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ORIGINAL PAPER

Successful cryopreservation of *Quercus robur* plumules

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Abstract Successful cryopreservation of *Q. robur* germplasm as plumules (i.e. shoot apical meristems of embryos) is described in this paper. After excision from the recalcitrant seeds and preliminary storage in 0.5 M sucrose solution (18 h), the plumules were subjected to cryoprotection (in 0.75 M sucrose, followed by 1.0 M sucrose and 1.5 M glycerol solutions), and next to desiccation (over silica gel or in nitrogen gas) and cooling (in slush at –210°C or in vials filled with liquid nitrogen, LN, −196°C), and were then cryostored for 24 h. High percentage of survival was obtained after cryostorage (21–67%, depending on pretreatment, assessed in vitro by greening plumules that increased in size). Desiccation of plumules over silica gel resulted in significantly higher survival after cryopreservation (58%) in comparison with desiccation in nitrogen gas (29%), with regrowth (shoots with leaves) 5–18%. The extent of plumule desiccation was comparable in both methods, in which drying of plumules for 20 min decreased the water content to 0.5–0.6 g H_2O g^{-1} dry weight before LN exposure. The type of LN exposure did not significantly influence plumule survival and regrowth after cryostorage. Plumules isolated from acorns of four provenances survived cryostorage after cryoprotection followed by desiccation over silica gel and direct cooling in vials with LN (survival 51–76%, regrowth 8–20%). Normal plants developed from the recovered shoots after rooting. The presented protocol for *Q. robur* plumule cryopreservation may offer a potential approach for establishing germplasm conservation in gene banks for *Quercus* species.

Keywords Pedunculate oak · Germplasm · Long-term storage · Liquid nitrogen · Desiccation

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Introduction

Conserving biodiversity in a changing environment is essential for human self-interest (Balmford et al. 2002; Barret and Schluter 2008; Geburek and Konrad 2008; Mace et al. 2003), especially in the natural forests that are key terrestrial ecosystems and major sources of biodiversity (Jump et al. 2008; Lindenmayer and Franklin 2002). Pedunculate oak (*Q. robur*) is one of the most economically and ecologically important forest tree species, especially in Europe (Stein et al. 2003). Forest decline, particularly of this species, was observed in Europe in the 1970s and 1980s (Liese and Siwecki 1991; Oleksyn and Przybył 1987). For more than a decade, invasive tree pathogens—like *Phytophthora ramorum*, causing sudden oak death—have been identified in the western United States (Brasier 2008; Jones and Baker 2007; Stein et al. 2003). In a similar manner, this pathogen now poses a serious threat to Japanese larch and other tree species in Europe (Brasier and Webber 2010). In 2003, symptoms of *P. ramorum* infection were observed in a mature (60 cm in diameter) American southern red oak (*Quercus falcata*) found in southeast England (Brasier 2008). Another fungus —*Ciboria batschiana*, causing black rot mummification of *Quercus* and *Castanea sativa* seeds during storage—was identified in Europe in the 1990s (Siwecki 1994).

Therefore, *ex situ* protection of the remaining stands of this important forest-forming species is needed.

Seeds of pedunculate oak are classified as "recalcitrant" (Roberts 1973) and after shedding cannot be desiccated below a water content (WC) of 40% (fresh weight basis) (Blomme and Degeyter 1986; Gosling 1989). Thus, they cannot be stored for more than three winters, at an optimal temperature of −1°C, without loss of germinability (Suszka and Tylkowski 1981).

Developing conservation practices for species producing recalcitrant seeds is important for scientific and practical reasons (Berjak and Pammenter 2008). Nowadays, plant cryopreservation is used to safeguard the biodiversity of such species and store elite materials that have shown genetic superiority in field tests (Park 2002). The method prolongs storage life and minimizes storage space requirements (Bouman and De Klerk 1990; Panis and Lambardi 2005). Moreover, the long-term storage of recalcitrant seeds in a germplasm bank is expected to rely on cryopreservation (Corredoira et al. 2004; Engelmann 2004; Engelmann and Sakai 2007).

Until now there have been no successful techniques for long periods of ex situ conservation of pedunculate oak genetic resources. However, for other recalcitrant species, many investigations in the past 20 years have shown that embryonic axes isolated from such seeds or intermediate seeds can be successfully cryopreserved after partial desiccation. Such investigations were performed on the embryonic axes of *Juglans regia* (cv. Franquette) (de Boucaud et al. 1991), *Quercus faginea* (Gonzalez-Benito and Perez-Ruiz 1992), *Corylus avellana* (hazelnut) (Gonzalez-Benito and Perez-Ruiz 1994), *Citrus suhuiensis* cv. Limau langkat (Makeen et al. 2005), and *Camellia sinensis* (L.) O. Kuntze (Kim et al. 2005). Corredoira et al. (2004) successfully cryopreserved embryonic axes of *Castanea sativa* (Fagaceae). Following desiccation in a laminar flow cabinet to a WC of 20–24% (fresh weight basis), 93–100% of axes survived cryostorage and 63% subsequently developed into whole plants.

