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From axenic spore germination to molecular farming

One century of bryophyte in vitro culture

Received: 19 July 2004 / Revised: 25 September 2004 / Accepted: 1 October 2004 / Published online: 19 November 2004
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Abstract The first bryophyte tissue culture techniques were established almost a century ago. All of the techniques that have been developed for tissue culture of seed plants have also been adapted for bryophytes, and these range from mere axenic culture to molecular farming. However, specific characteristics of bryophyte biology—for example, a unique regeneration capacity—have also resulted in the development of methodologies and techniques different than those used for seed plants. In this review we provide an overview of the application of in vitro techniques to bryophytes, emphasising the differences as well as the similarities between bryophytes and seed plants. These are discussed within the framework of physiological and developmental processes as well as with respect to potential applications in plant biotechnology.

Keywords Liverwort · Moss · *Marchantia* · Regeneration · *Physcomitrella*

Why in vitro culture of bryophytes?

The year 2002 was the 100th anniversary of the publication of Gottlieb Haberlandt's now famous treatise "Experiments on the culture of isolated plant cells" (Haberlandt 1902), a report celebrated as showing brilliant vision and being the starting point of plant tissue

culture in that it ultimately resulted in the development of manifold techniques and commercial applications. While several reviews and historical overviews have been published on the topic of plant tissue culture (see Laimer and Rucker 2003), these have usually included only experiments and publications on seed plants. Lal (1984), in his review on bryophyte culture, observed that "it is not commonly realised, sometimes not even by professed botanists, that the technique of culturing plant tissues and organs under sterile conditions was first established and profitably employed in bryophytes, especially mosses". Lal (1984) refers to a publication of Servettaz in 1913 as the first report on in vitro culture of bryophytes. Servettaz (1913) himself, however, cites Becquerel (1906) who described the development of pure cultures of protonema of *Atrichum undulatum* and *Hypnum velutinum*. Consequently, the conclusion can be drawn that bryophyte in vitro culture has a history of almost 100 years, which is indeed more than what has been reported for seed plants based on the fact that most chronologists agree that the first asymbiotic germination of orchid seeds reported by Knudson (1922) and the embryo culture of interspecific *Linum* hybrids published by Laibach (1925) represent the first successful in vitro cultures of seed plants. During this time, axenic cultures of the liverwort *Marchantia polymorpha*, which has been extensively studied with respect to the effect of various environmental conditions on its development (see below), appeared to be a routine technique, as described by Lilienstern (1927).

This raises the question of why in vitro techniques were first established in bryophytes, which, in turn, requires a definition of the term "in vitro culture". There is a plethora of different definitions given in the literature, most of which include three important characteristics. (1) The plants, plant tissues or cells are cultured axenically (although some people raise the question of whether axenic cultures are those whose contaminating endophytes still have to be identified). (2) Tissues or cells to be cultured are excised from the mother plant. Here we would like to include the in vitro culture of "complete" plants because in vitro culture does not stop after regen-

Communicated by P.P. Kumar

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eration and since the definition of what a complete plant is seems to be difficult when dealing with bryophytes. (3) Plants, plant tissues or cells are cultured on artificial (defined) nutrient media. Again, this point has to be modified, because from the early days of seed plant tissue culture up to and including the present time undefined medium supplements (e.g. coconut water and casein hydrolysate) have been used. Therefore, in this review we use the term “in vitro culture” to describe the wide range of techniques that are based on the axenic culture of complete plants, isolated tissues or cells.

