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Original Article

Evaluating the bioactivity of recalcitrant seeds by vital staining after freezing in two temperate tree species, *Quercus myrsinifolia* and *Q. glauca*

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Introduction

Seed preservation is a promising strategy for the conservation of endangered species. Some species produce a reasonable seed quantity only once every three to five years (ELLIS, 2003) and fruiting patterns of some species are unpredictable (e.g. NEWSTROM *et al.*, 1994; SORK *et al.*, 1993). To ensure seed availability for conservation, studies on extending the longevity of seeds are needed. Compared to maintaining tree nurseries, seed storage is space-saving, inexpensive, effective, and also a safe method for *ex situ* conservation of plant genetic resources (PHARTYAL *et al.*, 2002).

Seeds can be largely classified into three categories based on their storage behaviors: orthodox, intermediate, and recalcitrant (ELLIS, 2003; ELLIS *et al.*, 1990; ROBERTS, 1973). These categories are mainly based on levels of desiccation tolerance (FINCH-SAVAGE, 1992). Mature orthodox seeds are quiescent when they are shed (BERJAK and PAMMENTER, 2008), and can survive desiccation to a moisture content as low as 2-6 %. Because there is a negative relationship between temperature and seed longevity for a given moisture content (HONG and ELLIS, 1996), orthodox seeds can be stored for a relatively long period of time under conditions with low humidity and low temperature. Unlike orthodox seeds, recalcitrant seeds are metabolically active at maturity and lose viability below a relatively high moisture content (e.g. 15-20 %) when they are desiccated (HONG and ELLIS, 1996). Not only desiccation but also temperature below 16°C should be avoided when storing moist recalcitrant seeds because it may lead to chilling injury (KRISHNAPILLAY and TOMPSETT, 1998). Recalcitrant seeds can thus far be stored only for a relatively short period of time and successful procedures for long-term preservation of these seeds have yet to be established.

Cryopreservation is one of the most widely-used methods for the long-term storage of seeds. At the temperature of liquid nitrogen (-196°C), all biological processes are virtually suspended (PENCE, 1990; SAKAI, 1987; WALTERS *et al.*, 2004). Cryopreservation frees seed samples from damage by insects or fungi, with little or no genetic change (STYLES *et al.*, 1982), and allows samples to be stored semi-permanently (but see WALTERS *et al.*, 2004 for potential deterioration) without adjustments of temperature or humidity (STYLES *et al.*, 1982). Despite the lack of established methods, cryopreservation is considered to be one of the most promising ways of storing recalcitrant seeds, with some successful reports (e.g. BERJAK and DUMET, 1996;

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GONZALEZ-BENITO and PEREZRUIZ, 1992; PENCE, 1992).

Sample volume is an important consideration in cryopreservation. Cryopreservation of whole seeds is often lethal (GROUT *et al.*, 1983) and even orthodox seeds cannot be frozen whole. Excision of embryonic axis from the embryo before freezing and subsequent tissue culture is a common practice (e.g. HONG and ELLIS, 1996; WESLEY-SMITH *et al.*, 2001b). Reducing the sample volume from whole seed to embryonic axis is important in increasing the thawing rate, which affects the viability of frozen plant tissues, including seeds (JORDAN *et al.*, 1982; SAKAI *et al.*, 1968). When cells are sufficiently dehydrated, no destructive effect is observed in the process of thawing (SAKAI *et al.*, 1968). Otherwise, recrystallization occurs in cells that are frozen ultra-rapidly to the temperature of liquid nitrogen during a slow re-warming process, destroying cells internally (SAKAI and OTSUKA, 1967; SAKAI *et al.*, 1968; SAKAI and YOSHIDA, 1983). To avoid destruction of cells due to recrystallization, thawing must be rapid enough to bypass the temperature range between -40 and 0°C (SHIMADA, 1987).