Some authors exposed *Q. robur* embryonic axes to liquid nitrogen, but generally with little success (Chmielarz 1997; Berjak et al. 1999a, 2000; Pence 1990; Poulsen 1992). Berjak et al. (1999a, 2000) examined the tissues and cells of both root and shoot apices of *Q. robur* axes after storage in liquid nitrogen (LN). Microscopic results after rewarming, recorded after 6 days of in vitro culture, showed that 70% of axes initiated organized shoot development if they were fast-frozen and retrieved from cryostorage in a solution containing Ca^{2+} and Mg^{2+}. However, no data on their further development are available. Chmielarz (1999, 2000), in spite of relatively high initial survival++, obtained *Q. robur* plantlets from cryopreserved (24 h) embryonic axes isolated from acorns of only one tree. For long-term germplasm preservation of valuable clones of *Quercus* species, cryopreservation of the somatic embryos (embryogenic tissue) has also been used. Somatic embryos of *Q. petraea* were successfully cryopreserved using DMSO as a cryoprotectant and two-step cooling (Jörgensen 1990). In *Q. robur*, some authors cryopreserved somatic embryos in vitrification solution or on agar media with a different sucrose concentration 0.5–1.5 M (Chmielarz 1999; Chmielarz et al. 2005; Martinez et al. 2003). In *Q. suber*, high percentage of survival of cryopreserved somatic embryos was obtained when an encapsulation and dehydration procedure was applied (Fernandes et al. 2008). Vidal et al. (2005) reported 38–54% of shoot regrowth among five clones of *Castanea sativa* from in vitro grown shoot apices by using the vitrification procedure. In our experiments, similar explants (plumules, i.e. shoot apices of embryos) were used instead of whole embryonic axes, which appeared to be unsuitable for *Q. robur* germplasm cryopreservation in the literature and in our earlier experiments (Chmielarz 1997) with repeatable results.

To verify our hypothesis that *Quercus robur* plumular meristems of various Polish provenances can survive storage in liquid nitrogen, plumules isolated from acorns of four provenances were subjected to cryoprotection, followed by desiccation and LN exposure. Two drying techniques and two LN exposure methods were compared for plumule cryopreservation. Here, we present for the first time the successful cryopreservation of *Q. robur* plumules.

Materials and methods

Mature acorns of pedunculate oak (*Quercus robur* L.), (Fig. 1a) were collected after shedding in October 2007, at Błażejewko 52°15′N and 17°06′E, Krotoszyn 51°42′N and 17°27′E, and Radzewice 52°15′N and 16°51′E. After collection, acorns (WC 40%, based on dry weight 0.70 g H_2O g^{-1} dw) were initially stored in loosely closed containers at 3°C. Plumules (shoot meristem surrounded by leaf primordia) were excised from acorns no later than 6 weeks after collection because survival of plumules isolated from acorns stored in LN for longer periods was lower (Chmielarz et al., unpubl. data).

Storage of plumules isolated from acorns: preliminary experiment

Isolation of plumules from acorns was performed in two steps: isolation of embryonic axes (Fig. 1b) from acorns, followed by isolation of plumules (0.113–0.237 mg, about 1 mm in diameter on average, Fig. 1c) from embryonic axes (with a scalpel) under nonsterile conditions. During the isolation procedure, embryonic axes were placed on