So, let us return to the question of why was in vitro culture first established in bryophytes? The relative ease of starting axenic cultures using “aseptically packaged spores, ready to be inoculated onto sterilised media” has been mentioned as one of the reasons (Lal 1984). Another important reason is the unique characteristics of regeneration in bryophytes. Vöchting (1885) published detailed experiments on regeneration in liverworts. Goebel (1908) stated that “it is assumed that every protoplasma-containing cell of a liverwort is able to regenerate a new plant.” Therefore, developmental biologists working in the early years of the 20th century extensively studied bryophytes. Using the liverwort *M. polymorpha*, these researchers evaluated the effect of various environmental factors such as concentration and composition of nutrient solutions, temperature variations, intensity and quality of light and photoperiod on the development of gemmae. In this context, Voth and Hamner (1940) cite as many as 17 experimental reports published between 1874 and 1939. Although several of these publications describe a culture on nutrient solutions solidified with agar, most were not axenic—i.e. not “in vitro cultures”—according to our definition. For example, Förster (1927) described a “wash” of the agar, because “otherwise there would be a massive growth of fungi and bacteria, if one does not work under sterile conditions, which would certainly be a severe aggravation”. In contrast, Liliensern (1927) worked with axenic cultures and stressed that “problems (i.e. contradictory results regarding the effect of light on sexual organ development in bryophytes) can probably be solved most appropriately by use of axenic culture”.

The purpose of this review is to summarise the development and application of various in vitro techniques in bryophytes and compare these to the in vitro techniques applied in studying seed plants, thereby updating the review of Lal (1984).

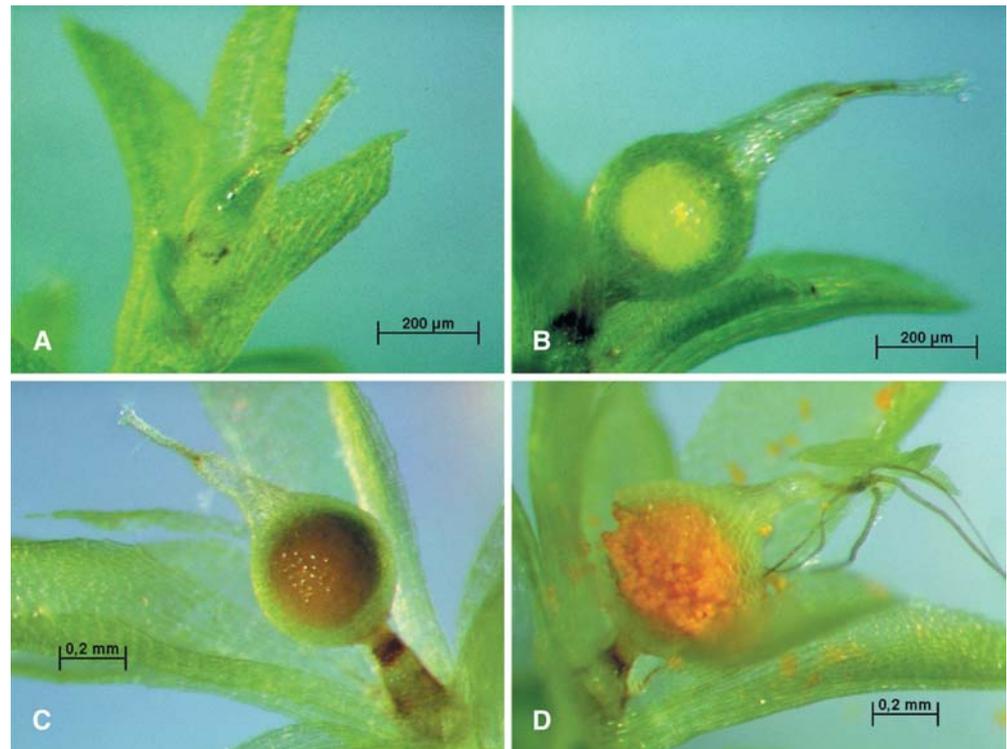
Culture conditions

In contrast to the majority of seed plant in vitro culture systems, in vitro cultures of bryophytes usually grow photoautotrophically. Thus, plant physiology and development in vitro is probably quite similar when compared to the plant in its natural environment. Correspondingly typical effects of in vitro culture of seed plants such as the reduced size of all organs or the problem of hyperhydricity have—to our knowledge—never been documented

