

PLANT REGENERATION FROM RICE ANTERS CRYOPRESERVED BY AN ENCAPSULATION/DEHYDRATION TECHNIQUE

MARÍA A. MARASSI*, ADRIANA SCOCCHI, AND ANA MARÍA GONZALEZ

Instituto de Botánica del Nordeste – Facultad de Ciencias Agrarias (UNNE), Casilla de Correo 209, 3400 Corrientes, Argentina

(Received 25 January 2005; accepted 8 November 2005; editor B. M. Reed)

SUMMARY

Anther-derived rice (*Oryza sativa* L. ssp. japonica variety Yerua P.A.) plants were obtained after cryopreservation by an encapsulation/dehydration technique. Immature anthers, excised from spikelets pretreated at 8°C for 8 d, were encapsulated in calcium alginate beads. The beads were cultured on N6 medium with 11.5 μ M naphthaleneacetic acid (NAA) and 2.3 μ M 6-furfurylaminopurine (KIN). Fifteen percent of the encapsulated anthers formed calluses when pretreated with sucrose for 3 d in liquid medium, desiccated on silica gel, slowly cooled to -30°C , immersed in liquid nitrogen (LN), thawed, and recultured. The cryopreserved encapsulated anthers produced 1.67 shoots/callus, in contrast to the control (non-cooled encapsulated anthers), which produced 6 shoots/callus. Eighty percent of the plantlets developed into normal plants after being transferred to greenhouse conditions. Histological observations showed that the origin of the plants was not modified by the cryopreservation process.

Key words: *Oryza sativa* L; cryopreservation; encapsulation/dehydration; androgenesis.

INTRODUCTION

The production of doubled haploid plants has been used to speed up the breeding cycle by achieving homozygosity in one generation. This has allowed an increase in the selection efficiency because of a better discrimination between genotypes within any generation, and the efficient retention of desirable genes in latter generations. The acceleration of the breeding cycle and increase in selection efficiency has made doubled haploid techniques very attractive, not only for conventional breeding but also for plant improvement through mutation induction. The use of anther culture has been limited by many factors (Afza et al., 2000). The dependence on the flowering time has been one of the major constraints in rice-breeding programs, especially in temperate regions, because of the very limited period of time when this technique can be used. Although a greenhouse may be used during the rest of the year to produce flowering plants, some negative consequences have been observed: (a) anther response to *in vitro* culture is affected, and (b) the large number of individuals needed to ensure the effectiveness of anther culture inbreeding programs makes the management of F1, F2, or F3 generations difficult. The production of callus and *in vitro* conservation of haploid cells might be considered as an option to extend the period of use of haploid culture, but when subcultured, cells undergo various chromosomal and ploidy changes that may result in genetic changes in the cultures (D'Amato, 1977). In view of the importance of haploid cultures in mutation, biochemical genetics, and haploid breeding programs, use of isolated anthers may be very important. Freeze preservation may be useful in anther conservation and needs to be developed.

Storage of cryopreserved anthers would facilitate an even distribution of labour, time, and space in the production of homozygous lines of each genotype. Physiological and biochemical studies also require anthers to be stored, without change over time.

Some attempts have been made to cryopreserve pollen (Towill and Walters, 2000), pollen embryos, and segments of anthers in *Triticum* sp. (Bajaj, 1990a), *Arachis* sp. (Bajaj, 1990b), and isolated microspores of *Brassica* sp. (Chen and Beversdorf, 1992). In rice, cryopreservation has been studied: embryogenic suspension cells (Huang et al., 1995; Jain et al., 1996; Wang et al., 1998; Zhang et al., 2001); callus and characterization of the cryopreserved plants (Moukadiri et al., 1999); and callus to evaluate the ability of the surviving cells to yield competent protoplasts for genetic transformation (Comejo-Martin et al., 1995). However, reports of the cryopreservation of rice anthers have shown limited success (Coulibaly and Demarly, 1979; Bajaj and Sala, 1991) in obtaining a low frequency of callus formation and only regeneration of albino plants.

The encapsulation/dehydration technique for cryopreservation was originally reported for shoot tips of *Solanum* sp. (Fabre and Dereudre, 1990). In this technique, shoot tips are encapsulated in alginate beads, precultured in liquid medium with a high sucrose concentration, and partially desiccated before cooling in liquid nitrogen (LN). The encapsulation/dehydration technique has been successfully applied to shoot tips from several plant species, including several genotypes of pear, apple, sugarcane, and potato (Engelmann, 1997).

