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ORIGINAL ARTICLE

Integration of tissue culture and cryopreservation methods for propagation and conservation of the fern *Osmunda regalis* L.

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Abstract A simple and reliable technique for in vitro multiplication and long-term preservation using liquid nitrogen was developed for gametophytes of *Osmunda regalis*. The effect of Knop's medium and various concentrations (1/2, 1/4, 1/8) of mineral salts provided in Murashige and Skoog (MS) basal medium, together in the presence or absence of both ammonium nitrate and a full complement of vitamins on gametophyte proliferation and sporophyte production was determined. Moreover, the effectiveness of gametophyte cryopreservation by vitrification and encapsulation–vitrification techniques was assessed. Maximum gametophyte proliferation (89 %) occurred on the ammonium nitrate- and vitamin-free [(-NH4NO3)(-vit)] MS medium with 1/2 or 1/4 strength mineral salts. The maximum production of sporophytes (30 plantlets per gametophyte clump) required 1/8MS (-NH4NO3)(-vit) medium. The flow cytometric analysis revealed that the sporophytes contained twofold more pg DNA than gametophytes. This confirmed that the sporophytes were obtained by sexual reproduction. The vitrification protocol and PVS2 solution were ineffective for cryopreservation. The greatest survival rate (81.6 %) following cryo-exposure occurred following treatment of encapsulated gametophytes with PVS3 solution for 3 h. This protocol allowed the recovery of gametophyte cultures following 6 weeks after rewarming. Finally, 100 % of sporophytes produced in vitro were successfully acclimated to *ex vitro* conditions. Application of the in vitro and cryopreservation methods made it possible to improve the number and time of *O. regalis* sporophyte production. Whole system of micropropagation can be completed in approximately 1 year. The protocols open new avenues for the mass propagation, germplasm conservation and resource management of the species.

Keywords Ammonium nitrate · Gametophyte multiplication · Hormone-free medium · Liquid nitrogen · Nuclear DNA content · Sporophyte production

Abbreviations

ABA	Abscisic acid
(-NH4NO3)(-vit)	Medium lacking ammonium nitrate and vitamins
LN	Liquid nitrogen
PGRs	Plant growth regulators

Introduction

To improve the ex situ conservation of plant biodiversity, new strategies had to be developed (Engelmann and Engels 2002). These include in vitro technologies and cryopreservation for mass propagation and long-term storage of plant germplasm. Until now, most have focused on crop plants of agricultural, horticultural or pharmaceutical value (Cruz–Cruz et al. 2013). Application of these technologies is still restricted to a limited number of rare and threatened species (Pence 2014; Kaczmarczyk et al. 2011). Similar research into both in vitro and liquid nitrogen (LN) techniques for the conservation of ferns is described in only

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few reports (Wu et al. 2010; Barnicoat et al. 2011; Marszał-Jagacka and Kromer 2011; Mikuła et al. 2011; Rybczyński and Mikuła 2011; Ibars and Estrelles 2012). The Red List of Threatened Species (IUCN 2014) clearly shows that more than half of fern species and their allies are threatened to some degree. Thus, there is urgent need for further research to improving and implementing novel methods for the conservation of fern biodiversity.

In vitro methods have been successfully exploited for many years for the micropropagation of plants, including ferns (Fernández and Revilla 2003; Bharati et al. 2013b). Tissue culture provides optimal nutritional and environmental conditions for large-scale fern production, often in the absence of exogenous plant growth regulators (PGRs), even for inducing somatic embryogenesis (Mikuła et al. 2015b). During the in vitro culture of ferns, many factors affecting the efficiency of multiplication of both gametophyte (Fernández et al. 1997; Sheffield et al. 1997; Goller and Rybczyński 2007; Wu et al. 2010; Moura et al. 2012) and sporophyte (Hirsch 1975; Materi and Cumming 1991; Teng 1997; Ambrósio and de Melo 2004; Kuriyama et al. 2004b; Moura et al. 2012; Mikuła et al. 2015a) have been investigated, besides PGRs. These reports focus on the type of explant donor (etiolation, age, length), as well as physical (light conditions, pH, various culture systems: dish, shake, immobilized, airlift) and chemical factors (sucrose and mineral salt concentrations). It has also been shown that poor culture conditions may favor apogamic sporophyte formation during the propagation of fern gametophytes (Raghavan 1989).

Another option for preserving the biodiversity of non-seed plants is cryopreservation. So far, this method has been used for the cryostorage of 69 fern species. However, for most of these (55 species), including *Osmunda regalis* L. and *O. japonica* Thunb. (Ballesteros et al. 2011; Li and Shi 2014, 2015; Mikuła et al. 2015c), investigation into the value of using LN focused on the preservation of spore material (Pence 2000b; Ballesteros et al. 2011; Barnicoat et al. 2011). Some previous studies have shown that the gametophytes of ferns (Pence 2000a, 2008; Mikuła et al. 2009, 2011; Barnicoat et al. 2011; Makowski et al. 2015) and mosses (Schulte and Reski 2004; Pence 2008; Rowntree and Ramsay 2009) can provide a novel and abundant source of material for long-term storage using LN. Gametophytes are highly regenerative following storage under in vitro conditions, and can be recovered from even single living cells of cryopreserved explants (Mikuła et al. 2009, 2011). Thus, the gametophyte generation serves an important complementary role and is a good candidate for cryopreservation.