in bryophytes. As already mentioned by Lal (1984), a wide variety of media is described in the literature. At one extreme are the relatively simple salt solutions, such as the one described by Knop (1884), which is still widely used, especially in biotechnical applications involving the moss *Physcomitrella patens* (see below). This medium does not contain any microelement supplementation, thus relying on impurities of the macroelement salts (which queries of course the term “defined medium”!). Interestingly, the same medium was also used by Haberlandt for his experiments on the culture of isolated cells of *Lamium purpureum* (Haberlandt 1902). At the other extreme are the very complex “full media” used for the culture of auxotrophic mutants. Such media have been described by Ashton and Cove (1977) and by Schween et al. (2003). In these media in addition to the macro- and microelement salts, amino acids, vitamins and a variety of other organic compounds that are known to be intermediates in several metabolic pathways are added. Some of these compounds, although they are not plant growth regulators, have been shown to exhibit a distinct effect on plant development. For example, when the moss *Physcomitrella patens* is cultured, the addition of ammonium tartrate to the medium promotes the development of chloronema and suppresses differentiation of caulonema cells (Ashton and Cove 1977; Jenkins and Cove 1983). The reason for this is still unknown, although it has been shown that this effect is partly mediated by the pH of the culture, which is lower in ammonium tartrate-supplemented medium, probably due to ammonium uptake (Hohe and Reski 2002; Hohe et al. 2002a). Hädeler et al. (1995) observed that the choice of the gelling agent used to solidify the media influenced cytokinin sensitivity during the in vitro culture of *Physcomitrella patens*. Gelrite mimicked cytokinin activity, and plants only responded to exogenous cytokinin when grown on agar-solidified medium.

Liquid cultures also have been developed for a variety of bryophytes. Liquid cultures of *Funaria hygrometrica* have been used extensively for physiological studies (e.g. Johri and Desai 1973; Handa and Johri 1976; Sharma et al. 1979). Ohta et al. (1977) reported the establishment of a cell suspension culture of *Marchantia polymorpha*, and Katoh (1983) published a detailed study on the growth kinetics of suspension cultures of *Marchantia polymorpha*. Liquid cultures of the moss *Physcomitrella patens* were described by Wang et al. (1981). One has to keep in mind, however, that moss liquid cultures are quite different from seed plant cell suspensions. The latter are mostly comprised of suspended callus cells or cell aggregates—i.e. cells in morphologically unorganised growth (although during the last decade there has been a rising number of publications on liquid cultures of differentiated propagules, often in so-called temporary immersion systems). This is in sharp contrast to those moss “suspension” cultures containing ruptured protonema filaments, as is the case for the above-mentioned cultures of *Funaria hygrometrica* and *Physcomitrella patens*. These cultures are kept as permanently growing suspensions by regular disintegration of the filaments and a complete

Fig. 1 Sporophytes of the moss *Physcomitrella patens* induced in vitro by the application of cool temperatures (15°C) and short-day length [8 h (light), 16 h (dark)]. **a** Young sporophyte with calyptra, **b** sporophyte with green-coloured spores, **c** sporophyte with ripe, brown spores, **d** bursting sporophyte releasing the spores



change of medium, both of which impede differentiation beyond the protonema stage by initiating chloronema regeneration and removing endogenously produced phytohormones.

Early in the 20th century, researchers revealed that development and reproduction in many bryophytes is dependent on light and temperature conditions (see Wann 1925; Lilienstern 1927; Förster 1927; Stephan 1928). To our knowledge, Lilienstern (1927) was the first to work explicitly with axenic cultures in this experimental context, and it was she who also emphasised the need of axenic cultures for obtaining reproducible results in these experiments on developmental biology. Benson-Evans (1964) summarised her results from “field collections as well as pure cultures” as follows: “... both, long and short day plants occur in the bryophytes and ... gametangia may be induced by one or the other condition. ... in many species the photoperiod is only operative over a certain temperature range”. The first experiments on photoperiodism in mosses were carried out by Benson-Evans (1961) and Hughes (1962). The physiology of sexual reproduction in mosses, including light and temperature effects, has been reviewed by Bopp and Bhatla (1990). However, new results are still being published in this “classical” field: Hohe et al. (2002b) describe the strong dependency of sporophyte development in *Physcomitrella patens* on daylength (short day) and temperature ($\leq 18^\circ\text{C}$), which they correlated with the expression level of a MADS-Box gene (Fig. 1). Yamaoka et al. (2004) generated by means of particle-bombardment mediated mutagenesis a mutant of *Marchantia polymorpha* that forms sexual organs constitutively in contrast to

the wild-type, which develops sexual organs only under long-day conditions. Genetic analysis revealed that the phenotype was caused by a mutation in a single autosomal locus which probably controls signal transduction in the transition to sexual reproduction.