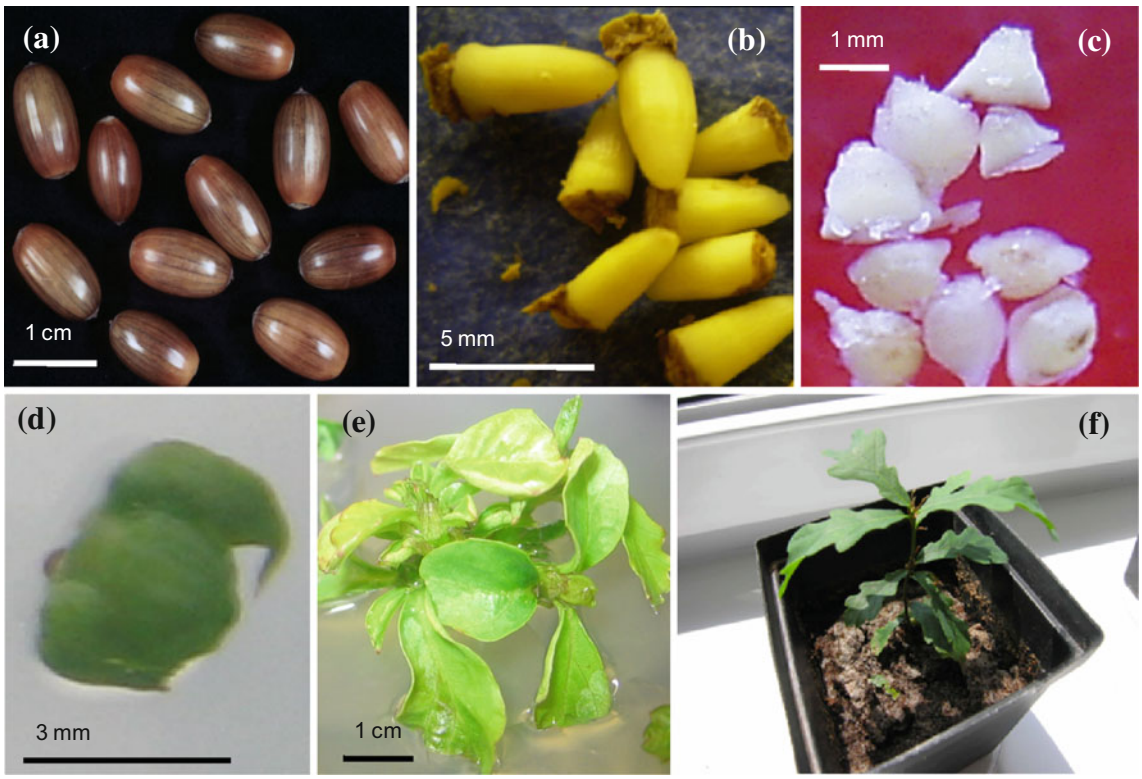

Fig. 1 Cryopreservation of *Q. robur* plumules. Acorns from which embryonic axes were isolated (**a**), embryonic axes from which plumules were isolated (**b**), plumules directly after isolation from embryonic axes (**c**), plumule recovered in vitro (after 2 weeks) after cryopreservation (**d**), shoots (3 months old) derived from recovered plumules after cryopreservation (**e**), rooted plantlet (6 months old) derived from a cryopreserved plumule (**f**)

filter papers moistened with distilled water within a closed Petri dish cooled on ice. Plumules were excised from embryonic axes under a binocular microscope and they were initially stored after excision for 18 h.

In this preliminary experiment, survival of plumules stored in various conditions was examined 18 h after excision from acorns. Plumules isolated from acorns were stored in water or in a 0.5 M sucrose solution in darkness, in both cases at 4 or 25°C. The optimal variant was chosen and applied in cryopreservation experiments.

Assessment of plumule water content (WC)

Plumule WC was assessed by air-drying at 90°C for 18 h (3 × 20 plumules), at which point the plumules had reached a constant dry weight. WC was expressed on a dry weight basis, in g H_2O g^{-1} dw (g g^{-1}). Plumule WC was also determined after drying for 0, 10, 20, 30, 40, 50, 60, and 70 min.

Cryoprotection

After initial 18-h storage in the 0.5 M sucrose solution, plumules were immersed in 0.75 M sucrose for 40 min followed by 1.0 M sucrose (40 min) and finally transferred into 1.5 M glycerol (40 min) solutions at 23°C.

Desiccation

To analyze the influence of various desiccation conditions on plumule survival (desiccation rate, lack of oxygen), we used drying under nitrogen flow and over silica gel at a constant temperature. Plumules were not desiccated in a laminar flow hood, where both temperature and humidity varied during the 3 weeks of experiments.

In nitrogen gas

After cryoprotection and following surface water removal using a paper towel, plumules were transferred to a drying apparatus (a loosely closed plastic box, 12 cm × 12 cm), placed on a net [a copper mesh (1 mm × 1 mm) covered with a cotton cloth], and exposed to nitrogen gas at 23°C. The nitrogen gas was taken from a nitrogen gas steel cylinder, where the pressure of the outflowing gas was reduced to 5,000 hPa (4–5 bar or 58–70 psi). Drying of plumules was performed for 0, 10, 20, 30, 40, 50, 60, and 70 min (Fig. 2). This curve showed that the optimal time of

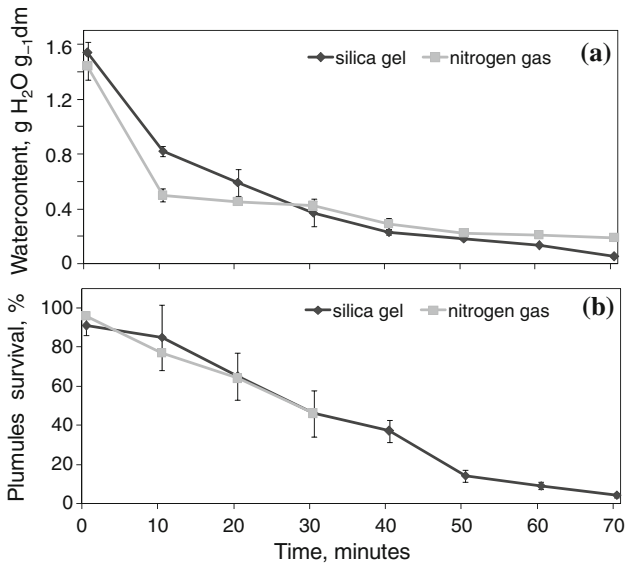

Fig. 2 Changes in water content **a** of *Q. robur* plumules and their survival **b** after desiccation over silica gel or in nitrogen gas at 23°C. Plumules were not treated with sucrose and glycerol solutions