The Royal Fern (*O. regalis* L.; Osmundaceae) is a species of very wide, almost cosmopolitan distribution, occurring both in temperate areas and the tropics (IUCN 2014). It is a basal leptosporangiate fern with semi-erect, trunk-like rhizomes, which can reach up to 1 m in height. The trunks were formerly an important source of fiber for the cultivation of orchids, but have since been replaced by conifer bark and coconut fiber, as well as several synthetic materials. The hairs of this species were also mixed with wool to produce textiles (Large and Braggins 2004). The Royal Fern is included on The IUCN Red List as a taxon of Least Concern (LC) (IUCN 2014). Globally, populations of *O. regalis* might be critically endangered within a particular region where numbers are very small or declining, for example: Norway, Switzerland, Croatia, Hungary, Iran. In Poland, this fern is included on the Red List of Vascular Plants of Poland as a vulnerable species (category V; Zarzycki and Szeląg 2006), and on the Red List of Lower Silesia as an endangered species (EN; Śliwiński and Szczęśniak 2008). The chlorophyllous spores of *O. regalis* lose their viability very fast at room conditions and do not survive for long-term when stored in a fridge or a freezer (Lloyd and Klekowski 1970; Ballesteros et al. 2011; Mikuła et al. 2015c). Therefore, developing ex situ conservation programs for the safe and effective preservation of *O. regalis* germplasm is of utmost importance. Liquid nitrogen storage of the spores of *O. regalis* have been shown to preserve their viability and the subsequent gametophyte development up to 7 years (Mikuła et al. 2015c), and longevity at such conditions have been projected in 1666 years (Ballesteros et al. 2011). However, LN storage of spores might not be enough to stop aging if spores are of low initial quality (Li et al. 2010; Ballesteros et al. 2011), as also has been observed for other plant germplasm such as seeds (Pritchard and Seaton 1993; Walters et al. 2004; Ballesteros and Pence 2014). For this reason, it is important to develop complementary ex situ conservation programs for the preservation of *O. regalis* using a different source of germplasm. In the present study, we describe an efficient method for the in vitro propagation of the fern species *O. regalis* and the cryopreservation of its gametophytes for ex situ conservation purposes.

Materials and methods

Plant material

Mature fertile fronds of *O. regalis* were obtained at the end of May 2012 from a field collection made by staff of the Polish Academy of Sciences Botanical Garden—CBDC in Powsin. Freshly collected fronds were stored for 2 days (24 ± 5 °C; RH ca. 45–80 %) until spores were released. They were then surface-sterilized by wrapping spores in filter paper and immersing the packages in 2 % hydrogen peroxide (H_2O_2) for 20 min. The packages with spores

were then washed three times in sterile distilled water. Sterilized spores were blotted onto the surface of half-strength Murashige and Skoog (1962) micro- and macronutrients (1/2MS) medium supplemented with a full complement of vitamins, 2 % (w/v) sucrose and 0.8 % (w/v) agar, at pH 5.8. Sterile distilled water (2 ml) was added to the spore culture to facilitate germination. All media and solutions used in the experiments were previously sterilized at 121 °C at a pressure of 1 atm for 20 min. The spores and gametophyte cultures were maintained in a growth chamber at 21 ± 1 °C with 16-h illumination provided by 50 μM m^{-2} s^{-1} daylight fluorescent tubes. For long-term maintenance of gametophyte cultures, the tissues were transferred to fresh medium every 6 months.

In vitro culture and efficiency assessment

Three-week-old spore-derived gametophytes were used as initial material for the study of the effect of various media on gametophyte multiplication (Fig. 1a–c). For sporophyte production, 6-week-old gametophyte clumps obtained on the 1/2 MS medium supplemented with full complement of vitamins and 2 % (w/v) sucrose were used.

The effectiveness of Knop's medium (1865) in conjunction with various concentrations of mineral salts (1/2, 1/4, 1/8) in MS basal medium, both in the presence and absence of ammonium nitrate (NH_4NO_3) and a full complement of vitamins [referred to as (-NH4NO3)(-vit)] was evaluated. The percentage of proliferating explants and the number of newly formed gametophytes per responding explant were calculated after 6 weeks of culture. The number of sporophytes per gametophyte clump was assessed after 6, 8 and 16 weeks of continuous culture (without any subculture).

Sporophytes with 5–8 leaves were used for acclimation. Acclimation was performed using sterile peat as substrate (pH 6.5). Plantlets were grown either in jars or in boxes, beneath polythene bags, under room conditions (4 weeks at +25 ± 5 °C; RH ca. 60–80 %). They were periodically exposed to the ambient atmosphere of the laboratory so as to maintain optimal relative humidity. Next, plantlets were transferred to greenhouse conditions, and subsequently, to the outdoor field collection of the Botanical Garden. Thirty plantlets were used per acclimation experiment.

Gametophyte cryopreservation and survival assessment

Secondary young gametophytes derived from those growing in vitro were chosen for these investigations. The explants were cryopreserved by means of vitrification and encapsulation–vitrification methods. Pre-culture consisted of a 2-week-long period on 1/2MS agar medium supplemented with 0.25 M sucrose and 10 μM ABA, and was performed according to a protocol described previously by Mikuła et al. (2009).

Vitrification Pre-cultured gametophytes were immersed in a loading solution containing 2 M glycerol + 0.4 M sucrose, at 22 °C for 20 min and then treated with PVS2 (on ice) or PVS3 (at 22 ± 2 °C) vitrification solutions for 0.5, 1, 2, and 3 h. The PVS2 consisted of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) DMSO and 0.4 M sucrose in 1/2MS culture medium (Sakai et al. 1990). The PVS3 solution contained 40 % (w/v) glycerol and 40 % (w/v) sucrose (Nishizawa et al. 1993). Samples were washed twice in fresh PVS2 or PVS3 solutions, loaded into 2-ml cryovials and placed directly into LN for 24 h. For rewarming, the cryotubes were plunged into a water bath at 35 °C for 1.5 min; the gametophytes were then transferred to 1.2 M sucrose solution for 0.5 h. The explants were subsequently placed on 1/2MS agar medium in darkness for 2 days. The cultures were then transferred to fresh medium, and after 5 days, they were transferred to light.

