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ORIGINAL PAPER

Desiccation and cryopreservation of actively-growing cultured plant cells and protoplasts

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Abstract Actively-growing cultured cells of *Pogonatum* and *Polytrichum* were desiccated and cryopreserved. Although *Pogonatum* was slightly more tolerant to desiccation, both species were cryopreserved with >90% survival rate. An examination of isolated protoplasts revealed that differences in desiccation tolerance were likely dependent on levels of injury of plasma membranes. Trehalose and sucrose provided some protective effects during protoplast desiccation, but mannitol and glucose were less effective when *Pogonatum* protoplasts were directly desiccated and preserved at various temperatures. The effectiveness of glucose was enhanced when combined with culture medium components.

Keywords Cryopreservation · Cultured cells · Desiccation tolerance · *Pogonatum inflexum* · *Polytrichum commune* · Protoplast

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
BA	6-Benzyl aminopurine
MS	Murashige and Skoog (1962) medium
CI	Callus induction medium (Takio et al. 1986)

Introduction

Cryopreservation of cultured cells or tissues is a dependable and long-established method for long-term conservation of plant genetic resources. Cells and tissues of various plant species have been preserved since Nag and Street (1973) first successfully preserved cultured carrot cells in liquid nitrogen (Kartha 1985). Although the agronomic importance of cryopreservation has long been recognized, recent studies have emphasized the importance of preserving rare wild plant species as well as transgenic cell lines and cultures that are particularly useful in experimental systems (Towill 2002).

Cryopreservation methods generally fall into one of three free-water removal categories: slow prefreezing by extracellular freezing, vitrification by plasmolysis, and desiccation by vaporization (Ishikawa 1994; Grout 1995). Desiccation is an ideal conservation method as it takes advantage of the innate ability of plant cells of some taxonomic groups to tolerate desiccation and does not require imbibition of toxic cryoprotective solutions (Ishikawa 1994). Cultured cells or tissues are often encapsulated in gel beads prior to desiccation to allow for slow and mild drying, which often increases survival rate of preserved specimens.

To expand desiccation technology to a wider range of plant specimens, basic studies on physiological and structural aspects of dehydration and rehydration are required. Those physiological and structural changes that occur in the plasma membrane are of particular interest (Steponkus 1984; Crowe et al. 1992; Bryant et al. 2001). Protoplasts have been used to study membrane behavior during freezing and freeze-induced dehydration (Gordon-Kamm and Steponkus 1984a, b; Uemura and Steponkus 1989, 2003). They should also serve as good tools for studying membrane behavior and other cellular events during desiccation. Protoplasts of pea embryos have been used to study the relationship between membrane damage and desiccation tolerance (Xiao and Koster 2001; Koster et al. 2003;

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Halperin and Koster 2006). Despite the usefulness of protoplasts as tools for studying desiccation tolerance, there is no report, to our knowledge, either of protoplast isolation from desiccated cells or desiccation and cryopreservation of isolated protoplasts. As cells encapsulated in gel beads are not suitable for protoplast isolation and microscopic observation, the development of a protocol that does not require encapsulation would be desirable for studying desiccation tolerance at the cellular and protoplast levels.

In this study, actively-growing cells of the mosses *Pogonatum inflexum* and *Polytrichum commune* (*Polytrichaceae*) were successfully desiccated without encapsulation.

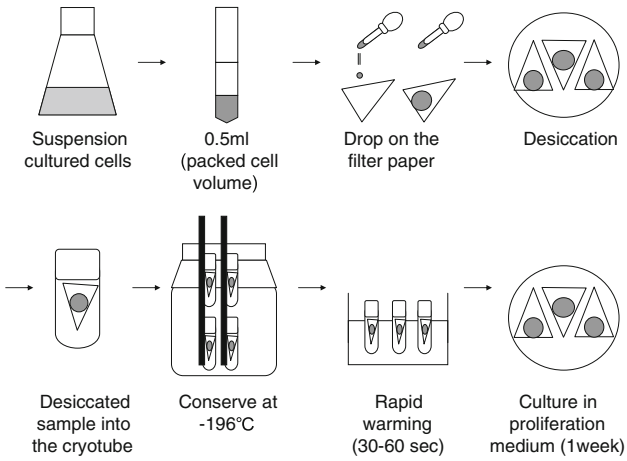

Fig. 1 Procedure for desiccation and cryopreservation of suspension cultured cells