The objective of the current study was to investigate rice anther cryopreservation using the encapsulation/dehydration technique, to recover plants from these anthers, and to evaluate by histology if the process of plant formation is affected by the cryopreservation technique.

*Author to whom correspondence should be addressed: Email marassi@agr.unne.edu.ar

MATERIALS AND METHODS

Plant material. Anthers from rice (*Oryza sativa* L. ssp. *japonica*) variety Yeruá P.A. were collected at the booting stage (immature anthers containing uninucleate microspores). The sheaves containing panicles were cut, placed in black plastic bags, and held at 8°C for 8 d. They were surface-sterilized in ethanol 70% (v/v) for 3 min and then 20% commercial bleach (1.6% sodium hypochlorite, final concentration) for 20 min. The panicles were then rinsed three times with sterile distilled water.

Histological studies. To determine the development stage of the cultured anthers and the origin of the callus and shoot, anatomical and morphological studies were carried out. For anatomical observations, anthers were fixed in formalin:acetic acid:ethanol (1:1:10), dehydrated in graded alcohol series, and embedded in paraffin. Sections 10 µm thick were stained with a safranin and alcian blue solution (Luque et al., 1996), and mounted in synthetic Canadian balsam (Biopur, Rosario, Argentina).

Morphological observations were performed under a scanning electron microscope (SEM). Anthers were removed from the flasks after different culture duration and fixed in 4% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer. Samples were kept at 4°C for 6 h and washed with phosphate buffer. All samples were dried to the critical point in an acetone series (Postek et al., 1980), sputter coated with gold, observed, and photographed using a JEOL 5800 LV SEM.

Effect of the anther culture density and encapsulation. An experiment was conducted to evaluate the effect of anther density on *in vitro* response of anthers following their encapsulation.

Anthers and encapsulated anthers were pretreated at 8°C for 8 d before culture and encapsulation. Encapsulation was carried out with 3% sodium alginate + 100 mM calcium chloride (CaCl₂) with 1 anther/bean and were cultured according to Marassi et al. (1993) on N6 culture medium (Chu et al., 1975) with 5% sucrose + 11.5 µM naphthaleneacetic acid (NAA) + 2.3 µM 6-furfurylaminopurine (KIN). The pH of the medium was adjusted to 5.8 before the addition of 0.8% agar (A-1296; Sigma Chem. Co., Miami, USA). The flasks (125 ml capacity), containing 25 ml of culture medium, and the tubes (12.5 ml capacity), containing 3 ml of culture medium, were covered with aluminum foil and autoclaved at 1.45 kg cm⁻² for 20 min. Cultures were incubated at 27 ± 2°C in dark for 40 d, until calluses or calluses with shoots were observed. Vessels were then transferred to light (116 µmol m⁻² s⁻¹, 14-h photoperiod) at 27 ± 2°C, where green shoots developed. When these shoots were 10 cm long (some of them showing poorly developed roots), they were transferred to MS basal medium (Murashige and Skoog, 1962) with 5% sucrose for rooting, in 375-ml flasks containing 75 ml medium.

The following treatments were used to study the effect of anther culture density and encapsulation on *in vitro* response: T0, 45 anthers/flask (control); T1, 10 anthers/flask; T2, 10 beads (with 1 anther each)/flask; T3, 1 anther/tube; T4, 1 bead (with 1 anther)/tube.

The control density (T0) of 45 anthers/flask was established by Marassi et al. (1993).

Each treatment consisted of 10 flasks/tubes, and the experiment was replicated three times. Comparisons of means were made by Tukey's studentized range (MSD) ($P < 0.01$).