Encapsulation–vitrification A 3 % (w/v) solution of sodium alginate (Sigma) and a 0.1 M solution of $CaCl_2$ were made up in a 2 % (w/v) aqueous solution of sucrose. Gametophytes were embedded in sodium alginate for 10 min, and hardened in $CaCl_2$ solution for 45 min, at room temperature. Capsules 4–5 mm in diameter were pre-cultured as described above, and then placed in the vitrification loading solution (2 M glycerol + 0.4 M sucrose) for 20 min. They were then placed in PVS2 or PVS3 solutions for up to 3 h and finally in LN, as described above. The procedure for rewarming was also as described above.

Explant survival was assessed by its capacity to regrow gametophytes. Percentage gametophyte survival was calculated following 7 days after rewarming.

Microscopic preparation

For the detection of the natural autofluorescence of chlorophyll in spores (Fig. 1a), an epifluorescence microscope (Vanox AHBT3; Olympus, Japan) equipped with computer image analysis system (cellSens Standard ver. 1.7) was used. Fluorescence was induced by blue-violet light (BV filter: 400–440 nm).

Pieces of gametophytes bearing archegonia (Fig. 1g) were fixed in 10 % (v/v) glutaraldehyde in phosphate buffer (PBS, pH 7.2) for 24 h, followed by dehydration in a graded ethanol series, and then embedded for histology in Technovit 7100 (2-hydroxyethyl methacrylate; Heraeus Kulzer) according to the protocol of Popielarska-Konieczna et al. (2011). They were sectioned at 5 μm using a rotary microtome (Microm, Adamas Instrumenten),

