Different Responses of Embryonic Axes and Cotyledons from Tea Seeds to Desiccation and Cryopreservation

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Summary

As important genetic resources, orthodox seeds from most herbaceous plant species can be usually cryopreserved in liquid nitrogen after desiccation. Recalcitrant seeds from some subtropical woody species, such as tea plants, however, are not feasible to cryopreserve, primarily due to the high moisture level and sensitivity to desiccation. Upon desiccation of tea whole seeds to moisture level below 50% (dry weight basis), viability declines abruptly. To elucidate the mechanism of the recalcitrance of tea seeds, we examined sensitivity of embryonic axes and cotyledons to desiccation and to subsequent cryopreservation using a Japanese cultivar, Yabukita. The excised embryonic axes were highly tolerable to desiccation, whereas cotyledons were highly susceptible to desiccation, suffering serious damage upon reduction of moisture level below 55%. This feature of cotyledons might be the major reason why tea whole seeds are so sensitive to desiccation. The excised embryonic axes previously desiccated to a moisture level of 15-30% survived subsequent cryopreservation to liquid nitrogen temperature. After the cryopreservation and thawing, embryonic axes grew and developed into normal plantlets on a recovery culture medium. Based on the results, we propose a practical protocol for cryopreservation of tea seeds.

Key Words: Camellia sinensis, cryopreservation, desiccation tolerance, embryonic axes, germplasm resource, recalcitrant seeds.

Introduction

Cryopreservation is an important technique for the genetic conservation of useful crops and plants. Several cryogenic techniques have been developed for preservation of shoot apices, meristems and tissue cultures (Sakai 1985). To date, techniques for cryopreservation have been established for small-sized seeds of grasses and vegetables (Stanwood 1985), most of which are highly tolerant to desiccation and exposure to liquid nitrogen temperature after reducing the moisture content below a critical level. For the cryopreservation of large-sized seeds, we are usually confronted with much difficulty due to the high moisture content and high sensitivity to desiccation (Chin et al. 1984). The sources of the recalcitrant seeds are many large-seeded species of tropical trees including rubber (Chin et al. 1981), cacao (Hor et al. 1984), coconut (Chin et al. 1989) and oil palm (Chin et al. 1988, Engelmann et al. 1988, Grout et al. 1983), all of which are of economic value. In recent years, successful cryopreservation has been achieved with embryonic axes excised from recalcitrant seeds of coconut (Chin et al. 1989), oil palm (Chin et al. 1988, Chin et al. 1989, Engelmann and Dereudre 1988, Grout et al. 1983), hazelnut (Normah et al. 1994, Reed et al. 1994, Gonzalez-Benito and Prez 1994) and olive (Gonzalez-Rio et al. 1994).

Tea plants (Camellia sinensis [L.] O. Kuntze) are indigenous to the subtropic and the seeds are thought to be recalcitrant (Chaundhury et al. 1991). The seeds are usually kept in semidry soil at 1°C and can be stored at least 7 years without significant loss of viability (Amm and Watanabe 1983). However, one may encounter several difficulties to keep the moisture and temperature in the soil at an ideal state, and therefore it is not suitable for permanent preservation. Takeda et al. (1991) reported that tea seeds from some cultivars with moisture content of 10-13% (fresh weight basis) survived freezing to −80°C for 2 days. Chaundhury et al. (1991) reported that excised embryonic axes of Camellia sinensis could be successfully cryopreserved after desiccation to a moisture content lower than 13% (fresh weight basis), while whole seeds could not. However, no explanation was given as to the reason why whole seeds can not be cryopreserved in liquid nitrogen after desiccation.

For providing basal knowledges on the cryopreservation of tea seeds, especially Japanese tea cultivars, the present study was attempted to investigate the sensitivity of whole seeds and seed parts, i.e., cotyledons and embryonic axes, to desiccation and subsequent cryopreservation to liquid nitrogen temperature.