Materials and methods

Plant materials

Spores of *Pogonatum inflexum* were obtained from Tokyo Denki University, and spores of *Polytrichum commune* were isolated from a forest in Nagano-ken (Japan). Capsules were sterilized by immersion in 70% ethanol for 1 min, and then in 1% solution of sodium hypochlorite for 10 min. Spores were germinated on MS basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose. Calli of *Pogonatum* were induced on CI medium (Takio et al. 1986) supplemented with 10 μM BA, 5 μM 2,4-D and 4% sucrose. A suspension culture of *Pogonatum* was established and maintained in CI medium supplemented with 10 μM BA, 5 μM 2,4-D, and 3% sucrose. Callus of *Polytrichum* was induced on MS medium supplemented with 10 μM BA and 4% sucrose. A suspension culture of *Polytrichum* was established in MS medium supplemented with 10 μM BA and 4% sucrose. All suspension cultures were grown at 26°C with shaking at 100 rpm under continuous light at 30 $\mu mol^{-2}\ s^{-1}$. All media were adjusted to pH 5.8 and autoclaved at 121°C for 15 min.

Desiccation, cryopreservation, and regrowth

Cells were subcultured onto fresh media for a period of 7 days, centrifuged to pack the cells, and dropped onto a filter paper for rapid desiccation under air flow on a clean bench (Fig. 1). Slow desiccation took place in Petri dishes at 70% relative humidity. Final water content of desiccated cells was determined gravimetrically by measuring the loss of water after drying at 100°C for 1 h. Desiccated and non-desiccated cells (controls) as well as those desiccated for different time periods, including 10, 20, 30, 40, 60 min and 6, 12, 18, 24 h were transferred to cryotubes, and plunged into liquid nitrogen.

Desiccated and cryopreserved cells were transferred to solid medium to assay for recovery and regrowth. Cell weights were measured after 7 days of culture, and survival of each sample was calculated as percent of control weight. Changes in morphology were observed by staining *Pogonatum* cells with 0.1% Calcofluor White ST, and microscopy (Olympus, IX70, Tokyo, Japan) under UV light.

Protoplast isolation

Protoplasts were isolated from *Pogonatum* and *Polytrichum* cells before desiccation, after desiccation, and during post-desiccation culture. *Pogonatum* cells were suspended in an enzyme solution containing 2% Driselase (Kyowa Hakko, Tokyo, Japan), 0.6 M mannitol, and 5 mM $CaCl_2$ (pH 5.8), and incubated at 27°C for 5 h on a reciprocal shaker (Iwaki Glass Co., SHK-U4, Tokyo, Japan) (100 rpm). *Polytrichum* cells were suspended in an enzyme solution containing 2% Driselase (Kyowa Hakko, Tokyo, Japan), 1% Macerozyme R-10 (Yakult Pharmaceutical Ind, Tokyo, Japan), 0.6 M mannitol, and 5 mM $CaCl_2$ (pH 5.8), and incubated at 27°C for 3 h on a reciprocal shaker (100 rpm). The incubated mixture was filtered through a nylon net with a pore size of 40 μm and centrifuged at 80*g* for 3 min. The pellet was washed three times with 0.6 M mannitol supplemented with 5 mM $CaCl_2$ and re-centrifuged. Protoplast yields were determined using a hemocytometer.