Cryopreservation protocol. After 8 d at 8°C, spikelets were extracted from the panicles and anthers were removed from the spikelets. For the encapsulation/dehydration technique, anthers were placed in 3% sodium alginate in distilled water. Beads (4–5 mm diameter) were formed by dropping the alginate solution into 100 mM CaCl₂ for 1 min. The encapsulated anthers were transferred, at 24-h intervals, to the liquid N6 medium with increasing sucrose concentrations (0.5, 0.75, 1M). Encapsulated anthers that were not pretreated with sucrose were harvested later, together with the controls, and encapsulated prior to culture or dehydration on silica gel. Beads were dehydrated on silica gel (30 g/flask) for 5 h. Desiccated encapsulated anthers were transferred to sterile 5 ml cryotubes (10 anthers/cryotube). Ten cryotubes were rapidly immersed in LN and held for 1 h (T5) and other 10 cryotubes were slowly cooled from 20°C ± 1°C (at 1°C min⁻¹) to -30°C (Controller Rate Freezing System, Gordinier Electronics, Inc. USA. Model 9000), and then immersed in LN (T6) and stored for 1 h. After warming (2 min in water-bath at 30°C), anthers were transferred (10/flask) directly to the recovery medium (N6 + sucrose 5% + 11.5 µM NAA + 2.3 µM KIN + agar Sigma A1296 0.8%). The controls were without LN treatment. Anther culture was performed as described before.

Also evaluated, in previous work, was the cryopreservation of the anthers using sucrose 5%, glycerol 5% and dimethylsulfoxide (DMSO) 5%, alone or in combination of two or three of the cryoprotectants, dehydrated or not with silica gel for 5 h, and slowly or rapidly, cooled.

Moisture content was determined and expressed as a percentage of the initial fresh weight (Janeiro et al., 1996). Samples consisted of encapsulated anthers with pretreatment at 24-h intervals in the liquid N6 medium, with increased sucrose concentrations (0.5, 0.75, 1M) and were or were not slowly cooled (at 1°C min⁻¹) to -30°C, and immersed in LN for 1 h after various dehydration periods. The moisture content was measured on 30 beads (each with one anther) per dehydration period (at 30-min intervals) from 0 min to 420 min.

The cryopreservation experiment consisted of six treatments with 10 flasks per treatment, and was replicated three times.

The following treatments were used for cryopreservation: C1, non-encapsulated anthers (45 anthers/flask); C2, encapsulated anthers, (10 beads/flask); C3, encapsulated anthers + sucrose (0.5M–0.75M–1M); C4, encapsulated anthers + sucrose (0.5M–0.75M–1M) + silica gel; T5, encapsulated anthers + sucrose (0.5M–0.75M–1M) + silica gel and to (-196°C); T6, encapsulated anthers + sucrose (0.5M–0.75M–1M) + silica gel to (-30°C) and to (-196°C).

Statistical analysis. Values for the growth response are the mean and standard error (± SEM) of three replications of the experiment. Comparisons of means were made by Tukey's studentized range (MSD) ($P < 0.01$).

RESULTS

Effect of the anther culture density and encapsulation. The effect of encapsulation on the response of anthers to *in vitro* culture was evaluated for callus induction and the number of shoots formed per callus. No significant difference ($P \leq 0.01$) was observed for callus induction when either 1 anther/tube or 10 anthers/flask were cultured (Table 1). There was also no significant difference between encapsulated and non-encapsulated anthers for callus induction. There were significant differences ($P \geq 0.01$) for the percentage of callus-forming shoots between the 10 unencapsulated (T1) and 10 encapsulated anthers/flask (T2). A significant increase in the number of shoots per callus (3.6) was observed in T2 when compared to T1 (2.3).

Encapsulation improved *in vitro* response of anthers. The percentage of calluses with shoots and the number of shoots obtained per callus were greater ($P \geq 0.01$) in the encapsulated anther treatments than the 1 anther/tube (T3) and 1 encapsulated anther/tube (T4) or when the comparison was made between 10 anthers/flask (T1) and 10 encapsulated anthers/flask (T2).

TABLE 1

CALLUS INDUCTION, CALLUSES WITH SHOOTS (% ± SEM) AND SHOOT PROLIFERATION (N° ± SEM) FROM RICE ANTHERS CULTURED AT DIFFERENT DENSITIES (ENCAPSULATED VS. NON-ENCAPSULATED)

Treatments	% Anthers w/callus	% Calluses w/shoots	N° shoots/callus
T0 (45 anthers/flask)	40.3 (± 5.7) a	82 (± 8.7) a	3 (± 0.8) ab
T1 (10 anthers/flask)	33.3 (± 6.6) b	52 (± 7.5) b	2.3 (± 0.65) b
T2 [10 beads (w/1 anther each)/flask]	26.7 (± 3.8) b	74 (± 6.4) a	3.6 (± 0.7) a
T3 (1 anther/tube)	30 (± 4) b	0 d	0 c
T4 [1 bead (w/1 anther each)/tube]	27.5 (± 3.5) b	35 (± 7.3) c	1.8 (± 0.6) b

The same letters within a column means no significant difference for Tukey's multiple comparison test ($P < 0.01$).