When working with a bryophyte in vitro culture for whatever reason, one has to bear these reactions in mind and carefully adjust the conditions in the growth chamber. It is very easy to judge a bryophyte in vitro culture to be sexually sterile if one does not mimic the changes in environmental conditions that usually occur in its natural environment. Nevertheless, we observed a drastic reduction in the number of developing sporophytes in axenic cultures of *Physcomitrella patens* even under inducing conditions if the plants were propagated vegetatively over several months without sexual reproduction (data not published). Thus, if a certain genotype is kept in vitro for a “longer” period of time, regular “renewal” of the cultures by sexual reproduction is recommended.

To date various bryophyte cultures have evolved as model systems in plant physiology (Wood et al. 2000). In addition to *P. patens* and *Funaria hygrometrica*, cultures of *Ceratodon purpureus* and *Tortula ruralis* are also widely used. The moss *Tortula ruralis* has been chosen for its desiccation tolerance to study stress tolerance mechanisms (e.g. Oliver et al. 2000; Proctor 2001). *Ceratodon purpureus* serves as model system for studies on phototropism and gravitropism (e.g. Esch et al. 1999; Kern and Sack 1999; Schwuchow et al. 2002).

Regeneration and derived culture techniques

Kreh (1909) emphasised that in liverworts the regeneration process resembles the development from the spore, only the germ tube was typically bypassed unless regeneration occurred from single cells. In surveying the literature on this topic, Knoop (1984) introduced the term of “redifferentiation”, meaning that regenerating cells of explants shift back to earlier stages of differentiation, depending on the size and the differentiation stage of the explant itself. Whereas small explants usually regenerate chloronema, larger fragments of, for example, the gametophyte might as well only shift back to intermediate stages of differentiation. These regeneration characteristics are different from those of seed plants, where indirect regeneration via a callus phase plays an important role in addition to pathways of direct regeneration. Moreover, regeneration events in seed plants are mostly triggered by the exogenous application of plant growth regulators. In bryophytes, the induction of stably growing callus cultures was first published by Allsopp (1957). Callus growth was initiated in the two liverworts *Fossombronia pusilla* and *Reboulia hemisphaerica* by adding 2% glucose to Knop’s medium. Following the transfer of the resulting callus to inorganic medium, the cultures began to differentiate. However, the author emphasised that other bryophytes undergo normal differentiation also on glucose-containing medium.

The “easy” direct (i.e. without callus phase) regeneration of bryophytes of course facilitates those in vitro techniques that rely on regeneration processes. This is certainly true in protoplast regeneration, which is by definition the true regeneration of whole plants from single cells from which the cell wall has been removed and, thus, have lost any organised contact to the tissue in which they were formerly embedded. The first attempts of protoplast fusion in seed plants were carried out in 1909 by Küster (1909). However the first successful plant regeneration from protoplasts was published only in 1971 (Takebe et al. 1971), with the first interspecific hybridisation using protoplast fusion being reported 1 year later (Carlson et al. 1972), both research groups working with tobacco. In addition to researchers developing a technique that successfully isolates the “naked” protoplast, rebuilds a cell wall and induces cell division, the technique also has to enable plants to be regenerated from the resulting microcallus. This last limiting step is omitted in techniques for bryophytes. Binding (1966) conducted experiments on protoplast regeneration and protoplast fusion with mosses (*Funaria hygrometrica*, *Physcomitrium eurystomum*, *P. piriforme* and *Bryum erythrocarpum*). He succeeded in the regeneration and fusion of the protoplasts, but not in the regeneration of the fusion products. Detailed reports on the successful regeneration and fusion (including regeneration) of protoplasts of the liverwort *Sphaerocarpos donellii* were published by Schieder and Wenzel in the early 1970s (Schieder and Wenzel 1972; Wenzel and Schieder 1973; Schieder 1974). These authors were also the first to work on enzymatic degradation

of cell walls in bryophytes. The regeneration of protoplasts of *Physcomitrella patens* was reported by Stumm et al. (1975). In these experiments, protoplasts were always isolated from differentiated protonema cells. In contrast, Ono et al. (1979) were the first to report the regeneration of whole plants from protoplasts isolated from (undifferentiated) *Marchantia polymorpha* cell cultures.