Desiccation before freezing can be effective in reducing the moisture in the seed, which destroys the cells when the seed is frozen. Although desiccation is deleterious for an intact embryo, the excised embryonic axis can withstand the degree of dehydration necessary to survive freezing, provided that water is lost sufficiently rapidly (PAMMENTER and BERJAK, 1999; WESLEY-SMITH *et al.*, 2001a). Both slow desiccation by natural air-drying (VARGHESE *et al.*, 2004) and rapid desiccation (PRITCHARD and PRENDERGAST, 1986) have been attempted as the pre-drying treatment. More studies have shown that rapid desiccation allows recalcitrant seeds to survive lower moisture contents than when desiccated slowly (FARRANT *et al.*, 1989; VARGHESE *et al.*, 2004; but see Finch-Savage, 1992). However, how desiccation and subsequent freezing affects viability in tissues within the embryonic axis is still largely unknown.

The objective of this study was to assess the potential for long-term cryopreservation of recalcitrant seeds in two oak species, *Quercus myrsinifolia* Blume and *Q. glauca* Thunb. (Fagaceae). They are common evergreen species that reach about 20 m in height, and are distributed over a large area in the warm temperate zone of East Asia. While trees in the genus *Quercus* are currently widely distributed throughout Japan and constitute a major proportion of the forest canopy layer, Japanese oak wilt incidence has caused widespread mortality of oaks, including the two focal species (KAMATA *et al.*, 2002). Both species generally provide stable seed production every year but their seeds are known to show recalcitrant behavior (KATSUTA, 1998). Thus, means for long-term preservation of these seeds is needed to prepare for the anticipated decline in *Quercus* population. However, studies on preservation of *Quercus* seeds, especially those of evergreen *Quercus* species, are scarce.

In this study, seeds of *Q. myrsinifolia* and *Q. glauca* (Fig. 1) were subjected to two processing treatments (seed coat removal and embryonic axis excision), followed by a freezing treatment. Desiccation treatments were also conducted on *Q. myrsinifolia*, first without freezing, and then as the pre-drying treatment before freezing. To examine loss of bioactivity in tissues in the embryonic axis, and whether bioactivity is retained in tissues that contain meristem cells, vital staining was conducted in both species for excised embryonic axes and for intact embryos when

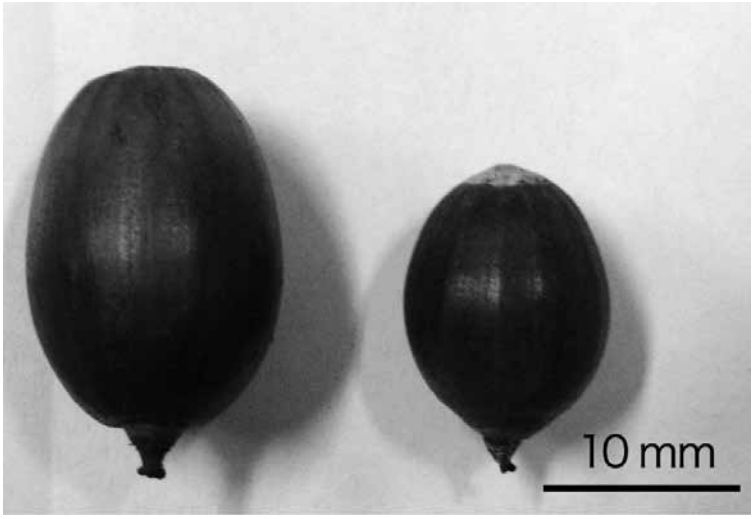

Fig. 1 Seeds of *Q. myrsinifolia* (left) and *Q. glauca* (right). In all experiments, the seed coat was removed unless otherwise indicated.

they did not germinate after desiccation or freezing treatment.

Materials and Methods

Seed samples

In November 2006, seeds (acorns) of *Q. myrsinifolia* (length: 17-19 mm, width: 11-14 mm, without cupule) and *Q. glauca* (length: 13-15 mm, width: 9-11 mm) were collected at the University of Tokyo Tanashi Forest, located in Tokyo, Japan (35°44' N, 139°32' E). The seeds were collected from mother trees by spreading a sheet under the crowns (*Q. myrsinifolia*: n = 2, *Q. glauca*: n = 3). These mother trees were adjacent to each other. After the cupules were removed, the seeds were immersed in the water for five minutes and only those that sunk were used in this study. The seeds that qualified were stored in large resealable polyethylene bags and kept at room temperature in the dark until use between November 2006 and February 2007. The bags were ventilated at least once a week and seeds with cracks or insect predation were removed as necessary.

