In vitro micropropagation and long-term conservation of the endangered moss *Splachnum ampullaceum*

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Abstract

Protonema explants of *Splachnum ampullaceum* Hedw. were grown *in vitro* on 10 different mineral media with different sources and contents of nitrogen, in each case with or without added sucrose (30 g dm^{-3}) and/or B5 vitamins. The cultures were maintained at day/night temperatures $24 \pm 4/20 \pm 2$ °C and a 16-h photoperiod (irradiance of 25 µmol m^{-2} s^{-1}). Sucrose had little or no effect on protonema diameter and bud number in nitrate-only media or in high-ammonium media but markedly reduced bud number in low-ammonium media. Sucrose markedly reduced one-year explant survival rate in the low-ammonium media. The presence of B5 vitamins in such media markedly improved one-year survival, suggesting that the best medium for long-term culture of *Splachnum ampullaceum* is a medium containing ammonium at relatively low concentration as ammonium phosphate or sulphate (*e.g*. Gamborg's B5 medium), with added B5 vitamins but without added sucrose.

Additional key words: bryophytes, *in vitro* germplasm conservation, nitrogen source, sucrose, vitamins.

Introduction

In recent years the conservation of bryophyte biodiversity and the cultivation of bryophytes as sources of secondary metabolites have attracted increasing attention (Ducket *et al.* 2004). Bryophyte germplasm conservation is becoming increasingly relevant, since of 15 000 known bryophyte species, almost 40 % are considered threatened (Hallingbäck and Hodgets 2000). Studies of *in vitro* culture have established the nutritional requirements of some species, and their developmental patterns (Sabovljevic *et al.* 2003). There have been numerous successful studies of long-term storage and preservation of wild vascular plant species (*e.g.* Gagliardi *et al.* 2002, Tyagi *et al*. 2004). At present, however, optimal conditions for the medium-term and long-term conservation of bryophyte germplasm by *in vitro* culture are scarcely known.

Splachnum ampullaceum is included in the Red Data Book of European Bryophytes. Despite its wide distribution in both Europe and America, it is threatened due to the extreme specificity and fragility of its microhabitat (Marino 1991). In Galicia (NW Iberian Peninsula), *S. ampullaceum* occurs in peaty mountain habitats, mostly on wet meadows around streams (Reinoso and Rodríguez 1984). In view of the increasing scarcity of peatbogs in Spain and Portugal, *S. ampullaceum* is classified as an endangered species in the Iberian Peninsula (Sérgio *et al.* 1994, Hugonnot and Bardat 2001, Reinoso *et al.* 2002).

In vascular plants cultured *in vitro*, the importance of sucrose and nitrogen source for culture growth and development have been known for some time (Welander 1976). In some bryophytes, sucrose has been reported as a good carbon source that contributes to increase of biomass (Mehta and Chopra 1991, Sarla 1992). The importance of nitrogen has been reported for some bryophyte species cultured *in vitro* (Basile 1975). The aim of the present study was to compare 10 different mineral media with or without sucrose and/or B5 vitamins (Gamborg *et al.* 1968) for one-month culture of *S. ampullaceum.* The long-term survival of *S. ampullaceum* cultures was tested in five of these media, again with or without sucrose and/or B5 vitamins, over a one-year period.

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Abbreviations: An - Anderson's *Rhododendron* medium; B5 - Gamborg's medium; H - Heller's medium; KM - Kao and Michayluk medium; MS - Murashige and Skoog medium; N - Nitsch's medium; N_6 - Chu's medium, SH - Schenk and Hildebrandt medium; W - White's medium.

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Materials and methods

An aseptic culture was established from protonema of *Splachnum ampullaceum* Hedw. collected in peatbogs of the Serra do Xistral (NW Iberian Peninsula) in June 2001. The protonema was sterilized in 5 % sodium hypochlorite (*Domestos*[R]) for 5 min, and then washed three times with sterile distilled water. The culture was established on a medium without mineral salts (agar at 7 g dm^{-3} in distilled water). After 3 months, the developed protonema was subcultured aseptically on Murashige and Skoog (1962; MS) medium supplemented with 30 g dm^{-3} sucrose and solidified with 7 g dm^{-3} microagar (*Duchefa*, The Netherlands); the pH was adjusted to 5.8 before autoclaving for 20 min at 121 °C.