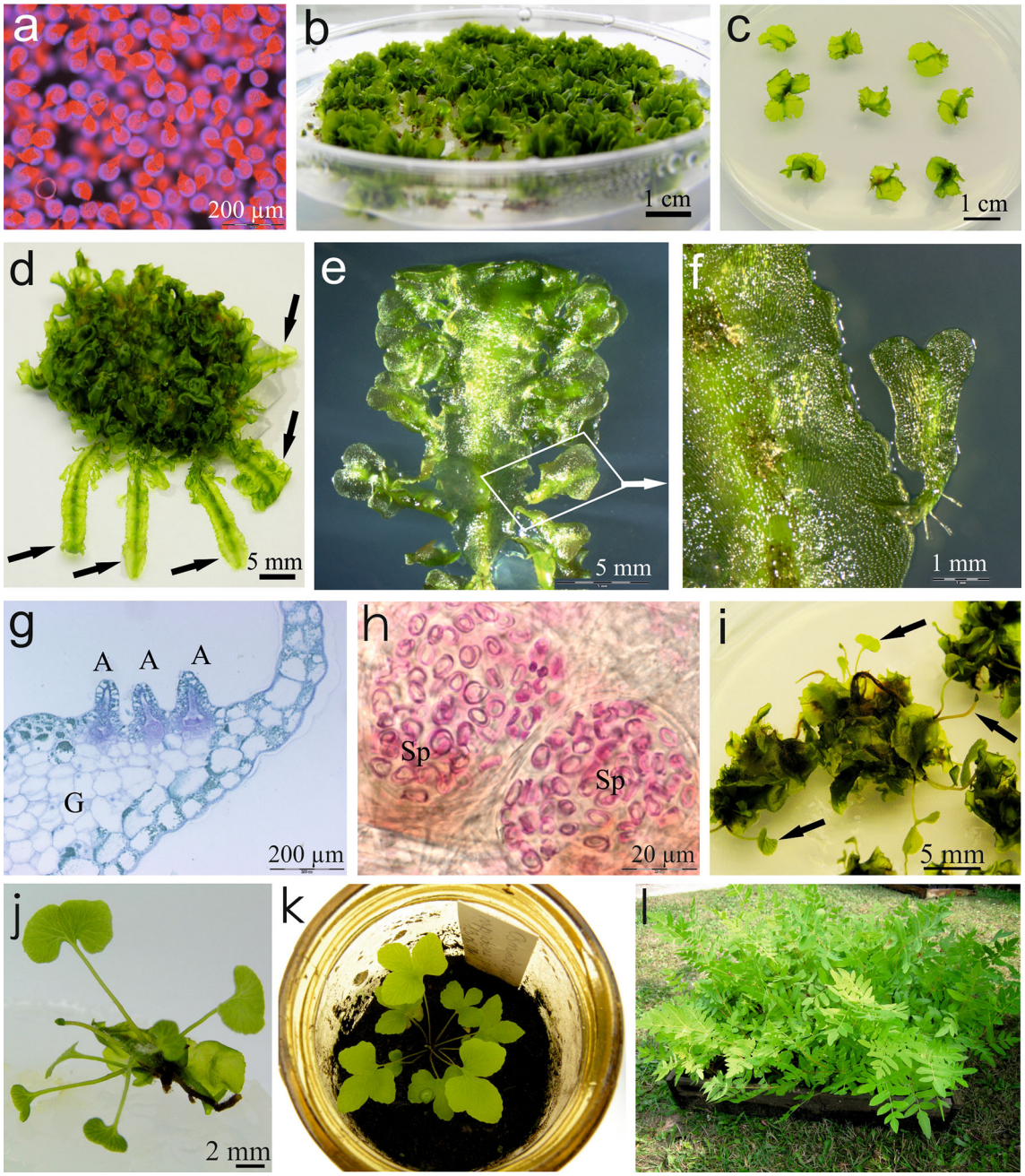

Fig. 1 Stages demonstrating the development of sporophytes from spores in *Osmunda regalis*. **a** Germination of spores 28 h after sowing on 1/2 MS agar medium. Blue autofluorescence of spore coat and red autofluorescence of chlorophyll was induced by blue-violet light (BV filter 400–440 nm). **b** Three-week-old gametophytes obtained from spores under in vitro culture conditions. **c** Single, three-week-old gametophytes used as a source of initial plant material for experimental studies. **d** Clump of gametophytes after 16 weeks of culture; *arrows* elongated gametophytes. **e** Secondary gametophyte production from the marginal cells of elongated gametophytes. **f** High-power view of newly developed secondary gametophyte. **g** Longitudinal section of archegonia (semi-thin section stained with toluidine blue) and **h** mature antherozoids in antheridia (squashed specimen; Feulgen stain) formed on heart-shaped gametophytes. **i** Production of sporophytes (*arrows*) on 1/8 MS (-NH4NO3)(-vit) after 6 weeks of culture. **j** Sporophyte with five juvenile fronds ready for acclimation. **k** Sporophyte after 1 month of acclimation in jar. **l** Sporophytes under *ex vitro* conditions during first growth season (July); after 4 months of acclimation and 1 year since sowing spores. *A* archegonium, *G* gametophyte, *Sp* antherozoids

stained with 0.1 % (w/v) toluidine blue dissolved in distilled water and mounted in Entellan synthetic resin (Merck). Microscopic sections were photographed using a Zeiss AxioCamMRe digital camera with Zeiss AxioVision 3.0 software and a Nikon DS-Fi2 with NIS-Elements D 4.00.00 4.0 software.

For demonstration of antheridia (Fig. 1h), pieces of gametophytes were treated as described by Tomiczak et al. (2015). Briefly, the explants were pretreated with 2 mM 8-hydroxyquinoline and fixed in ethanol–acetic acid (3:1, v/v). The tissue was then hydrolyzed in 5 M HCl for 50 min at 21 °C, and stained in Schiff's reagent (Sigma–Aldrich) for 2 h in the dark. Observations were carried out by means of a Vanox AHBT3 microscope.

Flow cytometry analysis

Profiles of relative DNA content in gametophytes and sporophytes cultured on 1/8MS (-NH4NO3)(-vit) medium were analyzed by flow cytometry following the protocol described previously (Mikuła et al. 2009). For each sample, at least 7000 nuclei were analyzed immediately following preparation by means of a Partec CyFlow SL Green (Münster, Germany) flow cytometer, equipped with an argon laser. Analyses were performed on 12 independent replicates of gametophytes or sporophytes. Histograms were analyzed using a Partec FloMax computer program. *Pisum sativum* (2C = 9.11 pg DNA) served as an internal standard. The nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *O. regalis*/*P. sativum* on the histogram of fluorescence intensities.

Statistical analysis

Statistical analyses were performed on two independent experiments, each comprising 75 gametophytes. Results were expressed as the mean ± standard deviation and analyzed by means of a one-way ANOVA analysis of variance and Fisher's least significant difference (LSD) procedure using Statgraphics Plus software. Significance was set at the 0.05 confidence level.

Results

In vitro gametophyte multiplication and sporophyte production

Freshly harvested disinfected spores of *O. regalis* showed 100 % germination on the 1/2MS agar medium supplemented with 2 % sucrose, 28 h after sowing (Fig. 1a). Under in vitro culture conditions, the development of gametophytes was rapid, and they were ready to be used for investigations within 3 weeks of initial culture (Fig. 1b, c).

The effect of seven media on *O. regalis* gametophyte multiplication was assessed after 6 weeks of culture. When gametophytes were cultured on 1/2, 1/4 or 1/8 MS medium, and Knop's medium, the percentage of gametophyte proliferation was as follows: 78, 75, 71 and 70 %, respectively. The average number of new gametophytes per explant ranged from 11 to 13 (Fig. 2a). Gametophyte formation was significantly more efficient on all ammonium nitrate- and vitamin-free media compared to the same media containing these supplements, and to Knop's medium (Fig. 2a). Under optimal experimental conditions [(-NH4NO3)(-vit)], more than 80 % explants formed new gametophytes, with productivity reaching as much as 15 during 6 weeks of culture.

After 6 weeks of culture (without subculturing), the production of new gametophytes accelerated rapidly, and clumps of gametophytes were formed on all media studied. Some gametophytes elongated (Fig. 1d). Their marginal cells formed secondary gametophytes (Fig. 1e, f). Although the gametophytes maintained on 1/2 and 1/4 MS media did not produce sporophytes until month 15 of culture (Fig. 2b), they formed archegonia (Fig. 1g) and antheridia (Fig. 1h) in large numbers.

The production of sporophytes strongly depended on the composition of the medium used (Fig. 2b). It was evident that ammonium nitrate- and vitamin-free media stimulated

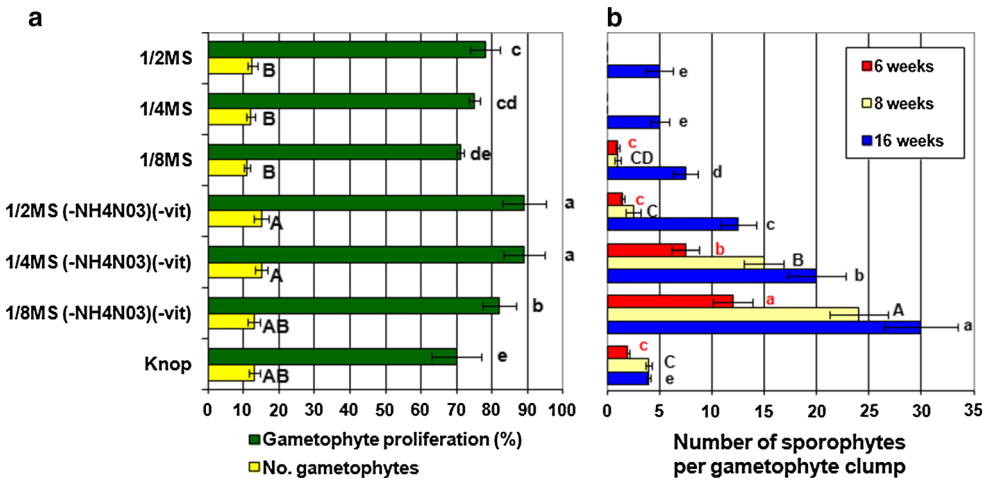

Fig. 2 Effect of various media tested on gametophyte multiplication (after 6 weeks of culture) (**a**) and sporophyte production per gametophyte clump after 6, 8 and 16 weeks of culture (**b**) media supplemented with 2 % (w/v) sucrose. *Values marked with the same letter* do not differ significantly at the 0.05 confidence level according to Fisher's least significant difference (LSD's) test