drying for plumule survival before cryopreservation was 10–20 min (about 50% weight reduction). To compare both methods of drying (silica gel and nitrogen gas) in the cryopreservation experiment with the same plumule WC, we have chosen the 20-min drying variant because there were no significant differences in WC between plumules dried under silica gel and under nitrogen flow for 20 min (Fig. 2a). Additionally, the survival of plumules after 10 and 20 min of drying was comparable and still relatively high (variation not significant, Fig. 2b).

Over silica gel

Plumules were placed on a filter paper in a tightly closed (sealed with Parafilm) Petri dish (30 plumules/Petri dish of 7 cm in diameter) containing 50 g of activated silica gel. The temperature and drying periods were the same as mentioned earlier (drying in nitrogen gas). Plumules were dried before cryopreservation for 20 min.

Cryopreservation of plumules

Plumules were cryopreserved by ultra rapid cooling with nitrogen slush (LN sub-cooled to −210°C under vacuum, Echlin 1992) or by rapid cooling of plumules in liquid nitrogen (direct immersion in LN in polypropylene cryovials). In both cases plumules had direct contact with liquid nitrogen.

For rewarming, the plumules were transferred immediately from LN into a 1.2 M sucrose solution at 42°C for 1 min and then the sample was placed into ice to decrease the temperature of the sucrose solution for 2 min. For slow rehydration, the plumules were placed into a 0.75 M sucrose solution for 40 min followed by a 0.5 M sucrose solution for 40 min both at 23°C.

The effect of provenance on plumule cryopreservation

Plumules were isolated from acorns of four provenances: Błażejewko 1 (90 plumules from one mother tree), Błażejewko 2 (90 plumules from one mother tree), Błażejewko 3 (90 plumules from three mother trees—bulked collection of acorns), and Radzewice (90 plumules from three mother trees—bulked collection of acorns), to determine the influence of provenance on plumule survival after cryopreservation. After successive cryoprotection in sucrose and glycerol solutions as mentioned earlier, plumules were dried over silica gel for 20 min and directly plunged into LN in vials and held for 24 h. Plumules were rewarmed as mentioned earlier.

Survival and regrowth of plumules

Survival, as a more sensitive indicator in the protocols tested, was chosen in addition to the regrowth of plumules. Three replicates of 30 plumules were used for each treatment. Survival of plumules was assessed after 2 weeks of in vitro culture. Plumules used as a control variant in cryopreservation experiment were not cryoprotected and not desiccated. Plumules were considered alive (survival) when they were green and had increased in size (Fig. 1d).

The regrowth, which is of practical importance, was assessed when leafy shoots (3–6 mm long) appeared after 8 weeks of in vitro culture. It was calculated in relation to the initial number of plumules used for in vitro culture.

Plumules were sterilized after rewarming in 10% commercial bleach (<5% sodium hypochlorite NaClO and <1% sodium hydroxide NaOH) for 5 min and then rinsed four times in sterile 0.5 M sucrose solution. Plumules were cultured in sterile plastic Petri dishes (55 mm) on Woody Plant Medium (WPM) (McCown and Lloyd 1981) containing 0.8 mg/l BAP (6-Benzylaminopurine), sucrose (30 g/l) and solidified with agar (7.0 g/l). The pH was adjusted to 5.7 before autoclaving. Plumules were cultured under a light intensity of 77 µmol m^{-2} s^{-1} and a 16 h/8 h light/dark photoperiod at 25°C. Shoots with small leaves formed bunches (Fig. 1e), so they were separated and transferred singly into Magenta vessels (Sigma) onto the same medium, and sub-cultured every 3–4 weeks. Shoots of up to 2.5 cm in height were rooted in Magenta vessels, on 1/2 WPM medium containing 0.5 mg/l IAA (indole-3-acetic acid), sucrose (30 g/l), and solidified with agar (7.0 g/l). After 5–6 months of culture, plantlets with well-developed roots (5–10 cm) were about 4–5 cm high, so they were transferred to flower-pots. The plantlets were planted in a

mixture (1:1) of peat and sand (Fig. 1f), and placed in a misthouse for 10 days (to harden them off).