Germination test

Germination tests were conducted in both species to ensure that inherent quality of the seeds used here was not poor, regardless of the treatment. The seed coats were first removed from the embryos to promote synchronous germination without delay. These embryos were immersed in deionized water overnight at a temperature between 23 and 28˚C. They were then immersed in 3.0% (v/v) hydrogen peroxide solution for 20 seconds for surface sterilization, and rinsed with running deionized water for 10 minutes. These sterilized embryos were then sowed in heat-sterilized silicate sand, kept at a temperature between 23 and 28˚C, and watered once every three

days. When the radicle reached a length of 3 mm within the three month time period of this study, the embryo was considered to have germinated. To assess the effects of treatment on embryo viability, germination tests were also conducted after the desiccation treatment in *Q. myrsinifolia* and after the freezing treatment in both species.

Processing of experimental samples

Seeds of both species were subjected to one of two processing treatments (Fig. 2): intact embryo (IE) and excised embryonic axis (EA). In the IE treatment, only the seed coat was removed. In the EA treatment, the embryonic axis (*Q. myrsinifolia*: 1.37 ± 0.54 mg, *Q. glauca*: 1.26 ± 0.38 mg fresh weight) was carefully excised from the embryo (*Q. myrsinifolia*: 1.37 ± 0.15 g, *Q. glauca*: 0.66 ± 0.11 g) by removing the cotyledons at the petioles (Fig. 2). Before freezing, samples from the EA treatment were kept in a moist paper towel to prevent desiccation during this short processing time.

Desiccation and determination of moisture content

Desiccation treatments were first conducted without freezing in *Q. myrsinifolia* to determine the time needed to reach the lowest moisture content without negative effects on the viability of the seeds. Pre-drying treatments were then attempted in *Q. myrsinifolia* to see whether such treatments improved the viability rate after freezing. A special apparatus was assembled following WESLEY-SMITH *et al.* (2001a) to achieve rapid desiccation using a stream of air and silica gel (Fig. 3). Embryonic axes from the IE and the EA treatments, and cotyledons from the IE treatment, were weighed before and after desiccation, and moisture content percentage was calculated on a fresh-weight basis (fwb). Moisture content of stored seeds did not change significantly during storage. The effects of desiccation on embryo viability were evaluated by germination tests in samples from the IE treatment. When embryos did not germinate, bioactivity

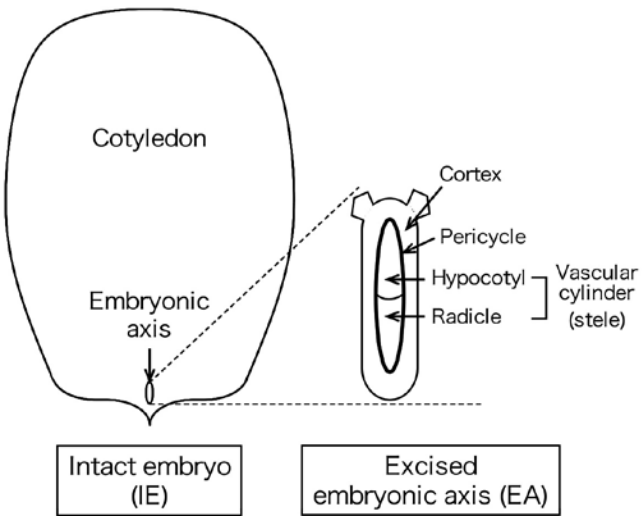

Fig. 2 Two processing treatments of seeds: intact embryo (IE) and excised embryonic axis (EA).