the development of sporophytes. After 16 weeks of culture, as many as 7.5 or 30 sporophytes were obtained in the presence or absence of both the ammonium nitrate and vitamins, respectively. Moreover, sporophyte production depended on the concentration of mineral salts in the MS medium. The greatest number of sporophytes was produced on the medium with the lowest concentration of MS mineral salts. On 1/8MS (-NH4NO3)(-vit) medium, an average of 12.5, 24 and 30 sporophytes were produced after 6, 8 and 16 weeks of culture, respectively. *Osmunda regalis* gametophytes were large, very elongated and produced multiple archegonia and antheridia simultaneously. However, one gametophyte produced only one sporophyte (Fig. 1j).

Young plantlets produced between 5 and 8 leaves that were entirely different from mature adult leaves, indicating their juvenile nature (Fig. 1i, j). This developmental stage was optimal for successful acclimation of sporophytes to *ex vitro* conditions (Fig. 1j). One hundred percent of sporophytes produced in vitro survived acclimation (Fig. 1k) and culture in soil (Fig. 1l). After 1 year of spore sowing, and a further 4 months of sporophyte acclimation, the plantlets grew strongly in the field collection of the Botanical Garden (Fig. 1l). In the third vegetation year (i.e., 2015), some plants were able to produce fertile fronds.

Flow cytometry revealed that gametophytes multiplied under in vitro conditions contained 1C = 14.66 pg nuclear DNA. The fronds of sporophytes contained almost twofold more DNA than gametophytes (2C = 28.15 pg) (Fig. 3).

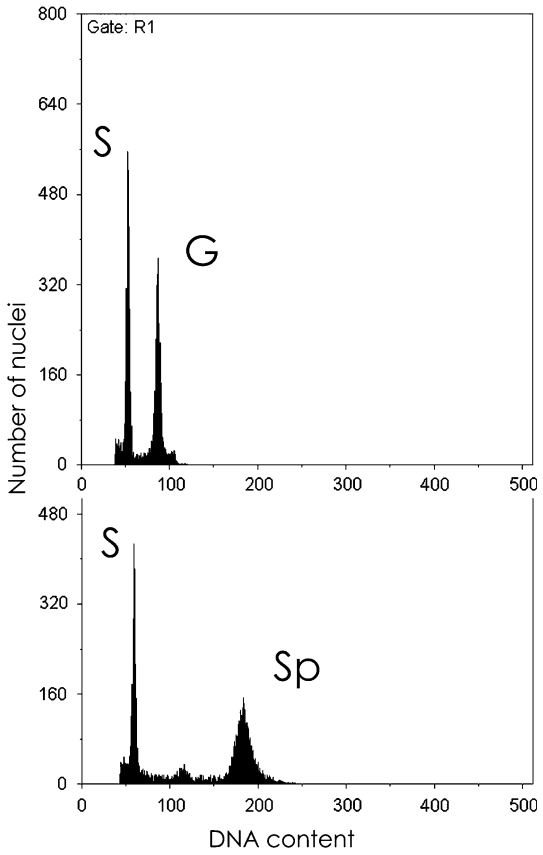

Fig. 3 Selected histograms of nuclear DNA content isolated simultaneously from leaves of *Pisum sativum* (internal standard) (*S*) and *Osmunda regalis* gametophytes (*G*) and sporophytes (*Sp*) regenerated on 1/8MS (-NH4NO3)(-vit) medium

Cryopreservation of gametophytes

The majority of cells of non-encapsulated gametophytes exposed to PVS2 or PVS3 vitrification solutions became irreversibly plasmolyzed within a few minutes (Fig. 4a). Only some cells within the apical notch survived 30-min-long PVS3 treatment. The cells were incapable of recovering in culture. Vitrified gametophytes died following rapid cooling with LN (Table 1).

Although 100 % of encapsulated non-frozen explants survived 0.5–3-h-long treatments with PVS2, only a few cells of each explant remained alive. Following cryopreservation, gametophyte survival reached 9 %, and decreased gradually with increasing length of exposure to PVS2 (Table 1). Recovery of gametophyte cultures occurred slowly, and despite 6 weeks of maintenance, only small, filamentous gametophytes were formed (Fig. 4b). Following cryopreservation by encapsulation–vitrification and 30-min treatment with PVS3, 62.5 % survival was achieved. The greatest percentage survival (81.6 %) of explants was obtained following treatment with PVS3 solution for 3 h. Moreover, large portions of gametophyte tissue were free of necrosis. This established cryopreservation protocol allowed the recovery of gametophyte cultures following 6 weeks after rewarming (Fig. 4c).

An application of 1/8MS (-NH4NO3)(-vit) medium for gametophytes recovered after cryopreservation allowed to obtain the sporophytes which survived acclimation similar to the non-cryopreserved plant material (Fig. 5).