Desiccation and cryopreservation of protoplasts

Protoplasts isolated from 7-day-old subcultured *Pogonatum* cells were suspended in a solution containing 0.35 M sugar, including mannitol (0.36 osmolarity), glucose (0.36 osmolarity), sucrose (0.38 osmolarity), or trehalose (0.38 osmolarity), and with or without CI medium. A drop of protoplast suspension (ca. 40 mm^3) was placed on a square section of aluminum foil in a 60 mm diameter petri dish,

and desiccated at 50% relative humidity for 24 h at 27°C. Desiccated protoplasts were preserved at −20, 4, 26°C or in liquid nitrogen. Protoplasts were resuspended in liquid medium and their viability was determined by staining them with Evans blue (Gaff and Okang'o-Ogola 1971).

Protoplast culture

Pogonatum protoplasts were suspended in liquid CI medium supplemented with 0.35 M glucose, 10 μM BA, and 5 μM 2,4-D. The protoplast suspension was plated on solid medium and cultured under the same conditions as those of suspension cells, but without shaking. The solidified medium contained the same composition as that of the liquid medium used for protoplast suspension plus 1% of each of agar and activated charcoal (Sugawara et al. 1983; Kuriyama et al. 1990). Cell division rates were determined after 14 days of culture.

Results

Desiccation and cryopreservation of cultured plant cells

Water accounts for a high fraction of the volume and weight of actively-growing cultured plant cells. Thus, both cell weight and volume should markedly decrease with desiccation. Dramatic changes in *Pogonatum* cell morphology during desiccation were observed using fluorescent microscopy, often with significant flattening of normally spherical cells (Fig. 2).

More than 90% of *Pogonatum* cells survived desiccation when moisture levels of tissues were below 1 g water per g tissue dry weight with either rapid or slow dehydration (Fig. 3). In contrast, *Polytrichum* cells did not survive rapid dehydration, but >75% of cells survived cryopreservation with slow desiccation over at least 18 h (Fig. 4).

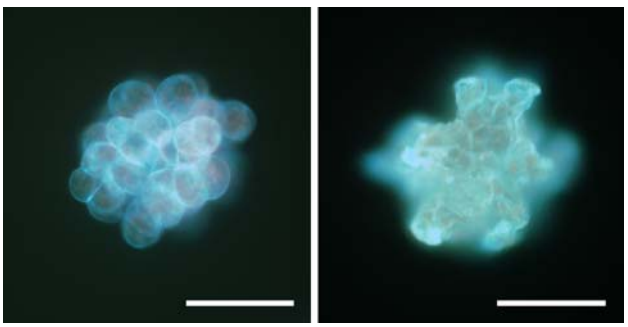

Fig. 2 Fluorescence-stained *Pogonatum inflexum* cells before desiccation (*left*) and after rapid desiccation for 30 min (*right*). Cells were stained with a drop of 0.1% Calcofluor White M2R and observed before or after desiccation. Bars = 100 μm