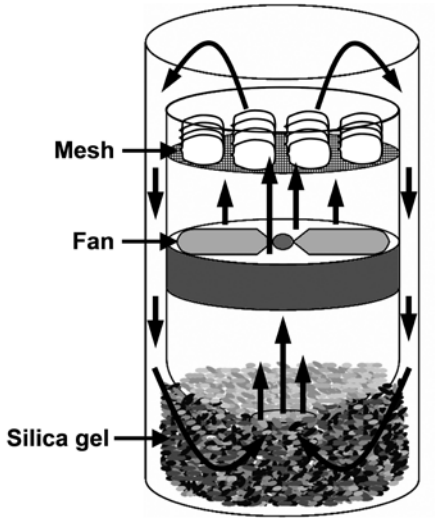

Fig. 3 Schematic diagram of the apparatus used in this study to achieve rapid desiccation. Arrows show the movement of air when seeds placed on the mesh (n = 9-16) were desiccated. Silica gel was packed under the main component and was recharged in an oven before each use.

in the embryonic axes from the IE treatment was also evaluated by vital staining, as with samples from the EA treatment.

Freezing treatment

For samples from the IE treatment, 15 ml polypropylene tubes were used and 1.2 ml polypropylene cryovials were used for samples from the EA treatments. The tubes and vials were immersed in liquid nitrogen for at least one minute, and then kept in a steel dipper suspended in the gaseous phase of the liquid nitrogen tank for 25 hours. In order to achieve rapid thawing, each tube or vial was double-bagged and placed in a 38˚C water bath for 50 minutes (IE treatment) or 30 minutes (EA treatment).

To assess whether germination failure is associated with bioactivity loss of certain tissues in the embryonic axis, germination tests and vital staining were conducted for samples from the IE treatment that were frozen without pre-drying treatment in both species. For the rest of the frozen samples in both species, viability was assessed only by vital staining.

Evaluation of bioactivity

Once the embryonic axis is excised, the embryo is not expected to germinate because cotyledons provide nutrients necessary for its growth, and subsequent tissue culture would be necessary for plant regeneration. Because tissue culture was beyond the scope of this study, vital staining was conducted in samples from the EA treatment to evaluate bioactivity of tissues in embryonic axes after desiccation in *Q. myrsinifolia* and freezing in both species. When intact embryos from the IE treatment did not germinate after freezing treatment, vital staining was also conducted separately to assess how bioactivity in tissues in the embryonic axis was lost.

Bioactivity of tissues in embryonic axes was evaluated by the red staining from insoluble triphenyl formazan (LAKON, 1949; PORTER *et al.*, 1947; SMITH, 1951). This formazan is produced when 2,3,5-triphenyltetrazolium chloride (TTC) is reduced by succinate-tetrazolium reductase, which is a part of the mitochondrial respiratory chain (RUF and BRUNNER, 2003). Embryonic axes were first immersed in deionized water for more than 20 hours for water absorption prior to vital staining in 1.0% (g/ml) TTC (Kanto Chemical Co., Inc., Japan) in 0.1 M phosphate buffer (pH 6.9). To enhance permeation of the stain, samples in the TTC solution were put under reduced pressure using a bell jar with vacuum pump (SUGAWARA, 1987). Samples were incubated between 23 and 28˚C for 24 hours in the test tubes shielded with aluminum foil to avoid degradation of TTC by ultraviolet light (LAKON, 1949), and then rinsed three times with deionized water.

Each embryonic axis was dissected and examined under a microscope, and red coloration was separately evaluated for cortex, vascular cylinder (stele), and pericycle (periphery of the vascular cylinder, which consists of hypocotyl and radicle; Fig. 2). Among these tissues, pericycle is the site for the initiation of lateral roots and the vascular cambium, which is a lateral meristem. Because the staining intensity is not discrete but rather continuous from colorless to vivid red, only tissue with a staining intensity equivalent to the controls (no desiccation or freezing) was considered to be active (Fig. 4).

Statistical analyses

Fisher's exact test (two-sided test) with Bonferroni adjustment for multiple comparison procedure was used to analyze data on bioactivity and germination after desiccation. All the analyses were conducted using SAS 9.4 software (SAS INSTITUTE INC., 2013).