Statistical analyses

Statistical differences between means were evaluated using analysis of variance (ANOVA), in combination with the post-hoc Tukey test for pair-wise comparisons. Three replicates of 30 plumules were used for each treatment (90 plumules). The replicates were carried out for 3 weeks: 30 plumules in each week.

The Tukey test was performed after arc-sine transformation, at a significance level of $P < 0.05$. The standard deviation is presented for plumule survival and water content.

Results

Changes in the plumule water content and their survival during drying

The initial water content of plumules (after excision from acorns) was approximately 1.5 g H_2O g^{-1} dw (g g^{-1}), but this rapidly decreased within the first 10 min of drying to 0.8 over silica gel and 0.5 g g^{-1} (statistically significant differences) in nitrogen gas (Fig. 2a). Further plumule desiccation with 20–30 min over silica gel or nitrogen gas did not significantly change the WC (0.4–0.6 g g^{-1}). Drying of plumules for 40–70 min in nitrogen gas did not decrease WC (0.2–0.3 g g^{-1}), unlike drying over silica gel where WC decreased after 70 min of drying to 0.05 g g^{-1} (Fig. 2a).

Survival (in vitro) of plumules after 20 min of drying (over silica gel and in a nitrogen gas) was reduced from 90% (control, undesiccated plumules) to approximately 70%, and to 50% after 30 min (Fig. 2b). Further drying of plumules over silica gel for 50 min decreased their survival to 18% and to almost 0% after 70 min (Fig. 2b).

Effect of preliminary storage conditions for 18 h (after plumule isolation from acorns)

Survival of plumules after storage in a 0.5 M sucrose solution (95% at 4°C and 83% at 25°C) was higher than that after storage in water (44% at 4°C and 15% at 25°C, Fig. 3a). The beneficial influence of 0.5 M sucrose in preliminary storage was also observed after plumules were cryoprotected using 0.75 M sucrose, 1.0 M sucrose, and 1.5 M glycerol solutions. Survival rate of plumules stored before cryoprotection in 0.5 M sucrose reached 74%, compared with 18% of plumules stored in water at 4°C.

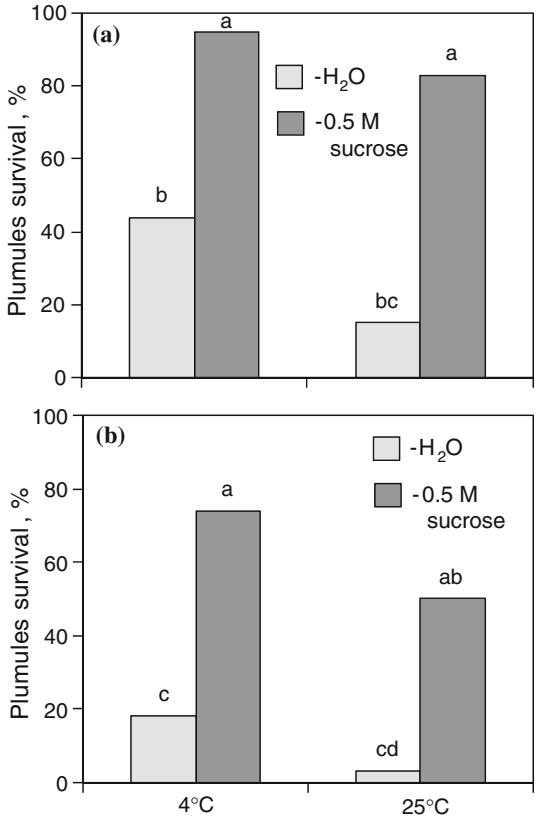

Fig. 3 Effect of 18-h initial storage of *Q. robur* plumules in water or in 0.5 M sucrose solution, at 4°C or 25°C on their survival after cryoprotection. **a** plumules not cryoprotected after initial storage; **b** plumules cryoprotected after initial storage in 0.75 M sucrose, followed by 1.0 M sucrose and 1.5 M glycerol, each treatment for 40 min at 23°C, and then rehydrated in 1.0 M sucrose followed by 0.5 M sucrose. Values marked with the *same letter* are not significantly different at $P < 0.05$, Tukey test

After storage at 25°C, their survival reached 50 and 3%, respectively (Fig. 3b).

Based on the aforementioned results, we used in cryopreservation experiments only plumules pretreated for 18 h in a 0.5 M sucrose solution at 4°C.

The effect of drying and cooling rates on plumule cryopreservation tolerance

Plumules desiccated to a WC of 0.35 g g^{-1} attained a survival rate of 21–67% after 24 h of cryopreservation (cooling in liquid nitrogen or in slush) (Fig. 4). There were significant differences in plumule survival following the two methods of desiccation (over silica gel or in nitrogen gas) when plumules were placed in vials of liquid nitrogen. Under this method, 67% of plumules desiccated over silica gel survived cryopreservation, but when desiccated in nitrogen gas to the same level of WC, only 21% of plumules survived (Fig. 4). The desiccation methods did not significantly ($P < 0.05$) affect plumule survival under

the second method of cryopreservation in slush (survival rate: 49% after desiccation over silica gel; and 37% after desiccation in nitrogen gas) (Fig. 4). The regrowth obtained from surviving plumules after 4 months of in vitro culture (shoots with small leaves, Fig. 1e) was 5–18%, and did not vary significantly among the treatments (Fig. 4).