For medium testing, developed protonemas obtained in this way were subcultured on 40 different media: specifically, the 10 mineral media considered in each case *a*) without supplements, *b*) with 30 g dm^{-3} of sucrose, *c*) with B5 vitamins, *d*) with both sucrose and B5 vitamins. The ten mineral media tested were Anderson's *Rhododendron* medium (Anderson 1980, An), Chu's medium (Chu *et al.* 1978; N_6), Gamborg's medium (Gamborg *et al.* 1968; B5), Heller's medium (Heller 1953; H), Kao and Michayluk medium (Kao and Michayluk 1975; KM), MS medium, Nitsch's medium (Nitsch 1969; N), NLN $Ca(NO_3)_2$ free medium (Lichter 1981; NLN), Schenk and Hildebrandt medium (Schenk and Hildebrandt 1972; SH), and White's medium (White 1963; W). All cultures were performed in 60-mm Petri dishes (*Sterilin*, UK) containing 15 cm^3 of medium, and 5 explants of 4 ± 1 mm in length per dish. Each medium was tested in triplicate, with two plates per assay. The cultures were maintained in a growth chamber for 4 weeks at 24 ± 4 °C (day) and 20 ± 2 °C (night), with a 16-h photoperiod and daylight irradiance of 25 µmol m^{-2} s^{-1}. Protonematal colony diameter (hereafter, protonema diameter) and number of buds formed were measured weekly. Cultures in five media (An, MS, B5, N_6 and SH) were maintained for a year (52 weeks), for evaluation of long-term survival, with no medium replacement, and under the same conditions of light and temperature. The nitrogen-source compositions of these media are detailed in Table 1.

Table 1. Nitrogen sources in the different media [mg dm^{-3}]. The codes used for each medium are listed. [1] $NaNO_3$, [2] $Ca(NO_3)_2$ and KNO_3, [3] KNO_3.

Group	Code	NO_3^-	NH_4NO_3	$(NH_4)_2SO_4$	$NH_4H_2PO_4$
I	H[1]	600	0	0	0
	W[2]	380	0	0	0
	NLN[3]	125	0	0	0
II	An[3]	480	400	0	0
	KM[3]	1900	600	0	0
	MS[3]	1900	1650	0	0
	N[3]	950	720	0	0
III	N_6[3]	2830	0	463	0
	B5[3]	2500	0	134	0
	SH[3]	2500	0	0	300

We consider the 10 mineral media used in this study grouped in three groups: group I containing only nitrates as nitrogen source (H, W, NLN), group II containing nitrate plus NH_4^+ as NH_4NO_3 (An, KM, MS and N), and group III containing nitrate plus NH_4^+ as ammonium phosphate or ammonium sulphate (N_6, B5, SH).

Data normality was assessed by Lilliefor's modified Kolmogorov-Smirnov test and the Shapiro-Wilks test; for normal data, treatments were compared by analysis of variance; for non-normal data, treatments were compared by Kruskal-Wallis nonparametric analysis of variance. The proportions of explants surviving after one year of culture were compared among media by χ^2 tests, and the strength of dependence of survival on medium characteristics was assessed using Cramér's V statistic.

Results

Growth and gametophyte development on the different media: At the end of the 4-week culture period, mean protonema diameter was highest for explants grown on media containing only nitrate: H, NLN and W media (Fig. 1). By contrast, mean number of buds was highest for explants grown on media containing nitrate plus NH_4^+ as ammonium phosphate (medium SH) or as ammonium sulphate (media B5 and N_6); though note also medium An (Fig. 1).

χ^2 tests (Table 2) indicated that protonema diameter and number of buds in most cases did not vary significantly within groups, but did show significant variation among groups; we therefore consider each group as basically homogeneous (though note in particular that number of buds was markedly and significantly higher in medium An than in the other nitrate-plus-NH_4NO_3 media).

The group-I media (Fig. 2) showed similar time-courses, both for protonema diameter and bud production. Protonema diameter increased rapidly between weeks 1 and 2, then more slowly between weeks 2 and 3, then rapidly again between weeks 3 and 4. The four group-II media (Fig. 2) showed broadly similar time-courses for protonema diameter, with a more or less steady increase over the 4-week period. The group-III media (Fig. 2) showed similar time-courses for protonema diameter, which increased steadily over the 4-week period.

In all group-I media, mean bud number increased markedly between weeks 3 and 4 of culture (Fig. 2). In contrast with protonema diameter, the time-courses of bud production differed rather dramatically among the group-II media. In group-III, number of buds increased throughout the period in media N_6 and SH, and until week 3 in medium B5.