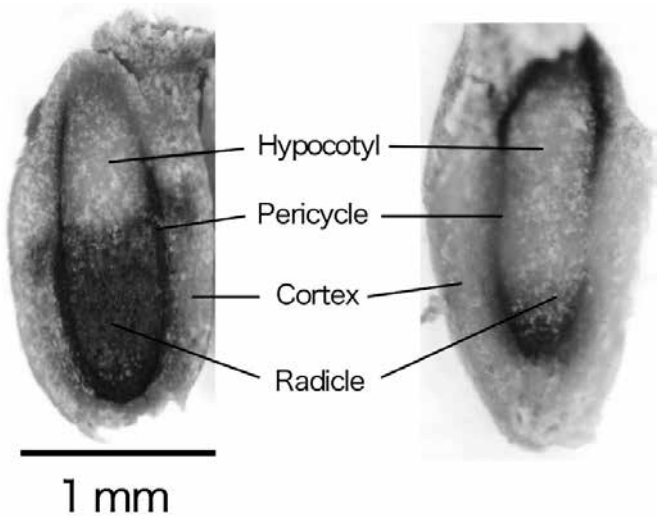

Fig. 4 Examples of embryonic axes from EA treatment after freezing in *Q. glauca* stained by TTC. The dark-colored tissues are considered active. The sample on the left shows an example of active radicle in the vascular cylinder. The sample on the right (taken from another seed) shows an example of active hypocotyl and radicle in the pericycle.

Results

Effects of desiccation and freezing on germination ability of seeds

Without desiccation or freezing, embryos from the IE treatment showed high germination percentages in both species (Table 1). When embryos of *Q. myrsinifolia* from the IE treatment were desiccated, germination percentages decreased with increasing desiccation time to nearly 50% after 60 hours of desiccation (Table 1). When embryos were desiccated, fungal infection was observed in some of the embryos that germinated. Freezing was also detrimental to embryos from the IE treatment in both species such that no seeds from either species germinated after freezing (Table 1).

Effects of desiccation and freezing on bioactivity of embryonic axes

Vital staining revealed no negative effect in embryonic axes of *Q. myrsinifolia* from the EA treatment when desiccation time was less than 25 minutes (Table 2). After 40 minutes of desiccation, moisture content dropped below 30%, and a loss of bioactivity was observed, especially in the vascular cylinder (40 minutes: $P = 0.033$) and the cortex (60 minutes: $P = 0.0031$). To achieve the lowest moisture content in the embryonic axis without losing viability, desiccation time for the pre-drying treatment was set to 0-25 minutes.

After freezing, bioactivity of tissues in the embryonic axis from both the IE and the EA treatments was severely reduced, especially in the cortex (Table 2), similar to the results from germination tests (Table 1). Notably, unlike in the desiccation treatment, bioactivity in the outermost cortex was almost entirely lost after freezing, regardless of the treatment or species (*Q. myrsinifolia*: IE, $P < 0.0001$; EA, $P < 0.0001$; *Q. glauca*: IE, $P < 0.0001$; EA, $P < 0.0001$). As with desiccation, the pericycle, the outermost layer of the vascular cylinder, was consistently the most resistant to freezing. In both species, bioactivity in the vascular cylinder was significantly reduced in samples from the IE treatment compared to those from the EA treatment (*Q. myrsinifolia*: $P = 0.0034$, *Q. glauca*: $P = 0.026$). In contrast, bioactivity in the pericycle was most retained such that bioactivity in the samples from IE treatment did not differ significantly from those from EA treatment in both species.

Table 1 Germination percentages of *Q. myrsinifolia* and *Q. glauca* embryos from the IE treatment. Proportion of embryos that germinated despite fungal infection is also shown when embryos were not frozen. Different letters for each column indicate significant difference at $\alpha = 0.05$ by Fisher's exact test with Bonferroni adjustment.

