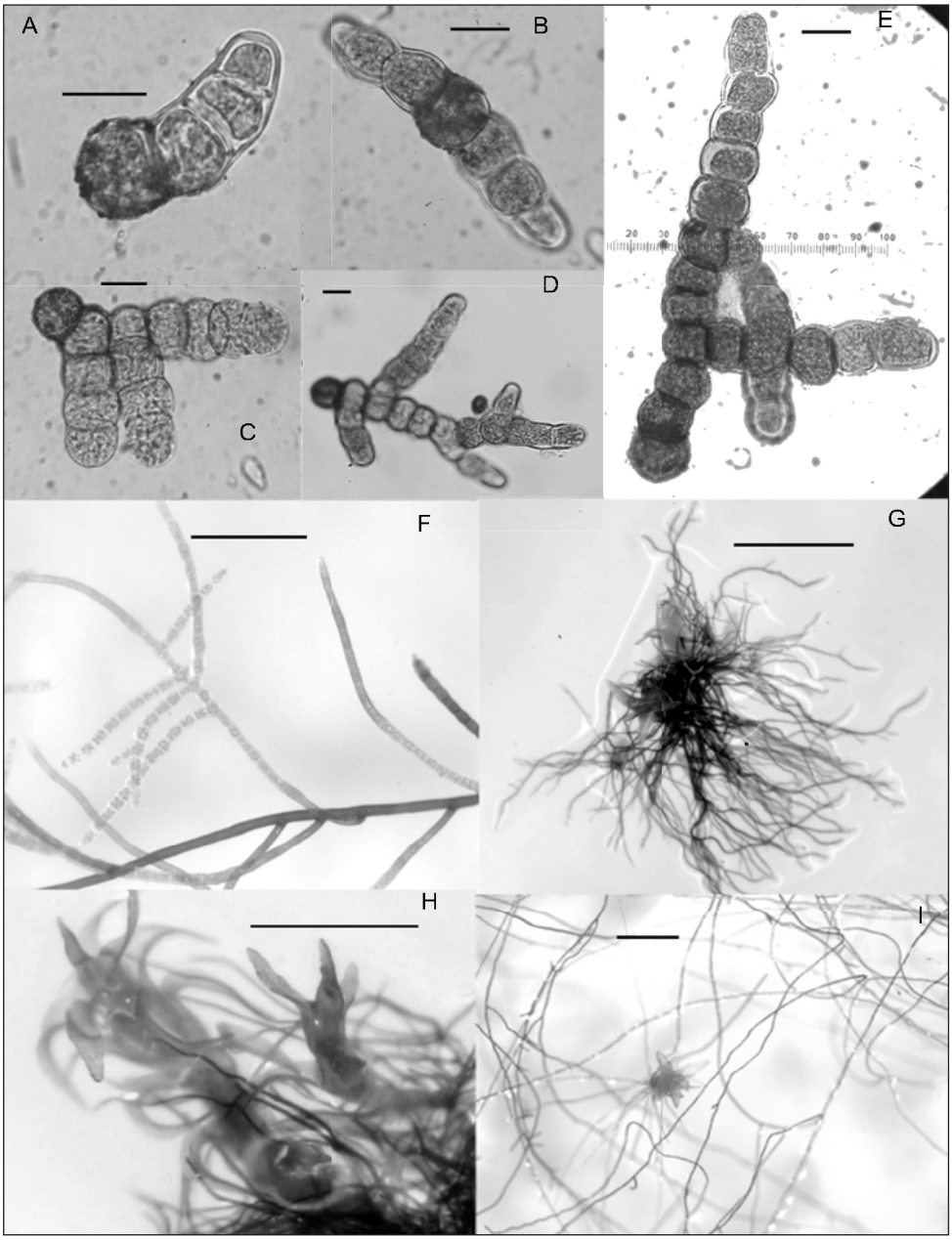

Fig. 2. Development of *Orthotrichum handiense in vitro* after inoculation of spores on axenic culture medium. **A.** Protonema obtained by *in vitro* culture after 46 days showing primary chloronema; **B**. *Idem* from another spore after 36 days; **C**. *Idem* after 55 days; **D.** *Idem* after 67 days; **E.** *Idem* after 76 days; **F.** Caulonema (brown) and secondary chloronema obtained after 90 days; **G.** Protonemata and gametophores; **H.** Detail of two gametophores after 5 months in culture. **I.** Young isolated plant growing from caulonema after 5 months in culture; Scale bars.- A-E: 30 μm; F: 200 μm; G-I: 0.5 mm.