Since the percentage of callus with shoots and number of shoots per callus showed no significant differences between 10 encapsulated anthers/flask (T2) and 45 non-encapsulated anthers/flask (T0), both treatments were selected as the control culture density to be used during cryopreservation assays.

Cryopreservation protocol. The best dehydration time was 300 min (5 h) for encapsulated anthers pretreated with sucrose (at 24-h intervals with 0.5, 0.75, and 1M sucrose each). At 300 min, beads had 19.7% moisture and showed a significant difference ($P \geq 0.01$) in callus induction when compared to other dehydration treatments after storage for 1 h in LN (Fig. 1). This dehydration time was selected for further cryopreservation experiments.

There was no need to extract the control and cryopreserved anthers from the beads prior to culture on standard recovery medium, since the callus grew and emerged from the alginate capsule without difficulty. In the dark, anthers developed calluses 21 d after thawing, and shoots formed 40 d after thawing (Fig. 2a, b, c). After 40 d, flasks with calluses and developing shoots were transferred to light, and after 10 d, green shoots with roots were observed (Fig. 2d). Plants were transferred to a rooting medium, and when a well-developed root system was observed, they were transplanted to pots and grown in the greenhouse. The morphology of 80% of the 39 potted plants that flowered and produced seed was similar to their respective mother plants according to the morpho-phenological rice descriptor (IRRI, 1988) used to compare the regenerants with the original mother plants; the remaining plants did not fit the descriptor, were smaller, and did not produce seeds or died after transplanting them to soil. The number of seeds per panicle and the morphology of the seeds were similar to the mother plant, suggesting that they were diploid. This similarity in morphological characteristics and fertility, associated with the $2n$ ploidy of anther-derived plants, was described by Chen and Li (1978) and Nakamura et al. (1994) and observed in our previous work with anther culture (Marassi et al. 1993).

Histological study. Anthers collected at the booting stage contained uninucleate microspores (Fig. 2e). Mitoses with successive cytokineses formed a small mass of cells that remained enclosed by the cryopreserved microspore wall (Fig. 2f). Further growth ruptured the microspore wall, eventually splitting the anther wall (Fig. 2g). Callus formation was asynchronous, as calluses of different sizes were seen simultaneously (Fig. 2h). Calluses continued to develop with a substantial size increment (Fig. 2i: control; 2j, k: cryopreserved). One to several vegetative meristems differentiated on the callus surface (Fig. 2l: control; 2m: cryopreserved). The meristematic apex differentiated into shoots, flanked by foliar primordia (Fig. 2n:

cryopreserved). Multiple shoots were observed on some calluses (Fig. 2o: cryopreserved). This androgenic initiation was similar for both cryopreserved and non-cryopreserved anthers and is similar to that reported by Mandal and Gupta (1996).

Among the cryopreservation treatments, we obtained callus and shoot proliferation only from (T6) sucrose pretreatment (0.5, 0.75, and 1M; 24 h in each concentration), desiccation on silica gel (5 h) followed by slow cooling and then immersion in LN for 1 h, warming at 30°C and cultured on the standard medium (Table 2). Significant differences occurred among T6 and the four controls (treatments C1, C2, C3, C4). For the number of shoots/callus, no significant differences were observed between C3, C4, and T6. These showed a decrease in the number of shoots per callus compared to C1 and C2.

DISCUSSION

Effect of the anther culture density and encapsulation. Cultured anthers release endogenous hormones and chemicals that affect embryogenesis (George, 1993). The effect of anther density on culture response was studied in *Triticum aestivum* L. (Orshinsky and Sadasivaiah, 1997), in which 20 anthers/plate produced a greater frequency of albino plants than 10 anthers/plate. Guo et al. (1999), working with *Phleum pratense* L., observed that a culture density of 50–125 anthers per dish produced the greatest embryo yield. They attributed this response to endogenous hormones and substances that affected *in vitro* embryogenesis and were released to the medium by the cultured anthers.