Species	Desiccation time (hr)	Germination without freezing (%)			Germination after freezing (%)	
		N	Total	With fungal infection	N	Total
Q. myrsinifolia	0	50	98^{A}	0^{b}	30	0
	20	30	83^{AB}	33^{a}	0	-
	40	42	57^{BC}	33^{a}	0	-
	60	40	52^{C}	28^{a}	0	-
Q. glauca	0	44	100	0	50	0

Table 2 Percentages of three kinds of tissues in the embryonic axis that were active when evaluated by vital staining after desiccation and freezing treatments in *Q. myrsinifolia* and *Q. glauca* seeds. Moisture contents listed for different desiccation times are for embryonic axes in both the IE and the EA treatments.

Species	Treatment	Desiccation time	Moisture content (% fwb)	Proportion active without freezing (%)				Proportion active after freezing (%)			
				N	Cortex	Vascular cylinder	Pericycle	N	Cortex	Vascular cylinder	Pericycle
Q. myrsinifolia	IE	0 hr	51.8 ± 2.2	0	-	-	-	20	0	5	40
		20 hr	47.8 ± 3.0	0	-	-	-	10	0	0	0
		40 hr	42.5 ± 16.7	0	-	-	-	10	0	0	0
		60 hr	39.0 ± 6.9	0	-	-	-	9	0	0	0
	EA	0 min	53.1 ± 2.8	15	93	100	100	20	5	55	75
		3 min	46.0 ± 2.4	14	100	100	100	10	0	20	30
		5 min	37.5 ± 11.0	15	90	100	100	4	0	25	25
		15 min	36.7 ± 5.3	10	100	100	100	11	0	0	0
		25 min	31.0 ± 7.2	10	100	100	100	10	0	0	0
		40 min	24.5 ± 5.4*	9	67	56	89	0	-	-	-
		60 min	16.1 ± 2.9	10	30	20	70	0	-	-	-
Q. glauca	IE	0 hr	58.0 ± 2.4	0	-	-	-	20	0	25	65
	EA	0 min	58.2 ± 3.4	10	100	100	100	20	0	70	75

*45 min.

Although pre-drying treatment was expected to be effective based on previous studies, it was rather detrimental when applied to *Q. myrsinifolia* (Table 2). In the EA treatment, the proportion of active embryonic axes was reduced to less than half of those that were not desiccated in either the vascular cylinder or the pericycle. No bioactivity was observed in any tissue for those desiccated more than 15 minutes before freezing. In the IE treatment, desiccation rate was much slower than in the EA treatment where moisture content was above 30% even after 60 hours of desiccation. Pre-drying treatments on samples from the IE treatment resulted in a total loss of bioactivity in all tissues, regardless of desiccation time.

Discussion

There is growing need for long-term preservation of recalcitrant seeds but successful methods for storage of these seeds have not been established. In this study, potential for long-term cryopreservation in recalcitrant seeds of two *Quercus* species was assessed by germination tests and vital staining. Two major findings from this study were: (1) in contrast to many previous findings, pre-drying treatment before freezing was rather lethal for the one species tested and (2) tolerance against desiccation and freezing differed among tissues in the embryonic axis.

A pre-drying treatment has been considered beneficial or even a prerequisite for cryopreservation for a range of recalcitrant seeds (e.g. ENGELMANN, 2011; GONZALEZ-BENITO and PEREZRUIZ, 1992; KAVIANI, 2011; PENCE, 1992; PRITCHARD and PRENDERGAST, 1986; VARGHESE *et al.*, 2004). In this study, however, the pre-drying treatment was only detrimental to survival of embryos and embryonic axes in *Q. myrsinifolia*. Germination percentages of embryos from the IE treatment decreased with increasing desiccation time (Table 1). Fungal infection was also observed in germinated embryos, regardless of desiccation time, suggesting that the integrity of the seeds was impaired by desiccation (Table 1). Results from vital staining showed that bioactivity of embryonic axes from the EA treatment also decreased after 25 minutes of