The effect of provenance on plumule cryopreservation tolerance

Survival of plumules isolated from acorns of four provenances after cryopreservation varied from 48% (Błażejewko 3) to 77% (Radzewice), but the variation was not significant (Fig. 5). Plumule regrowth (shoots with small leaves, Fig. 1e) after 8 months of in vitro culture was 8–20% and also did not vary significantly among provenances (Fig. 5). After the rooting of shoots (efficiency 80%) on 1/2 WPM with auxin (indole-3-acetic acid, IAA 0.5 mg/l) from such shoots, plantlets with correct growth of shoots, leaves, and roots (complete plants) were obtained after acclimatization from cryopreserved plumules) (Fig. 1f).

Discussion

This is the first study showing successful cryopreservation of *Q. robur* L. plumules, where normally growing plantlets were retrieved from cryopreserved explants of various provenances. For cryopreservation, plumules were dissected from mature embryonic axes, initially pretreated in 0.5 M sucrose for 18 h, cryoprotected in 0.75 M sucrose,

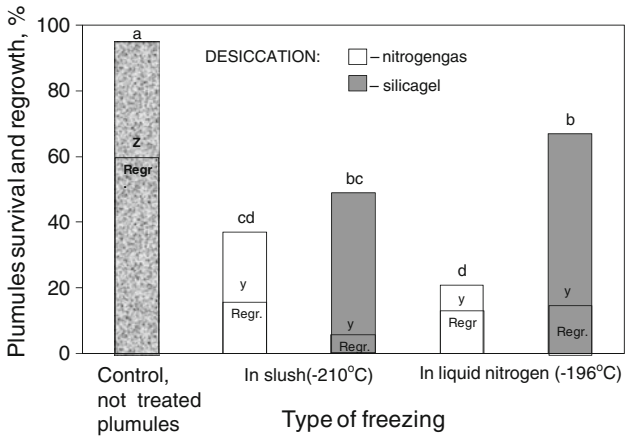

Fig. 4 Survival and regrowth of *Q. robur* plumules after desiccation over silica gel or in nitrogen gas to a water content of 0.5–0.6 g H_2O g^{-1} and cooling in slush or liquid nitrogen. Prior to desiccation and cooling the plumules were cryoprotected in 0.75 M sucrose, followed by 1.0 M sucrose and 1.5 M glycerol, each treatment for 40 min at 23°C. The values marked with the *same letter* are not significantly different at $P < 0.05$ (Tukey test)

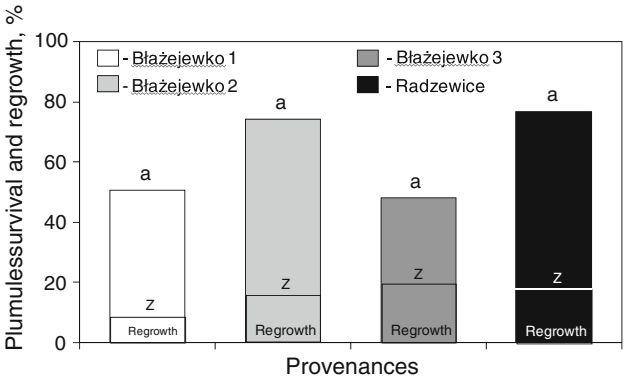

Fig. 5 Survival and regrowth of *Q. robur* plumules in vitro, isolated from acorns of four provenances after cryopreservation: Błażejewko 1 (plumules from one mother tree), Błażejewko 2 (plumules from one mother tree), Błażejewko 3 (plumules from three mother trees—the bulked collection of acorns), and Radzewice (plumules from three mother trees—the bulked collection of acorns). Plumules were cryoprotected in 0.75 M sucrose, followed by 1.0 M sucrose and 1.5 M glycerol, each treatment for 40 min at 23°C and dried over silica gel for 20 min. Plumules were stored in vials in liquid nitrogen for 24 h. The values marked with the same letter are not significantly different at $P < 0.05$ (Tukey test)

followed by 1.0 M sucrose and 1.5 M glycerol solutions, dried over silica gel for 20 min, directly cooled in LN, warmed at 42°C in 1.2 M sucrose solution, and slowly rehydrated in 0.75 M sucrose, followed by 0.5 M sucrose at 23°C.