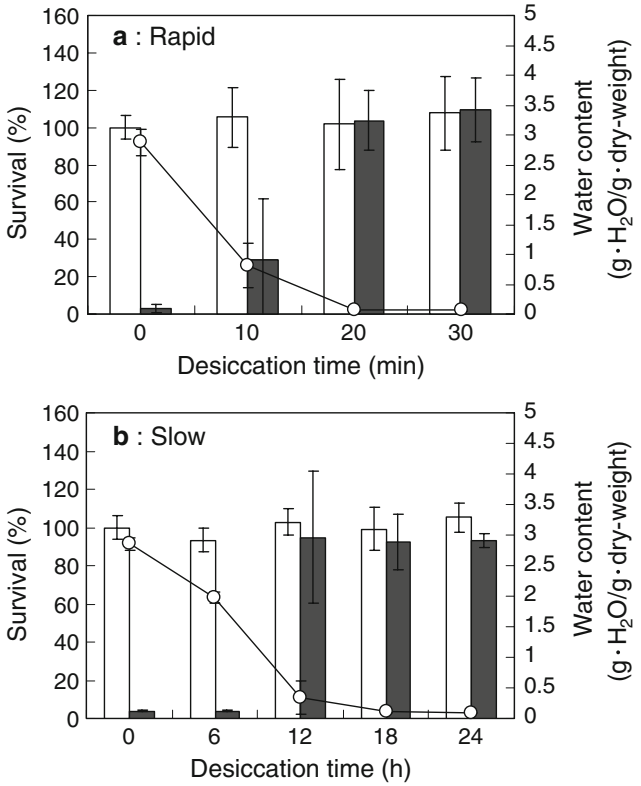

Fig. 3 Water content (○) and survival rates (*open* and *black bars*) of *Pogonatum inflexum* cultured cells after desiccation at 26°C for different times. **a** Rapid desiccation. **b** Slow desiccation. *Open bars* represent survival rates of desiccated cells. *Black bars* represent survival rates of cells preserved in liquid nitrogen after desiccation. Each data shows mean and SD of nine measurements

Protoplast isolation from cultured plant cells after desiccation

Both *Pogonatum* and *Polytrichum* cells appear to have some native desiccation tolerance, allowing them to be cryopreserved in liquid nitrogen without additional pretreatments such as growth in ABA supplemented or sugar-enriched media (Robertson et al. 1987; Sugawara and Hashimoto 2003; Hatanaka and Sugawara 2006, 2007). Protoplast yields from *Polytrichum* cells were very low compared to those of *Pogonatum* immediately after desiccation, with recovery to about 70% of controls by 3 days in culture (Fig. 5).

Desiccation of protoplasts isolated from *Pogonatum* cells

Protoplasts were isolated from actively-growing cultured cells, then directly desiccated and cryopreserved. Desiccated protoplasts were cultured to determine whether they retained their abilities to actively divide. Protoplasts isolated from *Pogonatum* cells were resuspended in solutions containing 0.35 M of various sugars (mannitol, glucose, sucrose or trehalose) with or without components of CI

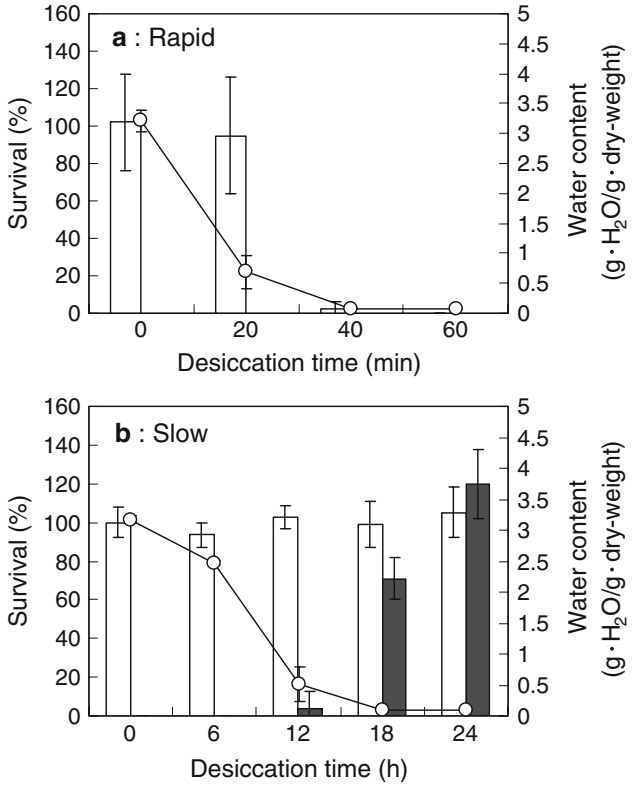

Fig. 4 Water content (○) and survival rates (*open* and *black bars*) of *Polytrichum commune* cultured cells after desiccation at 26°C for different times. **a** Rapid desiccation. **b** Slow desiccation. *Open bars* represent survival rates of desiccated cells. *Black bars* represent survival rates of cells preserved in liquid nitrogen after desiccation. Data correspond to means ± SD, of six measurements in (**a**) and nine measurements in (**b**)

basal medium, and then dried at room temperature. Survival rate of protoplasts suspended in 0.35 M mannitol solution was very low following desiccation, but it was higher than 70% in sucrose or trehalose, and 20% in glucose (Fig. 6). The addition of CI basal medium to 0.35 M glucose solution increased survival to nearly 90%. The highest survival rates were obtained with either sucrose or trehalose in combination with CI basal medium.