Cultures on W and NLN media, neither sucrose nor B5 vitamins had significant effects on either protonema diameter or bud number. In the medium H, protonema diameter varied significantly among the different treatments, but these variations were small in magnitude.

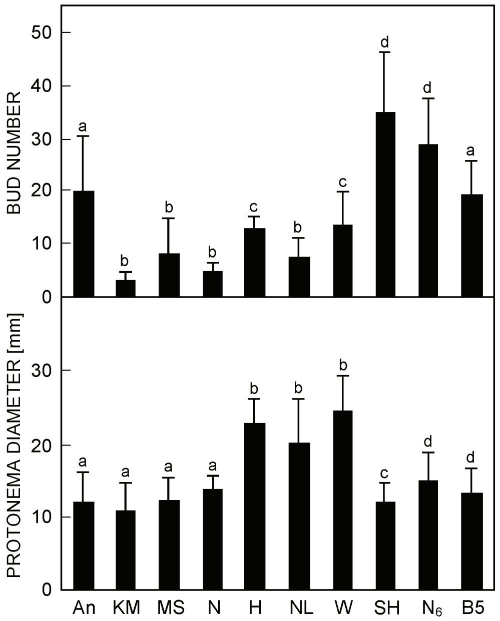

Fig. 1. Mean of both protonema diameter (n = 30) and bud number (n = 30) for explants cultured in each mineral medium without any supplements after 4 weeks. *Bars* indicate standard deviations. Means with the same letter do not differ significantly at the 5 % level (χ^2 tests).

Table 2. Results of χ^2 tests of homogeneity for the variables protonema diameter and number of buds, indicating within-group homogeneity in the nitrate-only and nitrate-plus-NH_4^+ groups, though not in the nitrate-plus-NH_4NO_3 group (see also Fig. 2). SH medium was not included in this analysis, since unlike media B5 and N_6 it contains ammonium phosphate, not ammonium sulphate. The analysis considers the data obtained at weeks 1, 2, 3 and 4.

Group	Variable	χ^2	df	P
I	diameter	1.598	2	0.450
	bud number	3.431	2	0.180
II	diameter	9.041	3	0.029
	bud number	26.301	3	0
III	diameter	2.135	1	0.144
	bud number	0.390	1	0.532

In all group-II and group-III media, sucrose and B5 vitamins had significant effects on both protonema diameter and bud number, with only two exceptions: bud number in medium N and protonema diameter in medium N_6. In general, sucrose markedly reduced bud number, but had only minor effects on protonema development (Fig. 2). Mean protonema diameter at week 4 was generally higher in the group-I (nitrate-only) media than in the group-II and group-III media. Mean bud number at week 4 was generally highest in the group-III media, though protonema diameter and bud number in the group-II medium An were similar to those seen in group III. These general patterns were basically unaffected by the presence of sucrose and/or B5 vitamins, indicating that they are dependent on the characteristics of the mineral medium (Fig. 2).

Morphological characteristics of cultures on the different media: Explants grown on the different media showed different morphological characteristics. Specifically, explants grown on the media containing only nitrate showed buds with yellowish green colour, larger than in the other media, and abundant protonema. Explants grown on the media containing nitrate plus NH_4^+ as NH_4NO_3 showed buds with dark green colour, and small phyllidia, though more abundant than in media containing nitrate only; protonema density (*i.e.* number of filaments per explant) was lower, and the buds were located exclusively in the central part of the explant. Explants grown on the media containing NH_4^+ as ammonium sulphate (B5 and N_6) showed similar morphological characteristics to those grown on media containing nitrate plus NH_4^+ as NH_4NO_3, though with the protonema showing extensive aerial growth perpendicular to the medium surface. Explants grown on the medium containing NH_4^+ as ammonium phosphate (SH) showed similar morphological characteristics to those grown on B5 and N_6, with aerial protonema growth, and additionally with buds growing in clusters from the initial explant.

In spite of the fact that general culture morphology was largely dependent on the characteristics of the mineral medium, sucrose and B5 vitamins had some effects on phenotype; these effects were generally similar independently of the mineral medium (Fig. 3). Sucrose induced an increase in protonema ramification, and higher filament density. Sucrose also intensified the reddish brown pigmentation of protonema and rhizoid cell walls, though the degree of intensification varied among the mineral media. In B5 medium sucrose induced a reddish rather than reddish brown coloration of the rhizoids (Fig. 3). The presence of B5 vitamins but not sucrose induced an intensification of the yellowish green coloration seen in the mineral media without complements.