Materials and Methods

Plant materials

Mature seeds of tea plants (Camellia sinensis cv. Yabukita) were obtained from the tea garden in Shizuoka Tea Experiment Station in October, 1991. Seeds were stored in a polyethylene bag at 2-4°C with a saturated humidity for about 2 months until use. After removing seed coats, embryonic axes and cotyledons were aseptically excised. The procedures of embryonic axes and sliced cotyledons were carried out in a sterilized condi-

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Effects of cooling on survival of seeds and seed parts

To determine the effects of cooling on survival of seeds and seed parts, ten of each intact seeds, excised embryonic axes and excised-sliced cotyledons (5×5×2 mm³) were, respectively, cooled to −25 °C at cooling rate of 2.5 °C/day in a Petri dish. During cooling, the excised seed parts were sealed in an aluminum foil bag to avoid desiccation. Samples were removed at −3, −5, −7.5, −10, −12.5, −15, −20 and −25 °C, then tested for survival after thawing at 4 °C for 6 hours. Survival was evaluated by germination in the case of intact seeds and embryonic axes and by greening in the case of sliced cotyledons on culturing on a recovery medium.

Desiccation of intact seeds, excised embryonic axes and sliced cotyledons

Seeds were desiccated at room temperature over silica gel. Batches of 30 seeds (approximately 50 g) were placed over silica gel in a Plastic box (1.3 liter capacity). To obtain seeds with desired moisture contents, ranged between 18 and 120 % (dry weight basis), the mass of silica gel in the Plastic box was varied from 20 to 300 g. After an appropriate period of desiccation, usually 6 days, the moisture level and percentage of germination were determined with different sample batches. For measurement of moisture content, seeds and seed parts were dried in an oven at 95 °C for 6 days and the dry weights were taken after cooling in a desiccator. Moisture level was expressed as a percentage of dry weight.

The excised cotyledons were further sliced into small pieces (1×1×2 mm³). The seed parts were desiccated at room temperature over silica gel. Thirty embryonic axes and thirty pieces of sliced cotyledons, approximately 600 mg each, were put on a small piece of aluminum foil, respectively. To obtain a desired moisture level, the samples were placed on a filter paper moistened with various amount of water, from 0 to 3.5 ml, and desiccated over 10 g of silica gel in a Plastic box (1.3 liter capacity). During desiccation, moisture levels were followed as in case of intact seeds and viability was tested as a function of moisture content.

Differential thermal analysis (DTA) of intact seeds

Low temperature exotherm was measured by a differential thermal analysis (DTA). Exotherms that correspond to a release of latent heat by the freezing of cell water or change in phase of other substances were detected with a pair of 0.2-mm copper/constantan thermocouples, and recorded on a potentiometric recorder (Chino AH 66). Thermocouples were attached to a seed sample and a completely dried seed as a reference, respectively, and each was mounted in a 10-ml test tube. Cooling was performed in a programmed freezer at a cooling rate of 0.1 °C/min and exotherms were recorded. Each analysis was performed with 5-10 replicates.

Evaluation of viability of whole seeds and excised seed parts

For germination of whole seeds before and after treatments with desiccation alone or combination of desiccation and cooling, seeds were placed on wet filter paper in a Petri dish and incubated at 26 °C under the continuous light condition. After desiccation and subsequent cryopreservation to liquid nitrogen temperature, survival of excised embryonic axes was evaluated by culturing on top of a piece of folded filter paper, the lower portion of which was submerged into a recovery culture medium added into the bottom of a culture tube (see Fig. 5). With this method the top portion of the filter paper was adequately moistened with the recovery medium during culturing. The recovery culture medium contained half-strength MS (Murashige and Skoog 1962) liquid culture medium supplemented with 0.01 mg/liter of indol butyric acid, 0.1 mg/liter of 6-benzylaminopurine and 0.5 mg/liter of gibberellic acid A₃ (Kuranuki 1992). Survival of embryonic axes was determined by growth and development into normal plantlets following culturing for 2 months. Survival of sliced cotyledons was visually determined by greening after culturing in the same way as in embryonic axes.