Discussion

Mass multiplication of fern gametophytes

For many fern species propagated under in vitro conditions, gametophyte multiplication increased when the medium was supplemented with PGRs (Bonomo et al. 2013; Bharati et al. 2013a; Das et al. 2013; Parajuli and Joshi 2014; Ravi et al. 2014). However, our previous studies showed that in many fern species, effective gametophyte proliferation occurred on 1/2 MS medium in the absence of exogenous hormonal stimulation (Goller and Rybczyński 2007;

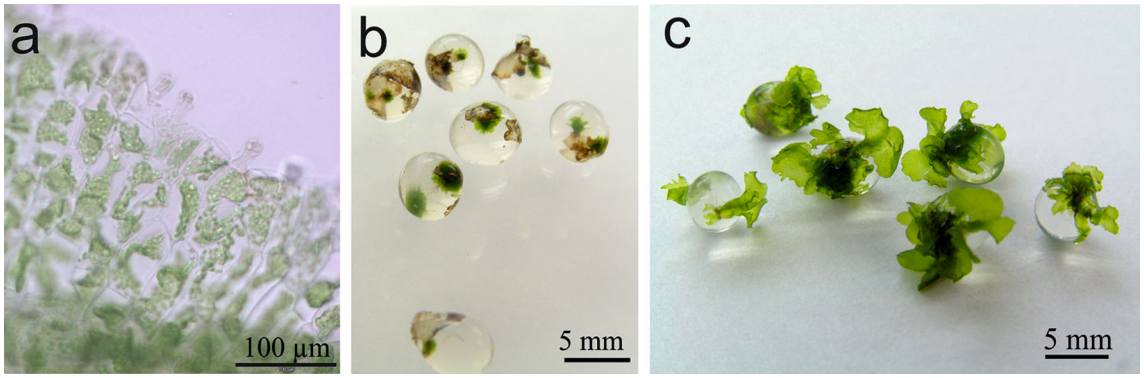

Fig. 4 Recovery of gametophyte culture after cryotreatments. **a** Irreversible plasmolysis of gametophyte cells following treatment with PVS2; **b** gametophyte cultures recovered after cryopreservation by encapsulation–vitrification prior to treatment with PVS2, **c** and PVS3; 6 weeks after rewarming

Table 1 Survival (%) of *O. regalis* gametophytes after cryopreservation by vitrification and encapsulation–vitrification techniques, with (+LN) or without (−LN) cooling with liquid nitrogen

Length of PVS2 or PVS3 treatment (h)	Vitrification		Encapsulation–vitrification			
	PVS2[1]/PVS3[2]		PVS2		PVS3	
	−LN	+LN	−LN	+LN	−LN	+LN
0.5	0/+	0	100.0	9.0 ± 0.8 a	100.0	62.5 ± 9.2 a
1.0	0	0	100.0	4.1 ± 1.3 b	100.0	64.4 ± 5.0 a
2.0	0	0	100.0	0.6 ± 0.5 c	100.0	69.5 ± 7.7 a
3.0	0	0	100.0	0 c	100.0	81.6 ± 1.6 b

Values marked with the same letter do not differ significantly at the 0.05 confidence level according to Fisher's least significant difference (LSD's) test. Data represent mean ± standard deviation of two independent experiments, each consisting of at least 75 explants

[1] PVS2 treated on ice

[2] PVS3 treated at room temperature

Mikuła et al. 2011; Rybczyński and Mikuła 2011). The present data revealed that a reduced concentration of mineral salts down to 1/8 MS basal medium led to a decline in gametophyte multiplication. It has been suggested that salt starvation inhibits this process. Furthermore, maximum gametophyte proliferation was achieved on the ammonium nitrate- and vitamin-free MS medium. In view of this research, we definitely cannot determine the role of vitamins, but according to data presented by others authors we would like to conclude that the ammonium nitrate is the main factor limiting the gametophyte proliferation in *O. regalis*. Fernández and co-workers (1997) were the first to show the inhibitory effect of the ammonium radical on the growth and development of *O. regalis* gametophytes. When ammonium sulfate was added to Knop's medium, gametophyte growth was inhibited (Fernández et al. 1997). Similarly, ammonium salts inhibited prothallus growth in *O. japonica* (Shin and Lee 2009). For this species, mass micropropagation of gametophytes using modified 1/8 strength MS medium is recommended. Similarly, a culture medium containing a low concentration of mineral salts (1/4 MS) was the most effective for the development of *Adiantum reniforme* var. *sinense* Y. X. Lin. (Wu et al. 2010). The number of spathulate- and heart-shaped gametophytes was greatest on this medium, whereas gametophyte development was inhibited on full- and half-strength MS medium. It was also demonstrated that nitrate is almost always required for the normal growth of photosynthetic fern gametophytes (Melan and Whittier 1990).

These results are contrary to those obtained for several other leptosporangiate ferns belonging to the genera *Asplenium, Blechnum, Dryopteris, Pteris* and *Woodwardia* (Fernández et al. 1997). Here, the ammonium radical has a stimulatory effect on the growth and development of gametophytes. However, more importantly, the ammonium radical improves the early development of non-photosynthetic gametophytes in mycorrhizal fern species such as *Botrychium dissectum* forma *obliquum* (Muhl.) Fernald (Melan and Whittier 1990).

Control of sexual reproduction in ferns

Although PGRs are involved in developmental processes, including gender expression in *O. regalis* gametophytes