Preservation of desiccated protoplasts at different temperatures

Protoplasts isolated from *Pogonatum* cells were also desiccated and stored at different temperatures in either CI plus glucose or CI plus trehalose (Fig. 7). Although differences in survival rates immediately after desiccation were low, these were significantly high after one month following desiccation. There was a general inverse correlation between temperature and survival rate in glucose-preserved protoplasts; whereas, preservation in trehalose was not dependent on temperature. Storage in trehalose is effective even at room temperature for at least a month (Fig. 7).

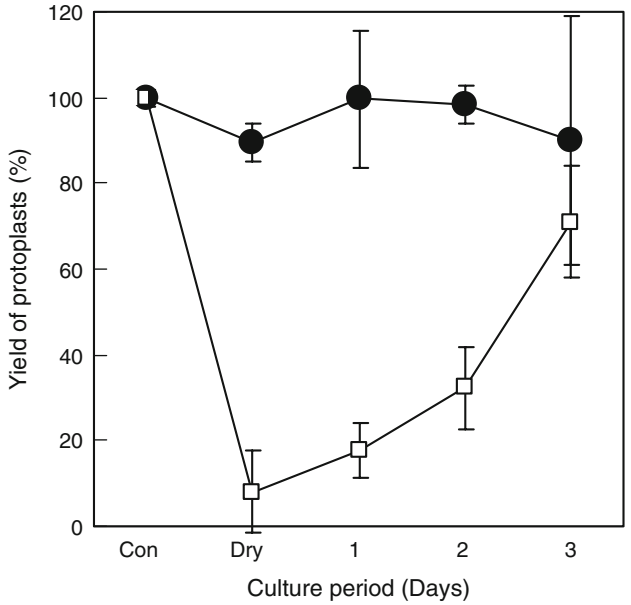

Fig. 5 Yield of protoplasts isolated from *Pogonatum inflexum* cells (●) and *Polytrichum commune* cells (□). *Pogonatum* cells were desiccated rapidly for 30 min and cultured for different lengths of time (days). *Polytrichum* cells were desiccated slowly for 24 h and cultured for different lengths of time (days). Con: Non-desiccated control cells. Dry: Desiccated cells. Data correspond to means ± SD of three measurements

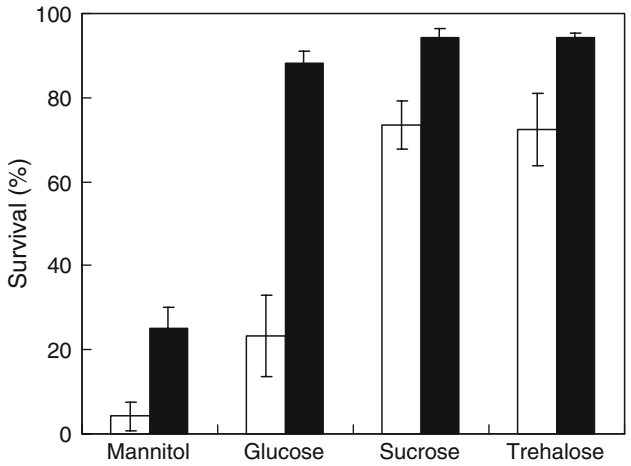

Fig. 6 Survival rate of *Pogonatum inflexum* protoplasts after desiccation for 24 h. Protoplasts were suspended in a solution containing only 0.35 M sugar (open bars) or 0.35 M sugar with CI medium (*black bars*) and desiccated for 24 h. Data correspond to means ± SD of four measurements