Desiccation of excised embryonic axes in a laminar flow cabinet and cryo-treatment

Excised embryonic axes were placed on an aluminum foil pan and the moisture level was equilibrated over wet filter paper in a Petri dish for more than 12 hours. Then, they were desiccated by exposing in a laminar-flow cabinet for various periods and changes in moisture level was followed with a batch sample by drying in an oven at 95 °C for 6 days. After desiccation for various periods, from 30 to 120 min, samples were placed in a Plastic cryotube and directly plunged into liquid nitrogen from room temperature and were kept frozen for 2 hours. Samples were then thawed at room temperature and tested for survival.

Results

Effects of cooling on survival of seeds and excised seed-parts

To determine the lowest temperature at which seeds or excised seed-parts can survive without any injury, they were slowly cooled to different temperatures. As shown in Table 1, there was a marked difference in the lethal temperatures between whole seeds and the excised seed parts. The lethal temperature of intact seeds was −7.5 °C, whereas that of the excised seed-parts was −20 °C. In this experiment, seeds and the excised seed-parts were cooled without any artificial ice-seeding, and therefore freezing might have been initiated spontaneously.

Differential thermal analysis

Upon removal of freezable water by desiccation, seeds may be successfully cryopreserved in liquid nitrogen without any substantial damage. To confirm this, the
temperature for onset of exotherm was determined as a function of moisture content of seeds. As shown in Fig. 1, the onset of exotherm in fresh seeds occurred around \(-8^\circ\text{C}\), however, the temperature for the onset of exotherm decreased as decreasing in the moisture content. The exotherm temperature decreased abruptly when moisture content was reduced below 40 % and reached \(-23^\circ\text{C}\) after desiccation to the moisture content of 20 %.

**Desiccation of whole seeds**

Fig. 2 shows effects of desiccation on viability of whole seeds. More than 80 % of seeds germinated normally unless the moisture content decreased below 50 % level. Viability declined abruptly after reduction of moisture content below 50 %, reaching zero when the moisture content was reduced to 20 %. Embryonic axes rarely survived in desiccated seeds with moisture content of 25–30 %, and cotyledons were heavily contaminated with bacteria during germination process.

**Survival of excised embryonic axes after desiccation and subsequent cryoexposure to liquid nitrogen temperature**

Unlike whole seeds, excised embryonic axes tolerated a severe desiccation after drying over silica gel. As shown in Fig. 3-A, all of the excised embryonic axes could tolerate desiccation to a moisture level of 15 %. Further desiccation below this moisture level caused a significant decline in survival. However, over 50 % of the axes could survive after desiccation to moisture content of 5 %. After desiccation to different moisture levels, the excised embryonic axes were directly immersed in liquid nitrogen from room temperature and tested for survival after thawing. As shown in Fig. 3-B, the survival was markedly dependent on the moisture content.

**Table 1. Effects of cooling on survival of whole seeds, excised embryonic axes and excised cotyledon slices**

<table>
<thead>
<tr>
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<th>Lowest temperature for survival (°C)</th>
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<tr>
<td>Whole seeds</td>
<td>(-7.5)</td>
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<tr>
<td>Embryonic axes</td>
<td>(-20.0)</td>
</tr>
<tr>
<td>Cotyledons</td>
<td>(-20.0)</td>
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**Fig. 1.** Onset temperature for exotherm in a whole tea seed as a function of moisture content.

**Fig. 2.** Viability of whole seeds of tea after desiccation to various moisture content.

**Fig. 3.** Effects of desiccation alone (A) and cryoexposure into liquid nitrogen after desiccation (B) on survival of excised embryonic axes.
level prior to the cryoexposure. No survival was found in embryonic axes with moisture level more than 55%. Survival increased as the moisture level decreased from 50% to 50%. The highest survival was obtained in embryonic axes with moisture level between 15 and 30%. After thawing from liquid nitrogen temperature and culturing on a recovery medium, the excised embryonic axes turned green after a few days and developed into normal plantlets (2-cm tall) after 2 months. When moisture level decreased below 15% prior to cryoexposure, survival rate was significantly decreased to 60% and no radicle emerged from the axes, but instead the epicotyls developed normally.