Long-term conservation and explant survival: One-year explant survival was studied in media An, MS, B5, N_6 and SH (Fig. 4). χ^2 tests indicated significant effects of sucrose and/or vitamins in all five media, and again in all five media Cramér's V indicated strong dependence (V at least 0.50, $P < 0.001$) on medium characteristics (*i.e.* presence of sucrose and/or B5 vitamins). In all five media, the highest survival rates were obtained when added B5 vitamins were present. The best survival rate (100 %) was obtained in B5 medium with added B5 vitamins. The worst survival rates were obtained in the media with NH_4^+ as NH_4NO_3 (media An and MS); in many cases explants survived only 5 weeks in these media. The presence of sucrose reduced the one-year survival rate to zero in media An, MS and N_6, and by 50 % or more in media B5 and SH. In all cases explants surviving one year could be subcultured normally in the same fresh medium, *i.e.* there was no loss of viability.

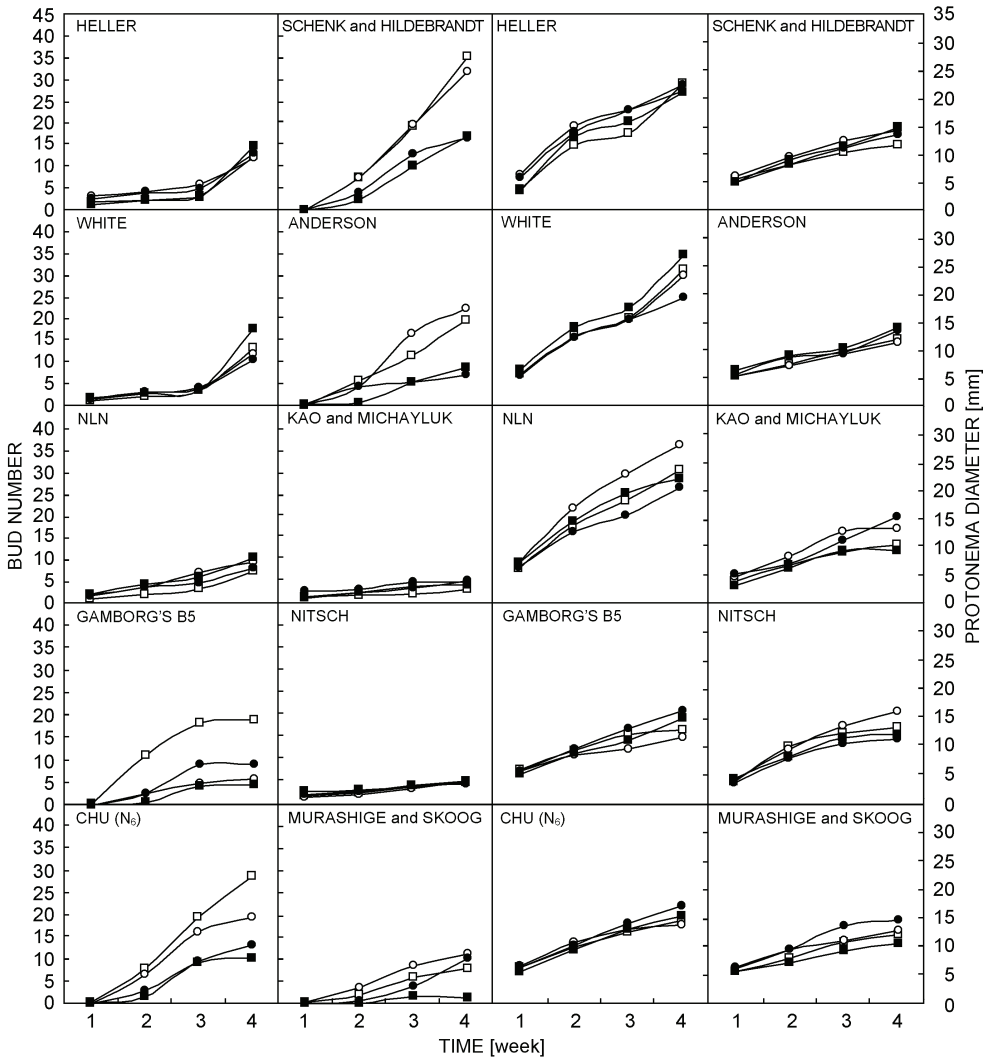

Fig. 2. Change of protonema diameter and bud number after 4 weeks of culturing on the media containing only nitrate as nitrogen source (media H, W and NLN), media containing nitrate plus ammonium nitrate (media An, KM, MS and N), and media containing nitrate plus ammonium sulphate or phosphate (media N_6, B5 and SH). Time-courses are shown for the mineral medium without supplements (open square), with added B5 vitamins (open circle), with added sucrose (closed square), and with added B5 vitamins and sucrose (closed circle). All values are means of the assays.