Culture of protoplasts following desiccation and cryopreservation

Protoplasts from *Pogonatum* cells were suspended in a solution containing 0.35 M glucose and CI, desiccated for 24 h, and cryopreserved in liquid nitrogen for another 24 h. Cryopreserved protoplasts were then cultured in CI

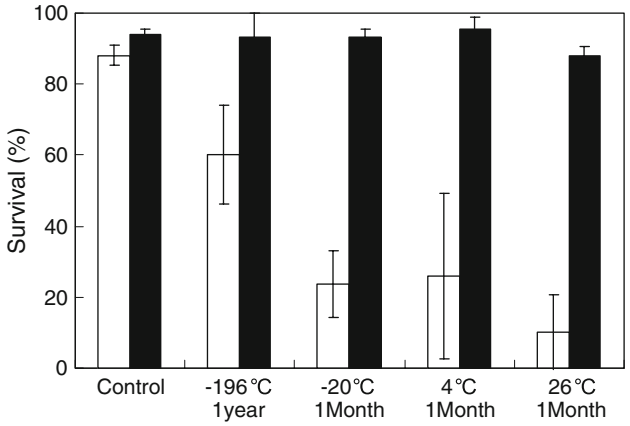

Fig. 7 Survival rates of *Pogonatum inflexum* protoplasts desiccated for 24 h and stored at different temperatures for 1 month or 1 year. Protoplasts were suspended in CI medium containing 0.35 M glucose (*open bars*) or trehalose (*black bars*). Control, Survival rate immediately after desiccation. Data correspond to means ± SD of four measurements

containing 0.35 M glucose and 1% activated charcoal, and observed microscopically. Cell wall regeneration was observed after 24 h of culture (Fig. 8a), and cell division was subsequently observed on the third to fourth day (Fig. 8b). After 14 days of culture, divided cells were frequently observed. About 25% of non-desiccated protoplasts were actively dividing, as compared to >10% in each of desiccated and cryopreserved protoplasts (Table 1). Cell aggregates initially formed after a month, and by 2 months, these were frequently observed (Fig. 8c).

Discussion

Desiccation methods have been developed for shoot-tips, somatic embryos, and callus or suspension cells for long-term preservation of these various tissues (Niino et al. 2006). These methods often involve encapsulating specimens in gel beads containing buffering compounds, such as calcium arginate. Unfortunately, encapsulation prevents analysis of cell structure. An important goal of this study was to

Table 1 Cell division in protoplast culture of *Pogonatum*

Treatment	Cell division rate
Non-desiccated	23.7 ± 5.5
Desiccated but non-cryopreserved	15.4 ± 3.1
Desiccated and cryopreserved	11.4 ± 3.0

Rate of cell division was determined after 14 days of culture

Each data shows mean and SD of three experiments

preserve suspension cultured plant cells without encapsulation. Based on the experiments conducted in this study, *Pogonatum* cells survived both rapid and slow desiccation; whereas, *Polytrichum* cells only tolerated slow desiccation. The qualitative differences in desiccation tolerance between *Pogonatum* and *Polytrichum* cells associated with the speed of dehydration suggested that there were likely differences in survival of isolated protoplasts before or immediately after desiccation, and during post-desiccation culture.

The plasma membrane is a primary site of damage in dehydration-sensitive cells (Steponkus 1984; Crowe et al. 1992; Bryant et al. 2001). Freeze-damaged cells preserved in liquid nitrogen by the pre-freezing method resulted in injury to plasma membranes due to dehydration stress, thereby few protoplasts were isolated from freeze-thawed cells (Sala et al. 1979; Cella et al. 1982; Kuriyama et al. 1997). In this study, few protoplasts were isolated from slowly desiccated *Polytrichum* cells, although many protoplasts were harvested from *Pogonatum* cells (Fig. 5). Based on these results, it is likely that plasma membranes of *Polytrichum* cells were irreparably damaged. However, slowly desiccated and cryopreserved *Polytrichum* cells demonstrated similar levels of regrowth as to non-desiccated control cells (Fig. 4b), thus indicating a sub-lethal level of damage must have occurred with slow desiccation. Protoplast yield from desiccated *Polytrichum* cells rapidly increased during the initial 3 days of regrowth culture. A similar finding was observed in rice cells cryopreserved using a pre-freezing method (Sala et al. 1979; Cella et al. 1982; Kuriyama et al. 1997). These results suggested that plasma membrane damage could be repaired during