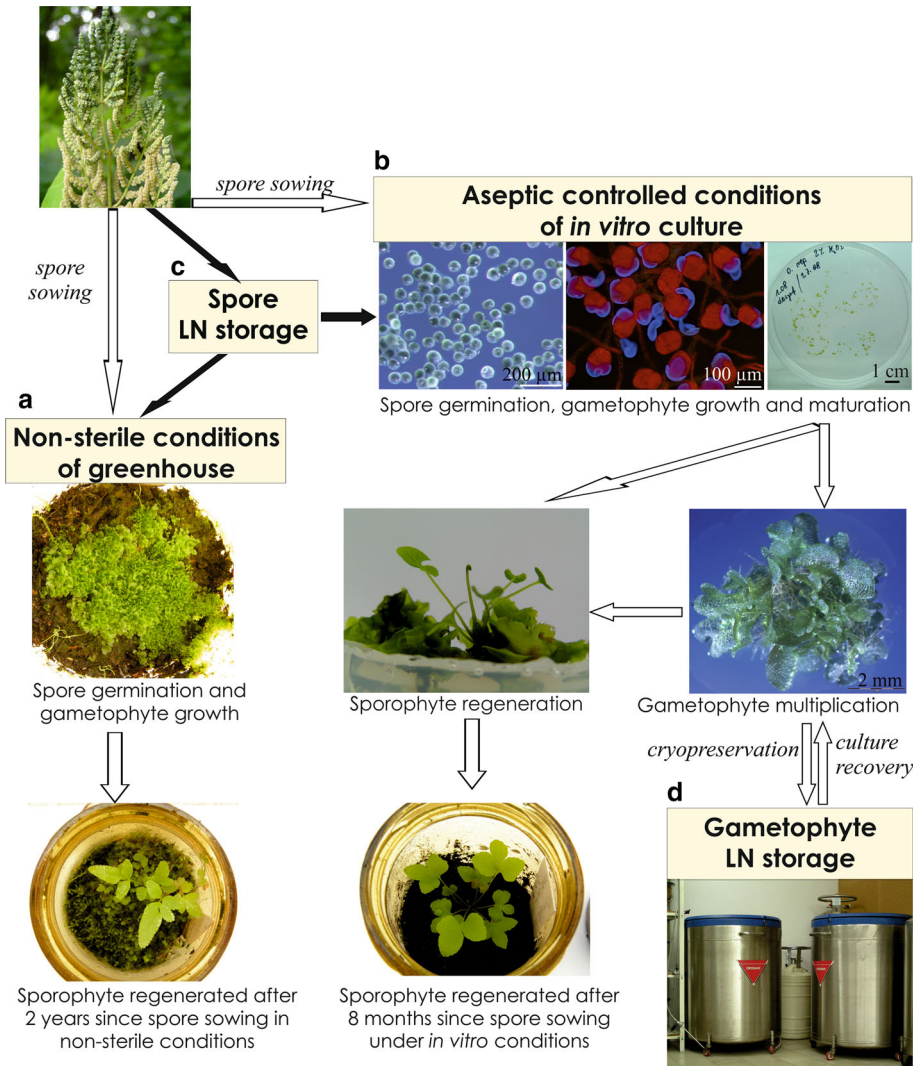

Fig. 5 Schematic diagram for the conservation of *Osmunda regalis* genetic resources. Compare the classical horticultural procedure (**a**) and newly developed procedure involving tissue culture technology (**b**) in combination with long-term storage of spores (**c**) and gametophytes (**d**) in liquid nitrogen (LN)

(Greer et al. 2012), our study showed that sporophyte formation can occur on hormone-free media. Gametophytes produced antheridia and archegonia on all media tested, but only those media which were low in mineral salts, and were ammonium nitrate- and vitamin-free were effective for sporophyte production in *O. regalis*. Since the number of sporophytes increased by more than sixfold under these culture conditions, it has been suggested that salt starvation may play a key role in sporophyte induction, whereas ammonium nitrate inhibits this process. Starvation is already being used for the initiation of fern sporophytes. Both *O. regalis* and *Pteris ensiformis* Burm. f. showed excellent sporophyte-producing capacity when gametophytes were cultured on plain agar gel (Fernández et al. 1999). Wu et al. (2010) showed that the rate of sporophyte production in *A. reniforme* var. *sinense* cultured in pure river sand was greater than that in a mixture of soil and sand. Again, the most effective propagation of *Cyathea lepifera* (J. Sm. ex Hook.) Copel. sporophytes occurred when gametophytes were grown on 1/80 strength MS basal medium (Kuriyama et al. 2004b). Our results also partly agree with those obtained by Kuriyama et al. (2004a) for *Adiantum capillus-veneris* L. They showed that reduction of both ammonium and potassium nitrate to 25 % of the MS medium promotes sporophyte formation, but the absence of a nitrogen source almost totally prevents it. Finally, ammonium nitrate was recognized as a critical factor in the inhibition of sporophyte production in *A. capillus-veneris*. The authors concluded that nitrogen might inhibit fertilization or the development of fertilized eggs (Kuriyama et al. 2004a). This tendency was also previously described for *Equisetum arvense* L., for which sporophyte initiation did not necessarily require a reduction in nitrogen levels, even though their further development required specific levels and types of nutrient nitrogen compounds (Kuriyama et al. 1992). However, contrary to *O. regalis, C. lepifera* and *A. capillus-veneris*, the formation of sporophytes on gametophytes of *E. arvense* was initiated by cytokinin (Kuriyama et al. 1992).

The time required for sporophyte induction tends to depend on the culture medium used. Morini (2000) showed that the first small *O. regalis* sporophytes developed on 15–20 % of gametophytes cultured for 3 months on a medium containing Hoagland and Arnon's macroelements and MS microelements. Fernández et al. (1999) reported that plain agar gel gave excellent results for *O. regalis* sporophyte production. They obtained about 20 sporophytes per gram fresh weight of gametophytes over 2 months. In our studies, the maximum number of sporophytes was obtained in 1/8MS (-NH4NO3)(-vit). This medium was also the most effective for the rapid induction and development of sporophytes. An average of 24 sporophytes per gametophyte clump was produced over 2 months of culture, and this figure increased to 30 over the next 8 weeks of continuous culture. The fresh weight of individual 6- and 18-week-old gametophyte clumps reached 120 and 500 mg, respectively. This demonstrates very clearly how efficient and effective this culture system is. The flow cytometric analysis revealed that the sporophytes had twofold more pg DNA than the haploid gametophytes. This confirmed that the sporophytes were obtained by sexual reproduction. Therefore, the present data support the recommendation to use 1/8MS (-NH4NO3)(-vit) medium for the mass propagation of *O. regalis*.