Experiments conducted on embryonic axes of *Trichilia dregeana*, a tree of the family Meliaceae (Berjak et al. 1999b), showed that tissue composition, organ, and cell structure are some of the most important factors during cryopreservation. We concluded from our earlier experiments that whole axes do not survive cryopreservation (Chmielarz 1997), so here we used plumules isolated from axes. In our opinion, the radicle is an unnecessary ballast during cryopreservation (desiccation, cooling of axes). Moreover, the heavy radicles are more tolerant to desiccation than the minute plumules (Pritchard 1991), so plumules tend to die first (before the radicles) at each step of cryopreservation of embryonic axes. If only radicles survive, their regeneration into whole plants is impossible (Chmielarz 1997). During cryopreservation of plumules, as described here, very small (0.5–1.0 mm) homogenous tissue of plumules can be successfully cryopreserved with repeatable results. Vidal et al. (2005) cryopreserved *Castanea sativa* in vitro shoot tips of various sizes (0.5–2.0 mm). In their experiment, the survival, shoot regrowth, and final shoot length of smaller shoot tips (0.5–1.0 mm) were significantly higher than those of larger tips (2.0 mm) (Vidal et al. 2005).

Berjak et al. 1999a, b, 2000, who studied *Q. robur* axis ultrastructure after rewarming from LN during in vitro growth (6th day), reported that 70% of such axes initiated

organized shoot development. In their studies, viability of axes was assessed mainly on the basis of microscopic results of explants after 6 days of in vitro culture. The results showed that dehydration should be carried out as quickly as possible, and the cooling should be also rapid (Berjak et al. 2000). Those authors did not present any data (Berjak et al. 1999a, b, 2000) on the regrowth of initially developed shoots, leaf development, shoot elongation, or plantlet growth. We presented such data in this article, backed with statistical analysis. In the present study, survival determined after 14 days (60–70%) refers to shoot development (as plumules are shoot apical meristems) and was repeated on various trees with the same good results. Regrowth in this paper (20% in the best variants) was determined after 8 weeks of in vitro growth. A similar period was used in shoot apices of *Castanea sativa* (Vidal et al. 2005). From such shoots, after rooting, normally growing plants were obtained.

Plumules appeared to be more successfully cryopreserved than whole embryonic axes, which had been tested previously (Chmielarz 1997). We observed a low level of phenol production and no callus formation in rewarmed plumules during in vitro culture in our experiments, unlike in the cryopreservation of embryonic axes, where some root growth and callus formation were observed in vitro after regrowth from LN (Chmielarz 1997). In our experiments shoots obtained from recovered plumules were rooted, and complete plants were hardened off in a greenhouse. There are two reports of the successful cryopreservation of plumular explants of coconut (*Cocos nucifera* L.) (Hornung et al. 2001; N'Nan et al. 2008). Both authors proved that cryopreservation was feasible through plumule storage in liquid nitrogen, thus avoiding the callus pathway. Corredoira et al. (2004) successfully cryopreserved embryonic axes of *Castanea sativa* desiccated to a WC of 20–24%, with 63% of regeneration into whole plants. We can assume that it was possible because embryonic axes of *C. sativa* are more desiccation tolerant than embryonic apical meristems of *Q. robur*. Only minor changes in *C. sativa* membrane permeability were observed after drying to a WC of 0.12 g g^{-1} (Leprince et al. 1999). By contrast, plumules of *Q. robur* desiccated only to a WC of 0.5 g g^{-1} showed a decrease in their survival in vitro in our research. We consider that different sensitivity of *C. sativa* and *Q. robur* tissues isolated from recalcitrant seeds can be one of the reasons of differences in regeneration after cryopreservation (63% for *C. sativa* vs 20% for *Q. robur*). Regrowth of cryopreserved in vitro shoot tips of *C. sativa* also reached a relatively high level of 34–58% in a study by Vidal et al. (2005). Here, we dissected plumules from embryonic axes, where a substantial wound arises during plumule isolation (1/3 of the plumule, at its base). This could be a reason for differences in regrowth of the tissues after cryopreservation.

There are some reports of successful cryopreservation of *Q. robur* embryogenic callus (Chmielarz et al. 2005; Martinez et al. 2003) and successful cryopreservation of *Q. robur* embryonic axes collected from a single tree (Chmielarz 1999). Unfortunately, the author failed to repeat these results on embryonic axes collected from other pedunculate oak trees (P. Chmielarz, unpubl. data). That is why plumules were chosen here as more promising explants. In this paper, a high percentage of survival and regrowth was obtained for plumules isolated from acorns collected from two individual mother trees as well as from bulked collections of three different provenances.