In addition to applying protoplast culture techniques for the production of somatic hybrids and for executing different physiological experiments, protoplast isolation and regeneration is of special interest because the genetic transformation of protoplasts is an important transformation method in bryophytes (see below). In this context, large-scale liquid culture has been established in the moss *Physcomitrella patens* for the mass production of protoplasts (Hohe and Reski 2002). Culture conditions during protonema growth in the suspension proved to be very important for both the successful isolation of protoplasts (Hohe and Reski 2002) and for efficient transformation (Hohe et al. 2004). An acidic pH of 4.5 greatly enhanced the number of protoplasts obtained by digestion with the enzyme mix Driselase. It is assumed that this effect was caused by a different constitution of the cell walls of cells grown in medium with an acidic pH.

Another culture technique that is highly dependent on the regeneration capacity of a culture is the cryopreservation of vegetative plant material. The first report on successful cryopreservation of *Marchantia polymorpha* was published by Takeuchi et al. (1980). Three years later Grimsley and Withers (1983) reported cryopreservation of the moss *Physcomitrella patens*. The most important process in the cryopreservation of tissue is the controlled removal of water from the cells in order to avoid the deleterious formation of ice crystals. This is achieved by preconditioning the plant material in a cryoprotectant followed by slow cooling at a precisely defined rate (Grout 1995). Several protocols have been published with respect to the application of various freezing procedures on different bryophyte species (Leverone and Pence 1993; Christianson 1998; Pence 1998). Burch (2003) compared a protocol that included pretreatment and encapsulation with a control protocol in which there was no pretreatment and found that desiccation-tolerant mosses survive the cryopreservation procedure without pretreatment. Recent publications focus on the development of large-scale methods. Burch and Wilkinson (2002) compared different cryoprotectant pretreatments for the long-term storage of protonemata of the endangered Cornish path moss, *Ditrichum cornubicum* as a prelude to a programme involving the in vitro conservation of rare and endangered UK bryophytes. Schulte and Reski (2004) reported the high-throughput cryopreservation of 140,000 mutants of the moss *Physcomitrella patens*. These researchers obtained regrowth rates of up to 100%, which can be attributed to both the moss’s high tolerance to stress and its high regeneration ability.

Genetic transformation

Compared to the other techniques described herein, genetic transformation is the youngest discipline. The first stable genetic transformation of plant cells was reported in tobacco by Krens et al. (1982), who used the technique of polyethylene glycol-mediated Ti-plasmid DNA uptake of protoplasts. The generation of stably transformed bryophytes was first documented in 1991 for the moss *Physcomitrella patens*, also using the technique of protoplast transformation (Schaefer et al. 1991). This protocol is rather similar to those published for the protoplast transformation of seed plants (Potrykus and Spangenberg 1995), and although it has undergone several slight modifications by different working groups, it is still applied in its basic form today and has also been adapted for the large-scale mass production of moss mutants (Nishiyama et al. 2000; Egner et al. 2002). In a slightly modified form, this protocol is also used for genetic transformation of the moss *Ceratodon purpureus* (Thümmler et al. 1992; Zeidler et al. 1999). A second method used for transforming bryophytes is particle bombardment. This has been applied to *Physcomitrella patens* (Sawahel et al. 1992; Cho et al. 1999) and *Marchantia polymorpha* cells (Irifune et al. 1996) and thalli (Takenaka et al. 2000).