Gametophytes for cryo-conservation

The cryo-methods chosen and their modification depend to a great extent on the tolerance of the species to desiccation and the types of tissues used for its conservation (Mikuła et al. 2011; Pence 2014). In the study presented here, the effect of two cryopreservation methods, based on vitrification and encapsulation–vitrification protocols, on *O. regalis* gametophyte survival was compared. Before cryopreservation, the explants were pre-cultured on medium supplemented with sucrose and ABA. For *O. regalis* gametophytes, 2-week-long pre-culture on that particular medium increased their viability from about 30–80 % following cryopreservation by encapsulation–dehydration (Mikuła et al. 2011).

Vitrification protocols are widely applied methods of cryopreservation, as they are easy to use. They have been exploited for the cell, shoot-tip and somatic embryo culture of a great many plant species (Sakai and Engelmann 2007). Indeed, the vitrification method may be considered a good alternative for the cryopreservation of mosses, because their protonema filaments display a very high level of tolerance to osmotic stress. This is probably correlated to the extreme natural environments where they occur (Schulte and Reski 2004). Consequently, a high-throughput protocol based on the use of cryoprotectant comprising DMSO (20 %) and glucose (25 %) was established to preserve 140,000 mutants of *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Schulte and Reski 2004). For *Splachnum ampullaceum* Hedw. 92.3 % brood cells, 60 % gametophores and 46 % protonemata survived direct exposure to PVS2 and LN (Mallón et al. 2010). However, the desiccation tolerance of ferns as a group is more limited compared with that of bryophytes (Pence 2008). Until now, the vitrification method had only been used on two fern species. Our previous study revealed that PVS2 and PVS3 were ineffective for the cryopreservation of gametophytes of tree-ferns such as *Cyathea smithii* J. D. Hooker and *C. delgadii* Sternb. (Mikuła et al. 2011). The results presented here confirm that the vitrification solutions used are lethal to gametophytes, including those of *O. regalis*. Owing to the sensitivity of this type of plant material to high osmotic stress produced by vitrification solutions, treatment with PVS2 and PVS3 induced rapid, extensive and irreversible plasmolysis. Research with ferns and bryophytes revealed that an encapsulation can significantly improve the survival of gametophytes dehydrated in air (Pence 2000a; Burch 2003). Our present findings showed that the encapsulation also decreases the toxicity of highly concentrated vitrification solutions. Encapsulated gametophytes of *O. regalis* which were not in direct contact with PVS3, survived treatment with this vitrification solution and cooling with LN excellently (81.6 %). However, by comparison, maximum survival following PVS2 treatment was only 9 %. This shows that PVS2 is also inappropriate for the cryopreservation of encapsulated gametophytes of *O. regalis,* probably because DMSO is so toxic. The encapsulation–vitrification method resulted in more than 50 % survival in *Dicksonia fibrosa* Colenso gametophytes following prior treatment with both PVS2 and PVS3 solutions (Mikuła et al. 2011). Similar viability (56.7 %) was demonstrated for encapsulated young sporophytes of the epiphytic fern *Platycerium ridleyi* H. Christ. following treatment with PVS2 and cooling with LN (Rodpradit et al. 2003). The effectiveness of the cryopreservation of *O. regalis* gametophytes by encapsulation–vitrification and treatment with PVS3, as described here, is similar to that previously obtained for encapsulation–dehydration (more than 80 %) (Mikuła et al. 2011). Rapid (about 4 h) encapsulation–vitrification may thus be considered an alternative for the slow (almost 4 days) encapsulation–dehydration method, and is recommended for the bulk, long-term gametophyte cryo-conservation of *O. regalis*.

Conservation of *O. regalis* germplasm: advantages of in vitro propagation and cryopreservation

The tissue culture system described in this paper enables the highly efficient propagation, starting from spore

material, of both *O. regalis* gametophytes and sporophytes. Furthermore, our observations showed that under favorable in vitro culture conditions, sporophyte propagation can be accelerated and completed in approximately 1 year (Fig. 5). Thus, this system has the capacity to be a very effective source of plant material for the multiplication of the species in horticulture, gardening and the plant production sector (including Botanical Garden collections), but also for conservation and restoration ecology works as the sporophytes are produced in a sexual way (helping to increase the genetic diversity of the plants produced). Attending to the previous literature and the results of this paper, conservation of the fern *O. regalis* can be possible using two diverse germplasms. Fern spore cryopreservation have been shown to be successful for *O. regalis*, maintaining viability of this germplasm type up to 7 years without apparent changes in germination and subsequent gametophyte development (Ballesteros et al. 2011; Mikuła et al. 2015c). In addition, gametophyte cryopreservation as described in this paper could serve as an additional conservation strategy using a diverse germplasm source. Considering all, we would like to propose a multi-strategy for conserving *O. regalis* genetic resources, which is presented in Fig. 5. This strategy would be based on the cryopreservation of spores and/or gametophytes, as well as tissue culture technology to speed up sporophyte production.

Conclusion

The hormone-free and effective in vitro propagation system described here appears to have the potential to be a useful tool for the mass production of both gametophytes for tissue banking and sporophytes for restoring degraded environmental resources of *O. regalis*. The use of gametophytes, together with an encapsulation–vitrification procedure following PVS3 treatment, ensures a high degree of tissue survival in *O. regalis* and the effective recovery of gametophyte cultures. This novel methodology now opens new avenues for the conservation and resource management of the fern *O. regalis*.

Author contribution statement Conceived the study and oversaw the research: DM, AM; performed the experiments: DM; antherozoids in antheridia assay: KT; prepared the figures and contributed to data analysis: DM, AM; manuscript preparation: AM; helping to interpret the results and discussion: KT, JJR. All authors approved manuscript content.

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