Survival of sliced cotyledons after desiccation and subsequent immersion in liquid nitrogen

The sliced pieces of excised cotyledons were desiccated over silica gel for 6 days and tested for survival. As shown in Fig. 4-A, the survival decreased when the moisture level fell below 50% and all of the cotyledon pieces died when they were desiccated to a moisture level of 20%. As shown in Fig. 4-B, cryoexposure to liquid nitrogen temperature after desiccation to different moisture levels was found to be lethal. Thus, there was a marked difference in sensitivity to both desiccation and cryoexposure between embryonic axes and cotyledons.

Cryopreservation of excised embryonic axes after desiccation in a laminar-flow cabinet

To explore the most effective way for cryopreservation, embryonic axes were excised and exposed in a laminar-flow cabinet for various length of time before immersion in liquid nitrogen. As shown in Table 2, the moisture level decreased as a function of the exposure time. The survival of axes after immersion in liquid nitrogen was closely related to the exposure time or the moisture level. Exposure time more than 90 min or reduction in moisture level below 30% was found to be the best condition for the cryopreservation of excised embryonic axes. The survival rate increased to 80% after 60-min exposure in a laminar-flow chamber, but 50% of the axes grew abnormally, forming into callus, and failed to develop any shoot and root. As shown in Fig. 5, after desiccation more than 90 min and subsequent cryoexposure, all of the axes showed normal growth, developing

<table>
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<th>Table 2. Survival of excised embryonic axes upon cryoexposure to liquid nitrogen temperature after desiccation in a laminar-flow cabinet</th>
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<tr>
<td>Duration time (min) of desiccation in a laminar-flow cabinet</td>
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<td>Moisture content (% dry weight)</td>
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<td>Survival (%)</td>
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<td>Normal growth (%)</td>
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<td>Abnormal growth (%)</td>
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<td>Survival was determined visually. Normal growth indicates that axes grew and developed into normal plantlets; Abnormal growth indicates that axes developed into callus and did not elongate any shoots and roots. Examined 2 months after cryoexposure.</td>
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![Fig. 4. Effects of desiccation alone (A) and cryoexposure into liquid nitrogen after desiccation (B) on survival of excised cotyledon pieces.](image)

![Fig. 5. Growth and development of excised embryonic axes after desiccation and cryoexposure into liquid nitrogen. Pictures were taken after culturing for 50 days. Desiccation time, 30, 45, 60, 90 and 120 min from left to right.](image)
into normal plantlets after culturing on a recovery medium.

Discussion

Roberts (1973) described two major categories of seeds based on their response to desiccation: (1) desiccation tolerant or "orthodox" seeds which can withstand drying to 2-5 % moisture content without substantial damage and (2) desiccation sensitive or "recalcitrant" seeds which are generally killed if they were dried below a certain moisture level, usually between 12 and 31 % moisture content. With regard to sensitivity to combination of desiccation and cryoexposure to liquid nitrogen temperature, Stanwood (1985) classified seeds into three categories: (1) tolerant both to desiccation and cryoexposure, (2) desiccation tolerant, but sensitive to cryoexposure and (3) sensitive both to desiccation and cryoexposure. Whole seeds of tea plants are considered to belong to the recalcitrant seeds with regard to the sensitivity to desiccation, and probably to the third category of Stanwood.

Whole tea seeds, in the present study, were found to be sensitive to freezing and could survive only to −7.5 °C (Table 1). On the contrary, the excised embryonic axes and sliced small-pieces of cotyledons could tolerate freezing to −20 °C. It was suggested that those seed parts with small sizes underwent an extracellular or an extraorgan freezing due to the large specific surface areas per volume, avoiding any occurrence of a fatal intracellular freezing. This result may indicate that the excised seed-parts, in particular the embryonic axes, are able to be effectively cryopreserved under appropriate conditions that include desiccation prior to freezing.