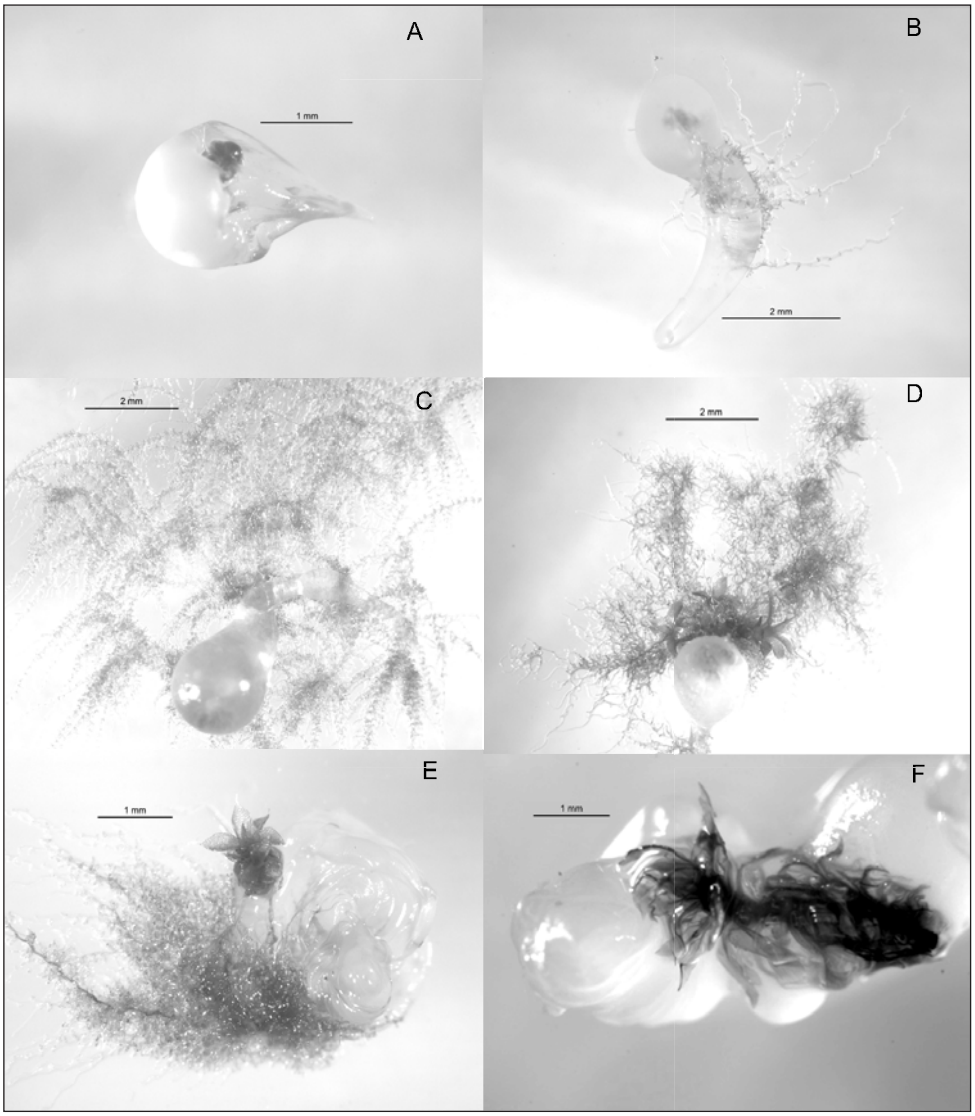

Fig. 3. Cryopreserved material. **A.** Alginate bead of *Entosthodon hungaricus* in good state of regeneration 5 weeks after freezing; **B.** Idem of *Entosthodon commutatus*; **C.** Alginate beads of *E. commutatus* in very good state of regeneration 5 weeks after freezing; **D.** *Idem* in *E. hungaricus*; **E.** *Idem* in *Funariella curviseta*; **F.** *Idem* in *Orthotrichum handiense*.

In this paper, the viability of the plant material was tested after a very short time on liquid nitrogen. But as in cryopreservation, freezing and thawing are the critical steps (Karlsson & Toner 1996), we conclude that for all the tested species the cryopreservation protocol followed could be suitable for long-term storage of the obtained germplasm.

Conclusions

The four species used in this work are easy to cultivate *in vitro* and survived after the process of cryopreservation, with a survival rate ≥ 54.5 ± 27.58%, meaning they can be preserved *ex situ* should reintroduction be necessary.

This procedure can also be applied to other Mediterranean mosses, once the red list of bryophytes of this area has been compiled.

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