Discussion

In bryophytes, the vegetative development of the gametophyte comprises two stages: the filamentous protonema stage, and the budding gametophore stage that involves more complex cell differentiation. The *in vitro* development of *Splachnum ampullaceum* varies considerably depending on the mineral medium used. It has been reported previously for other bryophytes that the pattern of protonema development *in vitro* varies depending on the mineral medium (Alcalde 1996).

In the present study, we evaluated growth of *Splachnum ampullaceum* on 10 mineral media with different types of nitrogen source. Explants showed growth on all of the media. However, there were differences in growth rates, bud production rates, and

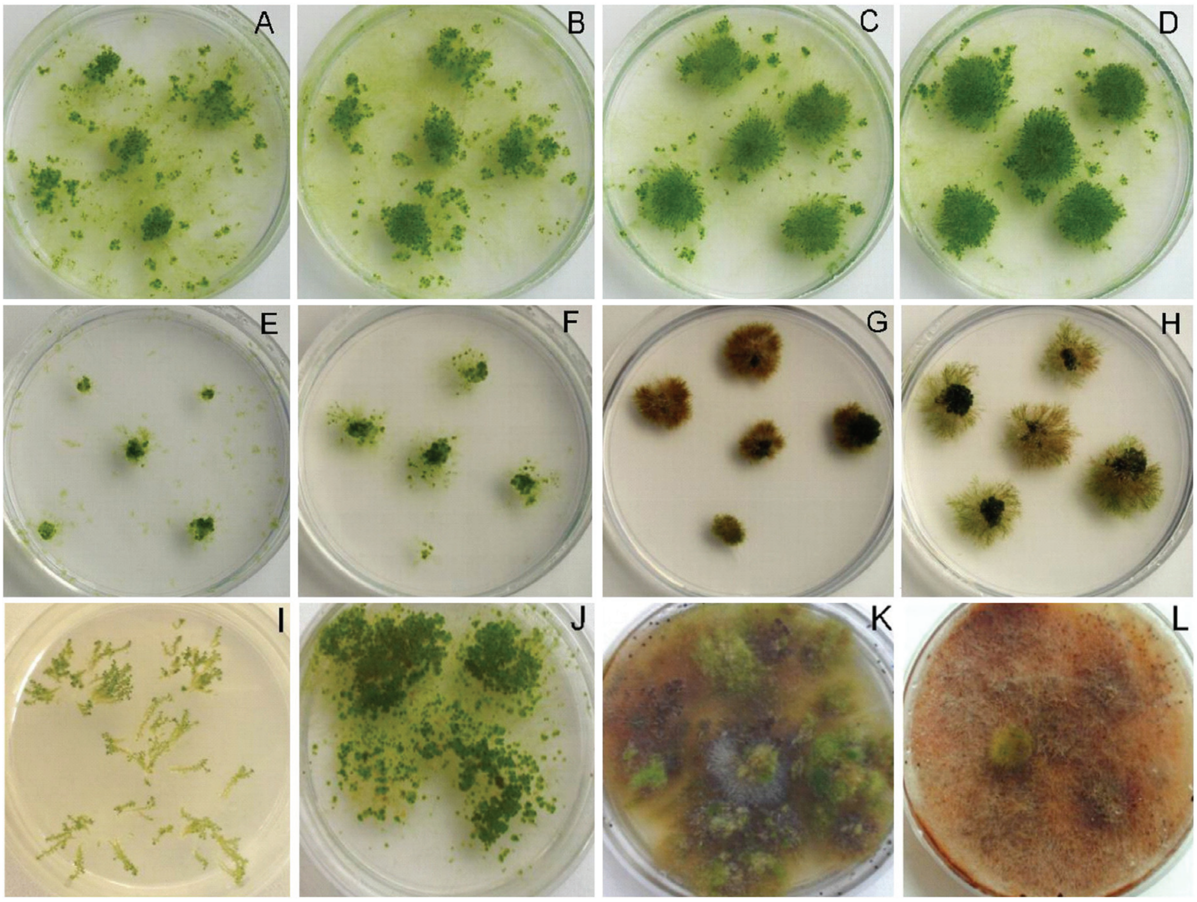

Fig. 3. *Splachnum ampullaceum* cultures were grown during two months on Heller (*A*,*B*,*C*,*D*), and Kao and Michayluk (*E*,*F*,*G*,*H*) media. Cultures were carried out without any supplement (*A*,*E*), with B5 vitamins (*B*,*F*), with sucrose (*C*,*G*), and with sucrose and vitamins (*D*,*H*). General appearance after one year of culture on medium B5: only mineral medium (*I*), with added B5 vitamins (*J*), with added sucrose (*K*), and with added B5 vitamins and sucrose medium (*L*).