desiccation without freezing (Table 2). The detrimental effects of desiccation were more pronounced after freezing. Pre-drying led to a total loss of germination ability in samples from the IE treatment, and substantial loss in bioactivity in samples from the EA treatment, especially in the cortex, even with a desiccation time of less than 25 minutes. Results from previous studies suggest that desiccation rates might not have been rapid enough in this study (e.g. Varghese *et al.*, 2004; Wesley-Smith *et al.*, 2001a). A total loss of bioactivity in samples from the IE treatment can be explained by slower desiccation rates due to a larger volume compared with samples from the EA treatment. In another study with several congeners, however, desiccation rates were slower and moisture content was higher but seeds still showed viability (Pence, 1992). Response to desiccation and freezing may thus be highly species-specific, even within a genus (Yao *et al.*, 2014). The two *Quercus* species in this study may be comparatively more susceptible to desiccation because they are both evergreen (Givnish, 2002), whereas congeners from other studies were all deciduous (Jörgensen, 1990; Pence, 1992).

When samples were frozen without pre-drying, bioactivity in the vascular cylinder in the EA treatment was higher than that in the IE treatment for both species. Such results are consistent with previous findings that recalcitrant seeds survive cryopreservation better when embryonic axis is excised (e.g. Gonzalez-benito and Perezruiz, 1992; Pammenter and Berjak, 2014). Both cooling (Grout *et al.*, 1978; Pammenter and Berjak, 2014; Sakai *et al.*, 1968; Wesley-Smith *et al.*, 2001b) and thawing (Jordan *et al.*, 1982; Sakai *et al.*, 1968) rates affect the viability of samples after cryopreservation. Because the volume of the samples from the IE treatment was much greater than that from the EA treatment, cooling and thawing rates were likely to be much faster in samples from the EA treatment and showed higher tolerance to freezing.

Different tissues within the embryonic axis showed differences in tolerance to desiccation and freezing, and the pericycle tissue was the most resistant to both, compared to the cortex or vascular cylinder in *Q. myrsinifolia* (Table 2). Such differences among different tissues are also known in other species (e.g. Finch-Savage, 1992; Pritchard and Prendergast, 1986), and studies suggest that these differences result from the differences in desiccation and cooling rates depending on where these tissues are located (e.g. Wesley-Smith *et al.*, 2001a; Wesley-Smith *et al.*, 1992). In a study using embryonic axes excised from recalcitrant seeds, water was preferentially withdrawn from the outermost tissues when the embryonic axes were desiccated rapidly, while inner tissues, such as the vascular cylinder, were relatively unaffected (Wesley-Smith *et al.*, 2001a). Conversely, when cortical parenchyma cells from twigs were frozen in another study, only the cells at the periphery of a tissue remained alive and the inner cells were completely destroyed due to slower cooling rate than cells at the periphery (Sakai and Otsuka, 1967). In another study using *Quercus* seeds, frozen tissues were destroyed due to tension caused by the large temperature difference between the inner and outer parts (Jörgensen, 1990). In this study, tolerance to desiccation also differed from the tolerance to freezing for a given kind of tissue in samples from the EA treatment, although this observation has not been reported elsewhere. In *Q. myrsinifolia*, the outermost cortex showed similar or even slightly higher bioactivity compared with the vascular cylinder in the desiccation treatment. In the freezing

treatment, in which tissue damages were more severe, bioactivity in the cortex was the first to be lost whereas bioactivity in the vascular cylinder and the pericycle was retained at higher levels. This pattern is consistent with that reported above (WESLEY-SMITH *et al.*, 2001a). Different tissues from recalcitrant seeds differ in the pattern of water loss and could also be species-specific (BALLESTEROS *et al.*, 2014). The observed differences in tolerance between desiccation and freezing for a given tissue in *Q. myrsinifolia* may thus be explained by the different patterns of water loss for desiccation and freezing.