Our results also showed that culture density affects the *in vitro* response of anthers. The optimum density for rice was 45 anthers/125 ml flask (Marassi et al., 1993), and fewer anthers cultured /125 ml flask decreased the number of anthers with calluses and calluses with shoots (Table 1). We also demonstrated that the density response is affected by encapsulation. When 10 encapsulated anthers were cultured per flask, the same callus frequency and an increased number of shoots per callus occurred compared to 45 anthers/125 ml flask. When one encapsulated anther was cultured per tube, shoot production occurred, but this did not occur when one non-encapsulated anther was cultured per tube (Table 1). Endogenous substances might be more slowly released to the medium because of the presence of the capsule, and remain around the anther for a longer time, affecting favorably the *in vitro* response.

Cryopreservation protocol. Cryopreservation of biological materials is a long-term objective in theoretical and applied science. Mature pollen is stored to facilitate crosses in breeding programs, distribute and exchange germplasm among locations, preserve nuclear genes of germplasm, and carry out studies about allergens and other objectives. Storage for use in haploid generation through pollen embryogenesis has also been mentioned, but storage of mature pollen is less important since microspores at the uninucleate stage are more responsive to embryogenesis in most systems. This may encourage long-term storage of immature pollen (Towill and Walters, 2000). Little research has been reported about microspore preservation; however, Chen and Bevensdorf (1992) reported *in vitro* embryo production from frozen microspores of *Brassica* sp.

Cryopreservation of rice anthers was first described by Coulibaly and Demarly (1979) using DMSO and reported 5% survival and 3% of anthers produced callus, but no mention of plant production. Bajaj and Sala (1991) cryopreserved excised rice anthers presoaked

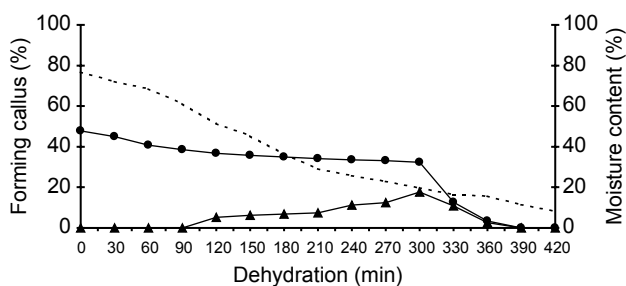


FIG. 1. Effect of dehydration duration on the percentage of encapsulated *O. sativa* L. anthers forming callus. Beads were sucrose pretreated, and were slowly cooled and immersed in LN for 1 h (+LN) or not (-LN). ---- Moisture content; ● -LN; ▲ +LN.