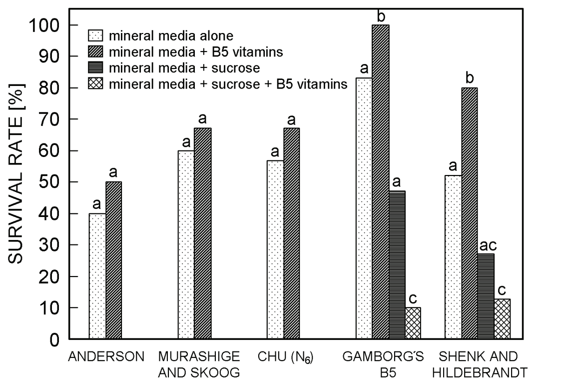

Fig. 4. Survival rates of *Splachnum ampullaceum* explants after 1 year of culture without medium replacement. Values are shown as means of percentage survival in each assay. Within each medium, bars with the same letter do not differ significantly at the 5 % level (χ^2 tests).

plant morphology. These differences were probably mainly attributable to the differences in nitrogen source, not to other differences in composition between the different media, since responses were basically homogeneous within each of the three groups of media (nitrate only, nitrate plus NH_4^+, and nitrate plus NH_4NO_3). Nitrogen source has previously been reported to be critical for determining growth in diverse bryophytes, significantly affecting net growth rate and/or the shape of the growth curve (Basile 1975). In the culture of many higher plants, the medium must contain not only nitrates but also a source of reduced nitrogen, or of the ammonium ion (George 1993). However, our results indicate that *S. ampullaceum* grows well - indeed best - on media containing only nitrate. Good bud production was likewise achieved on these media, though bud production was highest on the media containing nitrate plus NH_4^+ at low concentration, suggesting that low-concentration NH_4^+ may favour bud production. An appropriate nitrate/ammonium relationship for the plant can have stimulatory effects similar to those of cytokinins (George 1993). Indeed, bud formation in mosses, from specific protonema cells, is induced by cytokinins (Christianson and Hornbuckle 1999). Growth may be reduced by high concentrations of NH_4^+, even in the presence of high concentrations of NO_3^- (George 1993).

This may explain why explants grown on media KM, MS and N showed markedly lower bud production than explants grown on medium An (see Table 1, Fig. 1), which has similar NH_4^+:NO_3^- ratio but lower NH_4^+ concentration.

Some previous studies have found that some bryophytes show similar growth curves in nitrate-only media and in nitrate-plus-ammonium media, whereas other species show different growth curves in the two types of media (Basile 1975). We found that *Splachnum ampullaceum*, showed different growth curves in the two types of media. In nitrate-only media, growth (*i.e.* increase in mean protonema diameter) followed a double-sigmoidal-type curve, with practically zero growth between weeks 2 and 3; in nitrate-plus-ammonium media, by contrast, growth followed sigmoid-type curve. The time-course of bud production likewise differed between the two media: in nitrate-only media, hardly any buds were produced until the fourth week, when large numbers of buds were formed; whereas in nitrate-plus-ammonium media, bud production was more or less constant throughout the 4-week period. This different pattern in the three media containing only nitrate may be attributable to a need for reduced nitrogen for bud production. It has long been known that bud-forming cells compete to obtain resources from other protonema cells (Bopp 1963); if only nitrate is available, this will have to be reduced in the cells of the protonema, and will thus presumably become available to bud-forming cells more slowly and at lower concentrations. Likewise, the protonema cells will have to bear the increased metabolic cost of nitrate reduction and transport. On the other hand, mean bud size in media with nitrate only was larger than in the other media: this is perhaps attributable to the presence of ammonium in the other media, which may reduce pH and thus inhibit growth (Schofield 1985).

Thus the present results suggest that nitrogen form in the culture medium is of key importance for *in vitro* growth and development of the moss *Splachnum ampullaceum*. The differences detected within each group of media may be attributable to differences in the absolute or relative amounts of nitrate and NH_4^+, and/or to interactions with other components of the media.