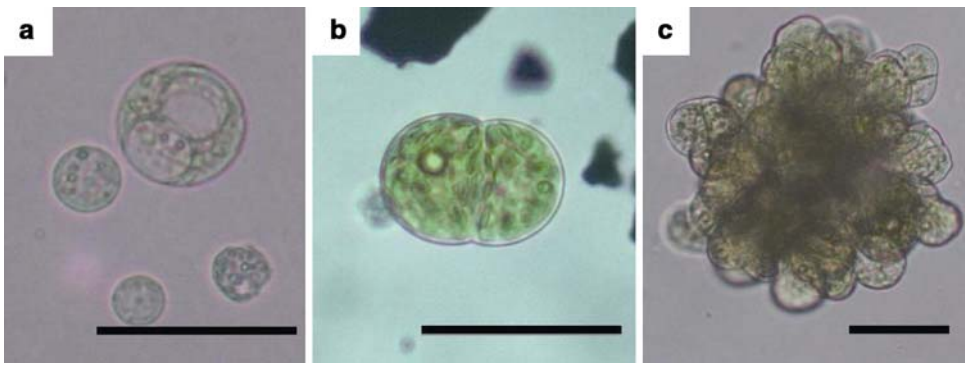

Fig. 8 Micrograph of cultured cells of *Pogonatum inflexum* protoplasts and dividing cells during different stages of culture. **a** Rehydrated protoplasts after desiccation and cryopreservation in liquid nitrogen. Bar = 50 μm. **b** Divided protoplast after 14 days of culture. Bar = 50 μm. **c** Cell cluster formed after 2 months of protoplast culture. Bar = 50 μm

recovery culture provided primary lesions were not lethal. In contrast, *Pogonatum* plasma membranes were stable during desiccation.

Pogonatum protoplasts suspended in 0.35 M mannitol with or without CI basal medium did not survive well, presumably because mannitol, a sugar alcohol, crystallized during drying, and destroying almost all protoplasts as previously reported (Xiao and Koster. 2001; Caffrey et al. 1988; Koster et al. 1996). Glucose, a reducing monosaccharide, was also less effective as a protectant when used alone. Monosaccharides generally failed to protect membrane vesicles and liposomes from desiccation damage in vitro (Crowe et al. 1986). On the other hand, the disaccharides sucrose and trehalose were effective even when used alone. The accumulation of di- and oligosaccharides, and particularly the natural desicco-protectant trehalose, has been associated with desiccation tolerance in many species (Crowe et al. 1984a, b; Hoekstra and Van Roekel 1988; Koster and Leopold 1988). The presence of CI basal medium constituents provided an additional margin of survival in combination with each of the sugars, but particularly with glucose. It is not known whether a specific CI component or several components acting together are responsible for the added protection. More experiments will be necessary to clarify this point.

Many species of seed, pollen, and spores survive for relatively long periods at relatively high temperatures in a gel matrix state (Burke 1986; Williams and Leopold 1989; Leopold et al. 1992; Sun and Leopold 1997). It is likely that *Pogonatum* protoplasts desiccated with sugar and CI constituents are in a similar gelling state at relatively high temperatures. However, protoplast preservation at different temperatures is not that simple, as indicated in Fig. 7. Protoplasts desiccated with trehalose and CI could be preserved over a large range of temperatures, but desiccation with glucose and CI decreased viability after preservation. The decreasing rate of viability is dependent on the preservation temperature, thereby indicating that the protective effect of glucose enhanced by CI is not as stable as that of trehalose. Glucose can initiate non-enzymatic glycosylation of free amines (Baynes et al. 1989; Kaanane and Labuza 1989), a process which has been suggested as a cause of seed deterioration during storage (Sun and Leolodd 1995; Wettlaufer and Leopold 1991) and for the loss of viable stored pea embryos (Xiao and Koster 2001).