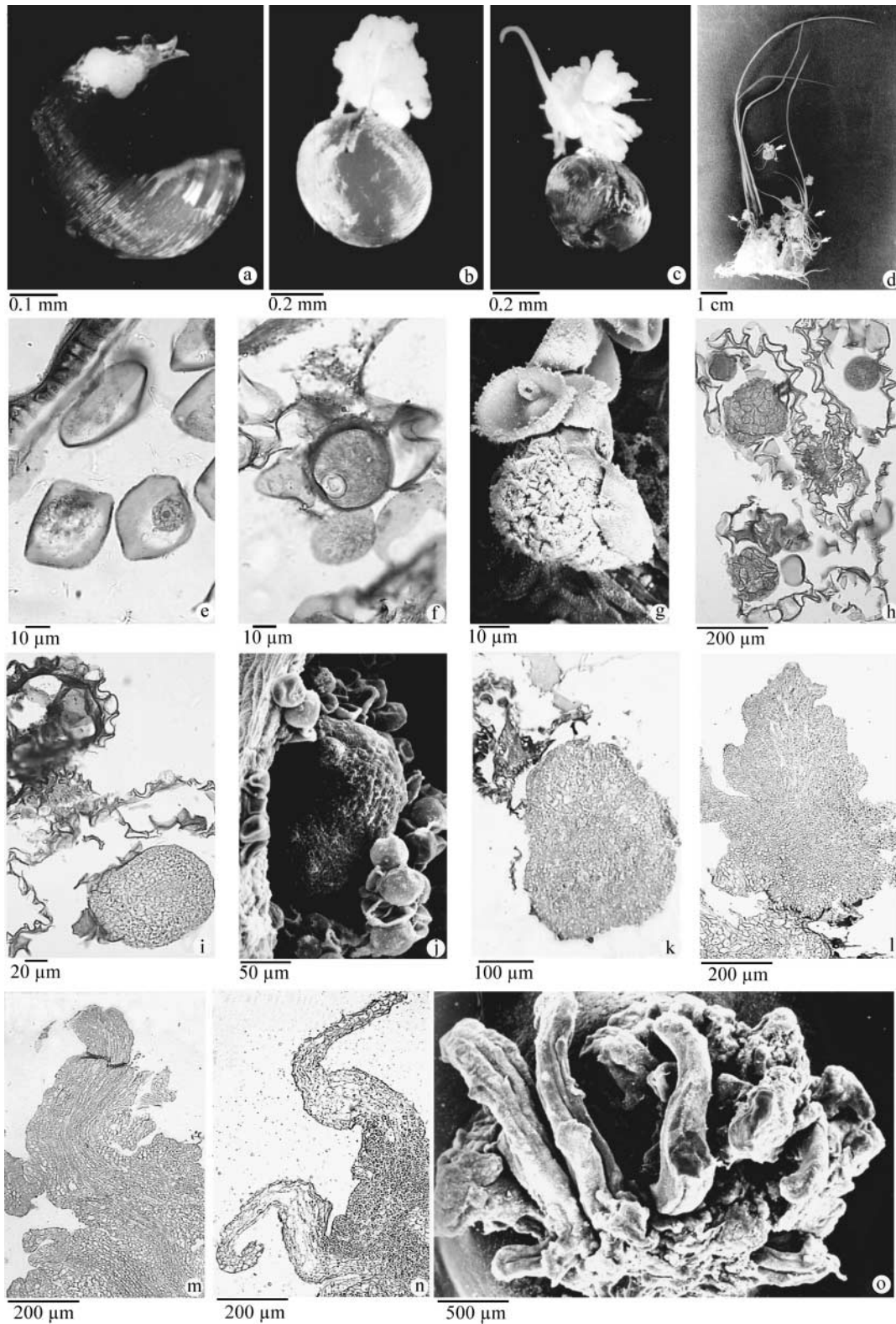


FIG. 2. *a*, Callus production from cryopreserved anthers 21 d after warming from LN. *b*, Callus growth from cryopreserved anthers 30 d after warming from LN. *c*, Shoot production after 40 d of warming from LN. *d*, Plant formation 50 d after warming from LN; *arrows* indicate portions of the remaining alginate capsules. *e*, Uninucleate microspores. *f*, Cryopreserved: small mass of cells inside the microspore. *g*, Microcallus breaks the wall of the microspore. *h*, Asynchronous division of microspores. *i*, Control. *j* and *k*, Cryopreserved: increasing of the callus size. *l*, Control and *m*, cryopreserved: differentiation of vegetative meristem. *n*, Cryopreserved meristematic apex, flanked by foliar primordia. *o*, Cryopreserved multiple shoots. *g*, *j*, and *o*, Scanning electron microscope images.

TABLE 2

CALLUS INDUCTION, CALLUSES WITH SHOOTS (% \pm SEM) AND SHOOT/CALLUS ($N \pm$ SEM) FOR RICE ANTHER CONTROLS AND CRYOPRESERVATION TREATMENTS

Treatments	Anthers w/callus (%)	Calluses w/shoots (%)	Shoots/callus (N)
C1 A w/o e	52.2 (\pm 6.9) a	80.4 (\pm 5.7) a	4.7 (\pm 0.6) b
C2 EA	60.5 (\pm 7.6) a	75.8 (\pm 6.7) ab	6.0 (\pm 1.0) a
C3 EA-SP	46.7 (\pm 5.8) b	66.9 (\pm 5.9) b	2.3 (\pm 0.6) c
C4 EA-SP-D	34.8 (\pm 6.7) b	64.7 (\pm 6.1) b	2.0 (\pm 0.7) c
T5 EA-SP-D-RC	0 d	0 d	0 d
T6 EA-SP-D-SC	15.2 (\pm 1.1) c	55.6 (\pm 3.2) c	1.7 (\pm 0.6) c

The same letters within a column means no significant difference for Tukey's studentized range (MSD) ($P < 0.01$). C1 (Control 1): anthers without encapsulation; C2 (Control 2): EA: encapsulated anthers without preculture desiccation and cooling; C3 (Control 3): EA-SP: encapsulated anthers with sucrose pretreatment but without desiccation and cooling; C4 (Control 4): EA-SP-D: encapsulated anthers, sucrose pretreatment and dehydration in silica gel 5 h but without cooling. T5: EA-SP-D-RC: encapsulated anthers; sucrose pretreatment: 24 h in each sucrose concentration (0.5M, 0.75M, and 1M); dehydration in silica gel 5 h.; rapid cooling (direct to liquid nitrogen); T6: EA-SP-D-SC: encapsulated anthers; sucrose pretreatment: 24 h in each sucrose concentration (0.5M, 0.75M, and 1M); dehydration in silica gel 5 h; slow cooling (1°C/min to -30°C and then to liquid nitrogen).

on a filter paper moistened with a mixture of cryoprotectants (5% each: DMSO, sucrose, and glycerol), and 6% of anthers formed callus and produced roots, but only one developed into a plant.