Although early reports describe the attachment of *Agrobacterium* to moss (*Phylloisella selwynii*) cell walls (Whatley and Spiess 1977; Spiess et al. 1984), it is commonly stated that mosses are not susceptible to *Agrobacterium* infection (Zeidler et al. 1999). In fact, there is only one publication—by Nasu et al. (1997)—on *Agrobacterium*-mediated transformation of a bryophyte, the liverwort *Marchantia polymorpha*. Here, undifferentiated cultured cells were transfected by coculture with *Agrobacterium tumefaciens*.

Two characteristics typify the genetic transformation of the moss *Physcomitrella patens*. Following transformation with non-linearised plasmids, Ashton et al. (2000) observed that the transfected moss cells and subsequent regenerating plants were able to replicate these extrachromosomal transgenic elements when subcultured repeatedly on selective medium. When the plants were placed on non-selective (“release”) medium between two selection rounds, these extrachromosomal elements were “cured.” Comparing the transformation efficiencies of circular and linearised DNA, Hohe et al. (2004) determined that only 0.2% of the plants that survived a first selection period following transformation with circular DNA also survived the second round of selection. This indicates that the plants had lost the extrachromosomal DNA carrying the resistance gene between the first and second round of selection. In contrast, 16% of the plants surviving a first selection round also survived a second selection round when they were transformed with linearised DNA. The difference in survival is due to two factors: for linearised DNA, the rate of stable integration of the transgene was higher and the total number of transiently transformed plants was lower, probably be-

cause extrachromosomal replication only works for circular elements.

The second and most important characteristic of *P. patens* is its ability to integrate foreign DNA into its genome by homologous recombination at very high rates, a trait that has to date not been described in any other plant. The phenomenon was postulated by Kammerer and Cove (1996) and first described by Schaefer and Zryd (1997). This natural genetic mechanism can be used efficiently for the generation of knockout plants, which facilitates the analysis of gene functions. Strepp et al. (1998) used this system to prove the function of a homologue of the bacterial cell division protein FtsZ in chloroplast division, while Girke et al. (1998) showed that the knockout of *PPDES6*, which encodes a $\Delta 6$ -acyl-group desaturase, dramatically altered the fatty acid patterns of the resulting plants. Transgenic plants with a desired loss and directed gain of functions have been proposed for use as production systems in molecular farming (Decker and Reski 2004). However, this “genetic” topic is exceeding the theme of the present article and has been summarised in various recent reviews (Reski 1998, 1999; Schaefer 2001, 2002; Hohe and Reski 2003). Interestingly, the reason for the existence of this unique characteristic is still unknown. Moreover, it is not known whether other bryophytes integrate foreign DNA via homologous recombination as well. Yamaoka et al. (2004) report on the random insertion of foreign DNA in *Marchantia polymorpha* following particle bombardment. However, as homologous recombination has only been described in *P. patens* following protoplast transformation, it cannot be excluded that the transformation procedure might also play a role.

Large-scale culture and biotechnological applications

The use of bryophytes for the production of valuable compounds requires large-scale in vitro culture systems since counterparts to the field or greenhouse culture systems for agricultural or horticultural crops have not been developed to date due to the very special ecological requirements of these plants (Becker 2001). Thus, a scale-up of in vitro cultures in bioreactors is necessary for the large-scale production of compounds by bryophytes (Fig. 2). First reports on the culture of various bryophytes in bioreactors were published in 1988 (Boyd et al. 1988; Katoh 1988; Rudolph et al. 1988). Wilbert (1991) described the cultivation of the moss *Leptobryum pyriforme* in a 250-l bioreactor. Bioreactor technology has to be adapted to the special requirements of the respective organism. In the case of bryophytes one has to keep in mind that the cultures are growing photoauto- or mixotrophically. Therefore, either relatively small (up to about 20 l) glass bioreactors are used or larger in situ sterilisable reactors have to be equipped with inside illumination (Wilbert 1991). Other constructions, such as a reactor made of glass tubes, can be scaled-up to virtually any volume with illumination from outside (Lucumi et al. 2003). Hata et al. (1997) developed a strategy to regulate