Upon drying of whole seeds of tea, the temperature for onset of exotherm decreased significantly as decrease in the moisture content, but a distinct exotherm was still observed around −23 °C even after drying to moisture level of 20 %, indicating a spontaneous intracellular freezing of tissues. Furthermore, due to the high sensitivity to desiccation, we were not able to cryopreserve the whole seeds after desiccation to an appropriate moisture level. On the other hand, the excised embryonic axes were highly tolerant to desiccation. No substantial loss of viability was observed even after drying to the moisture level of 15 %, although viability declined to 50 % level upon further desiccation to the moisture content of 5 %. After desiccation to the moisture content of 15-30 %, the excised embryonic axes can be safely cryopreserved in liquid nitrogen. After a direct immersion in liquid nitrogen from room temperature and thawing, the axes started to grow and to develop into normal plantlets. Similar results have been obtained preliminarily with excised embryonic axes from seeds of Camellia sinensis, composite tea stocks representing populations around Palampur (Kangra, Himachal Pradesh) (Chaudhury et al. 1991). In the present study, upon cryoexposure of embryonic axes excised from tea seeds, no survival was found with moisture content above 55 %. Although survival increased significantly as the moisture content decreased to 30 %, the axes grew abnormally and developed into callus. This may indicates that cells in the axes suffer a partial injury probably due to an abrupt crystallization of freezable water in the cells during cooling to liquid nitrogen temperature. All of the axes with moisture content less than 30 % can survive direct immersion in liquid nitrogen, grew and developed into a normal plantlet after culturing on a recovery medium. This may suggest that the water molecules in axes with moisture level less than 30 % are in a bound form and not freezeable upon a rapid cooling into liquid nitrogen temperature. Axes with moisture content less than 15 % had low survival rates after drying, and subsequent immersion in liquid nitrogen. This may imply that desiccation to such a low moisture level may cause partial removal of vital water molecules bound to macromolecules, leading to a lethal damage.

Cotyledons of tea seeds were found to be extremely sensitive to desiccation, being injured upon desiccation to moisture content below 30 %. Therefore, the cotyledons can not withstand drying and subsequent cryoexposure to liquid nitrogen temperature. This feature of cotyledons, which is typical for recalcitrant seeds, might be critical for cryopreservation of whole seeds. When intact seeds were previously desiccated and then directly immersed into liquid nitrogen, no germination was observed irrespective of the moisture content. Even though the embryonic axes in the seeds had been able to withstand desiccation and immersion into liquid nitrogen after drying, they would be secondarily injured by a lethal damage to cotyledons, which were severely infected by bacteria and fungi during the course of germination. Recently, Normah et al. (1994) have reported that the embryonic axes of hazelnut excised after drying and cryoexposure of whole seeds survived and were grown in tissue culture, although desiccated whole seeds did not survive.

For practical cryopreservation of embryonic axes of tea seeds, it is advantageous to desiccate them by air-drying in a lamina-flow cabinet for 2 hours prior to immersion into liquid nitrogen as suggested by Chaudhury et al. (1991). The moisture level decreased to a level, i.e., 18-20 %, within 2 hours, that is optimum for surviving direct immersion in liquid nitrogen. The axes after drying and cryoexposure can grow normally on a recovery medium (Kuranuki 1992) and developed into normal plantlets.

Although tea seeds as a whole are sensitive both to desiccation and cryoexposure and are considered to be categorized into recalcitrant seeds, the embryonic axes per se are desiccation tolerant and withstand cryoexposure to liquid nitrogen temperature, and, therefore, they behave like an orthodox one. In the late embryogenic process of the orthodox seeds, several genes which encode highly hydrophilic proteins such as dehydrins, late
embryonic abundant proteins (LEA) and early-methionine-labeled proteins (Em) are specifically expressed (review Skriver and Mundy 1990, Bray 1993). These proteins are considered to have an important role in protecting cells against desiccation. It may be interesting to examine whether such protecting proteins have already existed in the embryonic axes of tea seeds or are newly induced during the desiccation process. The evolutionary origin and the ecological significance of the several categories of seeds also remain to be elucidated. These are important problems to be investigated in future.

Literature Cited