In this study, vital staining was used to assess the chance of cryopreservation success. Because tissues will not be stained without cellular respiration and plant regeneration cannot be expected from non-active tissues without cellular respiration, no staining in a given tissue was considered loss of bioactivity in that tissue. Without desiccation or freezing, all the tissues were stained in embryonic axes (Table 2) and there was nearly 100% germination in both species (Table 1). When embryos from the IE treatment were frozen, not all tissues were stained, and such loss of bioactivity in some tissues likely led to no germination. While vital staining revealed how the bioactivity was lost and retained at the tissue level in this study, determining whether or not the bioactivity retained in certain tissues in embryonic axes leads to plant regeneration requires further work. Previous studies show that tissues that include meristems do not need to be alive in their entirety for regeneration into whole plants to take place (HASKINS and KARTHA, 1980). Using other *Quercus* species, cryopreservation using pericycle and hypocotyl (shoot meristems) excised from embryonic axes (PLITTA *et al.*, 2014) and entire embryonic axes (GONZALEZ-BENITO and PEREZRUIZ, 1992) has been successful. Thus, because the pericycle possesses the capacity for cell division, and the cambium is formed later in the vascular cylinder, which contains current and future meristem cells, plant regeneration may still be possible by the EA treatment that retained bioactivity in the vascular cylinder and the pericycle. Therefore, although the entire embryo lost germination ability after freezing, excised embryonic axes may still have a chance of plant regeneration after cryopreservation with subsequent tissue cultures for the two evergreen *Quercus* species used in this study.

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Summary

Recalcitrant seeds have a low tolerance for desiccation and cooling, and can be stored only for a relatively short period of time. This poses a major challenge in the conservation of plant genetic resources. Cryopreservation is a promising strategy for storage of recalcitrant seeds but successful procedures for long-term preservation are yet to be established. Using two evergreen

Quercus species (*Q. myrsinifolia* and *Q. glauca*) with recalcitrant seeds, this study assessed the potential for long-term seed cryopreservation by means of germination tests and vital staining after exposure to liquid nitrogen. Seeds were subjected to one of two processing treatments: intact embryo (IE) and excised embryonic axis (EA). Rapid desiccation treatments were also attempted with and without freezing in *Q. myrsinifolia*. Although seeds of both species showed high germination percentages, desiccation reduced germination percentages and bioactivity in the embryonic axis in *Q. myrsinifolia*. Bioactivity in the embryonic axis was further reduced after freezing, and in most tissues it was lower in the samples from the IE treatment than those from the EA treatment. Although a pre-drying treatment has been considered beneficial for cryopreservation based on previous studies, it was only detrimental in *Q. myrsinifolia*. Tolerance to desiccation and freezing differed among tissues in the embryonic axis, and tissue tolerance against desiccation also differed from tolerance against freezing. Tissues that retained bioactivity after freezing possess cell division capacity (e.g. pericycle) such that excised embryonic axes of the two focal species may have a chance of plant regeneration after cryopreservation with subsequent tissue cultures.

Keywords: Cryopreservation, desiccation, embryonic axis, recalcitrant seed, vital staining

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シラカシとアラカシ種子の超低温凍結後の生理活性評価

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要　旨

乾燥・低温耐性が低い難貯蔵性種子は，遺伝子資源保全のための長期的な保存ができない。このような種子の長期保存には，超低温凍結保存が有効であるとされているが，その方法は，まだ確立していない。本研究では，難貯蔵性種子であるシラカシとアラカシの種子を用いて，発芽試験と生体染色によって，種子の胚軸内の組織の生理活性を評価し，超低温凍結保存の可能性を探った。種子から種皮を除去した「胚」と子葉をも切除した「胚軸のみ」の二種類の供試体を調製した。また，凍結保存の際に耐凍性を高める効果があるとされる急速乾燥による予備乾燥の効果を，シラカシを用いて検証した。その結果，急速乾燥処理によって，発芽率と胚軸の活性が落ち，負の効果しか見られなかった。凍結後は，二種類の供試体の内，ほとんどの組織で，「胚」に比べ，「胚軸のみ」で活性が維持された。乾燥・凍結耐性は，胚軸内部の組織によって異なり，乾燥と凍結処理とでも異なった。予備乾燥を施さない場合，分裂組織を含むはずの内鞘は，凍結後も比較的活性が維持されることが明らかになった。このような組織から，組織培養技術によって個体再生ができれば，超低温凍結による保存が可能となるであろう。

キーワード：生体染色・超低温凍結保存・難貯蔵性種子・胚軸・予備乾燥