In previous work we used different cryoprotectants alone or in combination (sucrose, glycerol, DMSO) with or without dehydration. We could not obtain callus formation nor recover plants, either slowly or rapid cooled (unpublished).

Here we report that sucrose pretreatment increased the freezing tolerance of rice anthers. As reported for other explants and species (Jitsuyama et al., 2002; Scocchi et al., 2004), the callus formation, shoot induction, and plant development proceeded to maturity after cryopreservation with an encapsulation/dehydration technique. Fifteen percent of encapsulated anthers produced calluses, and 55.6% of the calluses produced single or multiple shoots.

Ontogenetic process. The androgenic initiation was similar to that reported by Mandal and Gupta (1996) and corresponds with the first model, where initiation occurred by several mitoses in the uninucleate microspore, forming a microcallus that breaks the wall, a callus is formed, and later the differentiation of vegetative apexes takes place.

The cryopreservation of the anthers by the encapsulation/dehydration technique showed no effect on the development of the plants from the microspore, as shown by the anatomical studies, ensuring that the origin of the recovered plants is the microspore and not the other tissues of the anthers.

This report opens the possibility of anther cryopreservation using the encapsulation/dehydration technique, with the recovery of fertile plants from these anthers, without modifications in the ontogenetic pathways, ensuring the haploid origin of the plants. This represents a first step to solve one of the limitations of the anther-culture technique, which is the short period of time in which flower explants are available.