Despite the importance of the mineral medium, interactions between its components and organic compounds, such as sugar, may affect culture development and growth (Welander 1976, Custódio *et al.* 2004). Vitamins, especially thiamine, also appear to be important determinants of various physiological processes in higher plants (George 1993, Vasudevan *et al.* 2004).

We observed a number of significant effects of sucrose (at 30 g dm^{-3}) on culture development, though interpretation of these effects is complex. Sucrose might be expected to have clearly positive effects on biomass increase, as it does in the culture of many vascular plant and bryophyte species (Metha and Chopra 1991, Sarla 1992); but in fact in this species sucrose had little or no effect (nitrate-only media, high ammonium-containing media), or negative effects (some ammonium-containing media). Notably, sucrose had negative effects on bud number and on survival rate in media containing ammonium at relatively low concentrations such as media An, N6, B5 and SH (Fig. 2); by contrast, it had no marked effects in the media containing high concentrations of ammonium. These results suggest *a*) that sucrose at 30 g dm^{-3} does not have strongly negative effects alone, but only in combination with ammonium and *b*) that at high ammonium concentrations the negative effects of sucrose are masked by ammonium toxicity. It has previously been reported that the use of sucrose at concentrations that are normal in the culture of higher plants may be excessive in bryophytes: in mosses like *Microdus brasiliensis*, and liverworts like *Riccia discolor*, it has been shown that the ideal sucrose concentration for *in vitro* culture is about 1 - 2 % (Mehta and Chopra 1991, Sarla 1992). There are several possible explanations for the negative effects of sucrose observed in the present study. First, cultured tissues may be affected by osmotic stress. Bryophytes are more prone to osmotic stress than vascular plants because of the lack of an effective protective cuticle. It has been shown that in many species of bryophytes osmotic stress inhibits protonema growth (Burch and Wilkinson 2002). Second, sucrose may reduce rates of photosynthesis. This possibility may explain why sucrose had negative effects only in the presence of ammonium. The assimilation of NH_4^+ takes place through the synthesis of carbonated compounds into which the nitrogen is incorporated (George 1993). Inhibition of photosynthesis by sucrose may lead to reduced fixation of ammonium, and thus to continuing ammonium toxicity.

The above results suggest that the best media are those with low concentrations of ammonium and no sucrose. In these media, B5 vitamins for culture of *S. ampullaceum* had no major effects on protonema development and no major effect or moderate negative effect on bud number (Fig. 2); more importantly, however, B5 vitamins increased survival rate (Fig. 4). Possible explanations for this positive effect on survival include *a*) that the vitamins themselves constitute a slow-release source of nitrogen and other essential elements; *b*) that they act as cofactors facilitating essential processes such as respiration; or *c*) that they help neutralize toxic molecules released by the plant.

In conclusion, the results of the present study indicate that the best medium for long-term culture of *Splachnum ampullaceum* is a medium containing ammonium at relatively low concentration as ammonium phosphate or sulphate (e.g. Gamborg's B5 medium), with added B5 vitamins but without added sucrose. Our results also suggest that long-term *in vitro* culture is a viable alternative to cryopreservation for long-term conservation of germplasm of bryophytes like *S. ampullaceum*: it is effective, has low cost, and cultures require little maintenance attention.

References

Alcalde, M., Abella, L., Estébanez, B., Ron, E.: Protonemal development under different culture conditions in *Bartramia* Hedw. (Musci). - J. Hattori bot. Lab. **79**: 107-114, 1996.

Anderson, W.C.: Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *R. occidentalis.* - Acta Hort. **112**: 13-20, 1980.

Basile, D.V.: A comparison of some macronutrient media used to culture bryophytes. - Bryologist **78**: 403-413, 1975.

Bopp, M.: Development of the protonema and bud formation in mosses. - J. Linn. Soc. Bot. **58**: 305-309, 1963.

Burch, J., Wilkinson, T.: Cryopreservation of protonemata of *Ditricum cornubicum* (Paton) comparing the effects of four cryoprotectant pretreatments. - CryoLetters **23**: 197-208, 2002.

Christianson, M.L., Hornbuckle, J.S.: Phenylurea cytokinins assayed for induction of shoot buds in the moss *Funaria hygrometrica*. - Amer. J. Bot. **86**: 1645-1648, 1999.