Fig. 2 Scaling-up of in vitro cultures of the moss *Physcomitrella patens*. **a** Cultures on solidified medium in petri dishes, **b** liquid cultures of protonema in Erlenmeyer flasks, **c** stirred tank glass bioreactors with a working volume of 5 l, **d** 30-l tubular photoreactor (Lucumi et al. 2003)



the external light irradiation of a bioreactor culture of *Marchantia paleacea* taking into account the profile of light intensity distribution as a variable dependent on the concentration of the suspended cells. The same group analysed the growth characteristics of photoautotrophic and photomixotrophic bioreactor cultures of *Marchantia polymorpha* (Hata et al. 1999, 2000a, 2000b; Hata and Taya 2000).

The high susceptibility to shear stress that is a major constraint in bioreactor cultures of seed plants (see Doran 1999; Sajc et al. 2000) is not a problem in bryophyte culture in the protonema stage. Moss protonema cultures even have to be stirred at 400–500 rpm (Reutter and Reski 1996; Hohe et al. 2002a) in order to maintain continuous disintegration of the protonema filaments, which would otherwise grow into large pellets. Using this technique, Hohe et al. (2002a) obtained a semi-continuously growing bioreactor culture of *Physcomitrella patens* and grew it stably for 7 weeks in a 5-l bioreactor with a total yield of 51 l of suspension culture. Boyd et al. (1988) using airlift fermentors obtained short (15 days) continuous cultures.

In contrast to seed plants, the analysis of bryophyte secondary metabolism commenced relatively late—around 1960. The reason for this according to Becker (2001) is twofold: (1) distinct medicinal or poisonous plants are missing within this group; (2) it is often difficult to obtain enough material for analysis. However, to date many detailed studies have been carried out on the chemical constituents of bryophytes, the results of which have been summarised in several recent reviews (Asa-

kawa 1995, 2004; Mues 2000). In contrast to seed plants, where the amount and stability of (undifferentiated cell) in vitro culture systems is a major problem, Becker (2001) reports that bryophyte in vitro cultures are producing qualitatively and quantitatively the same secondary metabolites as corresponding “wild” plants, which might be a consequence of the differentiated status of most bryophyte cultures. This is of course a good prerequisite for the in vitro production of secondary metabolites.

As mentioned above, the moss *Physcomitrella patens* might earn a role as a production system in molecular farming. Decker and Reski (2004) argue that the production of recombinant proteins in plants for pharmaceutical purposes requires controlled in vitro culture conditions in order to meet the requirements of good manufacturing practice (GMP) and complex legislation, especially in European countries. Seed plant cell cultures, however, might be sensitive to shearing, be genetically unstable and produce comparatively small amounts of recombinant proteins (Doran 2000). Consequently, Decker and Reski (2004) suggest that *Physcomitrella patens* is a good alternative, since in this in vitro culture system differentiated protonema filaments are grown. A major drawback of molecular farming, the plant-specific protein glycosylation, which may cause allergenic reactions in patients, might be circumvented in *Physcomitrella patens* by genetic engineering. By means of gene targeting (see above) the genes responsible for plant-specific glycosylation could be knocked out, resulting in the “humanised” gly-

cosylation pattern of the recombinant proteins (Decker and Reski 2004; Koprivova et al. 2004).

The purpose of this review was to relate the progression of seed plant tissue culture to the development of the same techniques in bryophytes, taking into account biological differences and their consequences on technological developments. Due to the multifaceted nature of the present review topic choices had to be made, and we omitted mentioning some aspects of bryophyte tissue culture and presented others in lesser detail; for this we express our regrets. In the part of our review relating the history of bryophyte in vitro tissue culture we attached importance to citations from the original articles. We cannot exclude, however, that additional and older articles also exist, especially those not published in English, French or German. We will be grateful for any feedback of such information.

We hope that our review will help draw more attention to bryophyte in vitro culture by showing that it is not a field of mere basic research on less well-known plants but also an area of recent biotechnological developments.

Acknowledgements Figure 2d was kindly provided by Alexander Lucumi and Iris Perner (Karlsruhe University, Institute for Mechanical Engineering and Mechanics, Bioprocess Engineering)

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