REFERENCES

- Afza, R.; Shen, M.; Zapata, F. J.; Xie, J.; Fundi, H. K.; Lee, K. S.; Bobadilla-Mucino, E.; Kodym, A. Effect of spikelet position on rice anther culture efficiency. *Plant Sci.* 153:155–159; 2000.
- Bajaj, Y. P. S. Cryopreservation of germplasm of wheat. In: Bajaj, Y. P. S., ed. *Biotechnology in agriculture and forestry*, vol. 13 Wheat Berlin, Heidelberg, New York: Springer-Verlag; 1990a:670–681.
- Bajaj, Y. P. S. Cryopreservation of germplasm of legumes and oilseed crops. In: Bajaj, Y. P. S., ed. *Biotechnology in agriculture and forestry*, vol. 10, Legume and oil seeded crops I. Berlin, Heidelberg, New York: Springer-Verlag; 1990b:49–62.
- Bajaj, Y. P. S.; Sala, F. Cryopreservation of germplasm of rice. In: Bajaj, Y. P. S., ed. *Biotechnology in agriculture and forestry*, vol. 14 Rice Berlin, Heidelberg, New York: Springer-Verlag; 1991:553–571.
- Chen, J. L.; Beversdorf, W. D. Cryopreservation of isolated microspores of spring rapeseed (*Brassica napus* L.) for *in vitro* embryo production. *Plant Cell Tiss. Organ Cult.* 31:141–149; 1992.
- Chen, Y.; Li, L. T. Investigation and utilization of pollen derived haploid plants in rice and wheat. In: *Symposium on plant tissue culture*. Peking: Science Press; 1978:199–211.
- Chu, C. C.; Wang, C. C.; Sun, C. S.; Chen, H.; Yin, K. L.; Chu, C. Y.; Bi, F. Y. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sin.* 18:659–668; 1975.
- Cornejo-Martin, J.; Wong, V. L.; Blech, A. E. Cryopreserved callus: a source of protoplast for rice transformation. *Plant Cell Rep.* 14:210–214; 1995.
- Coulibaly, Y.; Demarly, Y. Androgenese *in vitro* chez *Oryza sativa* (var. Cigalon) a partir d'antheres conservees dans l'azote liquide (-196°C). *L'Agronomie Tropicale* 34(1):74–79; 1979.
- D'Amato, F. Cytogenetics of differentiation in tissue and cell culture. In: Reinert, J.; Bajaj, Y. P. S., eds. *Applied and fundamental aspects of plant cell, tissue and organ culture*. Berlin, Heidelberg, New York: Springer-Verlag; 1977:343–357.
- Engelmann, F. *In vitro* conservation methods. In: Callow, J. A.; Ford-Lloyd, B. V.; Newbury, H. J., eds. *Biotechnology and plant genetic resources: conservation and use*. Wallingford, UK: CAB International; 1997:119–161.
- Fabre, J.; Dereuddre, J. Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot-tips. *CryoLetters* 11:413–426; 1990.
- George, E. F. *Plant propagation by tissue culture*. Part I. The technology. London: Exegetics Ltd; 1993.
- Guo, Y. D.; Sewon, P.; Pulli, S. Improved embryogenesis from anther culture and plant regeneration in timothy. *Plant Cell Tiss. Organ Cult.* 57:85–93; 1999.
- Huang, C. N.; Wang, J. H.; Yan, Q. S.; Zhang, S. Q.; Yan, Q. F. Plant regeneration from rice (*Oryza sativa* L.) embryogenic suspension cell cryopreserved by vitrification. *Plant Cell Rep.* 14:730–734; 1995.
- International Rice Research Institute (IRRI). *Standard evaluation system for rice*, 3rd edn. Los Baños: Philippines; 1988.
- Jain, S.; Jain, R. K.; Wu, R. A. Simple method for conservation of embryogenic cells of aromatic indica rice varieties. *Plant Cell Rep.* 15:712–717; 1996.
- Janeiro, L. V.; Vieitez, A. M.; Ballester, A. Cryopreservation of somatic embryos and embryonic axes of *Camellia japonica* L. *Plant Cell Rep.* 15:699–703; 1996.
- Jitsuyama, Y.; Suzuki, T.; Harada, T.; Fujikawa, S. Sucrose incubation increases freezing tolerance of asparagus (*Asparagus officinalis* L.) embryogenic cell suspensions. *CryoLetters* 23:103–112; 2002.
- Luque, R.; Sousa, H. C.; Kraus, J. E. Métodos de coloração de Roeser – modificado – e Kropp (1972) visando a substituição do azul de astra por azul de alcião 8 GS ou 8 GX. *Acta Botanica Brasilica* 10(2):199–212; 1996.
- Mandal, N.; Gupta, S. Studies on histomorphological course of events during androgenesis in rice (*Oryza sativa* L.). *Phytomorphology* 42(2):99–107; 1996.
- Marassi, M. A.; Bovo, O. A.; Lavia, G. L.; Mroginski, L. A. Regeneration of rice double haploids using one step culture procedure. *J. Plant Physiol.* 141:610–614; 1993.

- Moukadiri, O.; Connor, J. E.; Cornejo, M. J. Phenotypic characterisation of the progenies of rice plants derived from cryopreserved calli. *Plant Cell Rep* 18:625–632; 1999.
- Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15:473–497; 1962.
- Nakamura, K.; Suzuki, H.; Hattori, K.; Futsuhara, Y. Identification of ploidy level of the regenerated plants by anther culture in rice. *Breeding Sci.* 44(1):19–22; 1994.
- Orshinsky, B. R.; Sadasivaiah, R. S. Effect of plant growth conditions, plating density, and genotype on the anther culture response of soft white spring wheat hybrids. *Plant Cell Rep.* 16:758–762; 1997.
- Postek, M. T.; Howard, K. S.; Johnson, A. H.; McMichael, K. L. *Scanning electron microscopy – A student's handbook.* Williston VA: Ladd Research Industries, Inc; 1980.
- Scocchi, A.; Faloci, M.; Medina, R.; Olmos, S.; Mroginski, L. A. Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. *Euphytica* 135:29–38; 2004.
- Towill, L. E.; Walters, C. Cryopreservation of pollen. In: Engelmann, F.; Takagi, H., eds. *Cryopreservation of tropical germplasm. Current research progress and application.* Ibarak, Japan: JIRCAS/IPGRI; 2000:115–129.
- Wang, J. H.; Ge, J. G.; Liu, F.; Huang, C. N. Ultrastructural changes during cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells by vitrification. *CryoLetters* 19:49–54; 1998.
- Zhang, Y. X.; Wang, J. H.; Bian, H. W.; Zhu, M. Pregrowth-desiccation: a simple and efficient procedure for the cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells. *CryoLetters* 22(4):221–229; 2001.