Chu, C.C., Wang C.C., Sun C.S., Hsu C. Yin K.C., Chu C.Y., Bi F.Y.: Establishment of an efficient medium for anther culture of rice, through comparative experiments on the nitrogen sources. - Scientia sin. **18**: 659-668, 1975.

Custódio L., Martins-Louçao M. A., Romano A.: Influence of sugars on *in vitro* rooting and acclimatization of carob tree. - Biol. Plant. **48**: 469-472, 2004

Duckett, J.G., Burch, J., Fletcher, P.W., Matcham, H.W., Read, D.J., Russel, A.J., Pressel, S.: *In vitro* cultivation of bryophytes: a review of practicalities, problems, progress and promise. - J. Bryol. **26**: 3-20, 2004.

Gagliardi, R.F., Pacheco, G.P., Valls, J.F.M., Mansur, E.: Germplasm preservation of wild *Arachis* species through culture of shoot apices and axillary buds from *in vitro* plants. - Biol. Plant. **45**: 353-357, 2002.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

George, E.F.: Plant Propagation by Tissue Culture. - Exegetics Limited, Edington 1993.

Hallingbäck, T., Hodgetts, N.: Status Survey and Conservation Action Plan for Bryophytes: Mosses, Liverworts and Hornworts. - I.U.C.N., Gland - Cambridge 2000.

Heller, R.: Researches on the mineral nutrition of plant tissues. - Ann. Sci. nat. Bot. Biol. vég. **14**: 1-223, 1953.

Hugonnot, V., Bardat, J.: Données sur l'écologie de quatre bryophytes rares dans le Massif Central. - J. bot. Soc. France **14**: 37-40, 2001.

Kao, K.N., Michayluk, M.R.: Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. - Planta **126**: 105-110, 1975.

Lichter, R.: Anther culture of *Brassica napus* in a liquid culture medium. - Z. Pflanzenphysiol. **103**: 229-237, 1981.

Marino, P.C.: Dispersal and coexistence of mosses (Splachnaceae) in patchy habitats. - J. Ecol **79**: 1047-1060, 1991.

Mehta, P., Chopra, R.N.: The effect of some chemical factors on growth and fertility in male clones of the moss *Microdus brasiliensis*. - Cryptog. Bryol. Lichénol. **12**: 55-62, 1991.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio-assays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nitsch, J. P., Nitsch, C.: Haploid plants from pollen grains. - Science **163**: 85-87, 1969.

Reinoso, J., Rodríguez, J.: [*Splachnum ampullaceum* Hewd. in Spain.] - Bol. Soc. Broteriana **57**: 213-218, 1984. [In Spanish.]

Reinoso, J., Rodríguez-Oubiña, J., Viera, M.C.: [Red list of Bryophytes of Galicia (N.W. Spain)] - Nova Acta Cient. Compostelana (Biol.) **12**: 83-93, 2002. [In Spanish.]

Sabovljevic, M., Bijelovic, A., Dragicevic, I.: *In vitro* culture of mosses*: Aloina aloides* (K.F. Schultz) Kindb., *Brachythecium velutinum* (Hedw.) B.S. & G. and *Grimmia pulvinata* (Hedw.) Sm. - Turk. J. Bot. **27**: 441-446, 2003.

Sarla: *In vitro* studies on male clone of *Riccia discolor*: effect of sugars. - J. Hattori bot. Lab. **72**: 141-150, 1992.

Sérgio, C., Casas, C., Brugués, M., Cros, R.M.: Red List of Bryophytes of the Iberian Peninsula. - I.C.N., Lisboa 1994.

Schofield, W.B.: Introduction to Bryology. - MacMillan, New York 1985.

Schenk, R.U., Hildebrandt, A.C.: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. - Can. J. Bot. **50**: 199-204, 1972.

Tyagi, R.K., Yusuf, A., Dua, P., Agrawal, A.: *In vitro* plant regeneration and genotype conservation of eight wild species of *Curcuma*. - Biol. Plant. **48**: 129-132, 2004.

Vasudevan, A., Selvaraj, N., Ganapathi, A., Kasthurirengan, S., Ramesh Anbazhagan, V., Manickavasagam, M.: Glutamine: a suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L. - Biol. Plant. **48**: 125-128, 2004.

Welander, T.: Effects of nitrogen, sucrose, IAA and kinetin on *Beta vulgaris* grown *in vitro*. - Physiol. Plant. **36**: 7-10, 1976.

White, P.R.: The Cultivation of Animal and Plant Cells. 2nd Ed. - Ronald Press, New York 1963.