In the experiments presented here, the small size of *Q. robur* plumules allowed us to achieve a higher rate of drying during plumule desiccation over silica gel and in a nitrogen gas in comparison with whole embryonic axes (Chmielarz 1997, 1999), which ensured the high percentage of plumule survival. As reported previously (Beardmore and Whittle 2005; Pammenter et al. 2000; Wesley-Smith et al. 2001), faster desiccation of cryopreserved tissue appears to be more beneficial to the tissue of recalcitrant seeds. We observed in our experiments that plumule desiccation under nitrogen flow for 60–70 min did not cause any further decrease in their MC, while drying above silica gel did. This could be due to technical parameters of nitrogen gas used (from the bottles) or to the unsealed desiccation boxes, where some moisture from the outside could get into. This did not happen during desiccation under silica gel, where Petri dishes were sealed with Parafilm.

After fast desiccation, *Q. robur* plumules could survive liquid nitrogen exposure at a WC of 25%, a higher percentage in comparison with the WC of embryonic axes of *Hevea bragen* at 16% and *Araucaria hunsteinii* at 20% (Normah et al. 1986; Pritchard and Prendergast 1986), and similar to that for *Aesculus* and *Castanea* at about 20–30% (Pence 1992). Survival of plumules after desiccation to a WC of 0.5 g g^{-1} (20 min) in our experiments amounted to 70%. After cryoprotection (in sucrose and glycerol solutions) and then cryopreservation, about 60% of plumules survived, while regrowth was observed in 20%. The relatively high level of plumule WC (0.5 g g^{-1}) before cryopreservation could cause ice crystal formation during cooling. However, longer drying (for 30 min) decreased plumule survival to 45%. We can speculate that after an intermediate time of plumules drying (25 min), a higher survival rate could be obtained.

It is clear that there is different sensitivity to the desiccation of root and shoot axes among species. The root (radicle) is more sensitive in *A. hippocastanum* and the shoot is more sensitive in *Aesculus glabra*, *Castanea sativa*, and *Araucaria husteinii* (Pence 1992; Pritchard et al.

1995). Whole embryonic axes of *Q. rubra* survived desiccation to a WC of 40%, but epicotyls could be dried to 10% (Pritchard 1991). Removal of radicles from the embryonic axes during cryoprotection and desiccation in the experiments presented here allowed successful cryopreservation of *Q. robur* plumules.

Q. robur plumules appeared to be very sensitive to desiccation in our experiments, although they survived 50% water loss. Therefore, it is suggested that some protection from desiccation-induced injury could be provided by plumule cryoprotection in sucrose solutions. First, non-reducing sugars may facilitate tolerance to desiccation by protecting membranes and proteins (Scott 2000) and by inducing vitrification (Suzuki et al. 1997).

We found no statistically significant differences between cooling by direct immersion of plumules in vials with LN (−196°C) and cooling in slush (−210°C), nor was the difference in plumule survival significant between the two temperatures applied during cooling. Partial dehydration reduces the requirements for extremely rapid cooling because it lowers the cooling temperature (Farrant and Walters 1998; Pritchard and Prendergast 1986; Vertucci 1990; Walters et al. 2001; Wesley-Smith et al. 1992), elevates the glass transition temperature (Buitink et al. 1996; LePrince and Walters-Vertucci 1995; Williams et al. 1993), and increases the intracellular viscosity.

Pretreatment and rehydration medium composition also played an important role in the regrowth of surviving plumules in our research. Only plumules that were pretreated directly after dissection from axes in 0.5 M sucrose, instead of water, and rehydrated after rewarming survived cryopreservation. Makeen et al. (2007) showed a similar effect during rehydration of seed *Citrus suhuiensis* cv. Limau madu. In contrast for other species like *Artocarpus heterophyllus*, *Podocarpus henkelii*, and *Ekebergia capensis* fast rehydration ensures better survival of embryonic axes (Perán et al. 2004). For amaryllid species fast rehydration of embryonic axes in CaMg solution after partial desiccation gave better results in terms of axis viability in comparison to slow rehydration on filter paper (Sershen et al. 2007).

We achieved similar results in terms of final regrowth to N'Nan et al. (2008) who obtained 20% of regrowth of coconut plumules after cooling them in liquid nitrogen. Our protocol employed for cryopreservation of *Quercus* germplasm should be improved to increase plumule regrowth, and we have already initiated experiments aimed to reach this goal.

Our investigations show that cryopreservation of plumules gives new possibilities for conserving the biodiversity of pedunculate oak, which produces large seeds with very sensitive plumule tissue. Among the other black and white oak species, *Q. robur* embryonic axes are relatively highly sensitive to desiccation (Chmielarz and Walters 2007). We think that the protocol presented here, which was successfully repeated using several provenances of *Q. robur*, should be verified for other species of the *Quercus* family and other recalcitrant species. The cryostorage of plumules requires very little space in cryo-containers, which is important especially when large amounts of material have to be stored in gene banks in order to collect all the genetic variation of the species (Humphries et al. 1995). This procedure for *Q. robur* plumule cryopreservation offers a way to improve *Quercus robur* genetic resource conservation in gene banks.

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