Cryopreservation of plant protoplasts from either cultured cells or intact plants (Grout 1995), and survival following preservation in liquid nitrogen have been reported (Takeuchi et al. 1980; Langis and Steponkus 1991; Chen and Wang 2003). However, cell division and regrowth post-preservation have not been reported except in algal protoplasts preserved by vitrification (Liu et al. 2004). Thus far, there are no reports on regrowth culture of protoplasts isolated from actively-growing cultured plant cells and cryopreserved by desiccation.

In this study, activated charcoal was added to the culture medium to promote cell division of *Pogonatum* protoplasts. The beneficial effect of activated charcoal on protoplast cell division was demonstrated in *Marchantia,* a liverwort (Sugawara et al. 1983) and in *Equisetum*, a horsetail (Kuriyama et al. 1990). Activated charcoal is known to absorb some substance(s) inhibitory to protoplast division. Our study also shows that the addition of activated charcoal to the culture medium stimulates the initial stage of protoplast division. Protoplasts cultured on media without activated charcoal had a cell division rate less than 1% of cells grown with activated charcoal after 14 days of culture whether or not protoplasts were previously desiccated. Cell division rates of desiccated and cryopreserved protoplasts are lower than those of desiccated control protoplasts. This is likely due to lower survival rate of desiccated and cryopreserved protoplasts when compared to those that were only dessicated.

The phenomenon of desiccation tolerance is well known in many species of bryophytes (Hosokawa and Kubota 1957; Alpert 2006). Vascular plants also have potential capacities for desiccation tolerance during one or more stages of development (Ishikawa 1994; Ishikawa et al. 2005). The ability of embryos, pollen, or spores to survive severe water stress suggests that large numbers of plant species have conserved mechanisms for desiccation tolerance. Such tolerance genes, however, would not generally be expressed in actively-growing cultured cells wherein free water is abundant. In both bryophytes and vascular plants, cultured cells require additional treatments such as preculturing with high concentrations of sugar (Fabre and Dereuddre 1990; Paulet et al. 1993; Niino and Sakai 1992; Uragami et al. 1990; Blakesley et al. 1996; Gonzales-Arnao et al. 2003; Walters et al. 2002; Sugawara and Hashimoto 2003) or abscisic acid (Shimanishi et al. 1991; Kim and Janick 1989; Senaratna et al. 1989; Fang et al. 2004) to induce desiccation tolerance pathway genes. Desiccation and preservation of cultured plant cells at high survival rates requires the same close control of the speed of dehydration, final water content and relative humidity during storage as in seed preservation (Vertucci and Roos 1990, 1993; Vertucci et al. 1994). In this study, we demonstrated two culture and preservation systems for *Pogonatum* and *Polytrichum*. Although significant differences rates of desiccation are observed between these two species, both species have not required preculturing or encapsulation in alginate beads, thus rendering this simple and amenable protocol for further cell manipulation. Our culture systems may be useful for developing additional technical improvements in desiccation and preservation of actively-growing cultured cells.

We have also isolated protoplasts from desiccated cells and cultured protoplasts after desiccation and cryopreservation. Protoplasts isolated from actively-growing cultured cells provide a new system for studying desiccation tolerance at the cellular level. Differences in responses to dehydration between protoplasts and cultured cells with intact cell walls can provide insights into the role of cell walls in desiccation tolerance. Furthermore, microscopic observations of protoplasts may reveal more knowledge on the response of plasma membranes to desiccation than those of cultured cells with their intact cell walls. Further studies on protoplast behavior during dehydration and rehydration may provide additional insights into approaches for improving cryopreservation techniques for cultured cells and protoplasts by desiccation.

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