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The regeneration of plants from frozen pollen embryos and zygotic embryos of wheat and rice

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Summary. Anther culture derived pollen embryos and immature zygotic embryos of wheat and rice, frozen in liquid nitrogen in the presence of dimethyl sulfoxide, sucrose and glycerol, have been revived. The retrieved cultures proliferated and/or regenerated shoots and plantlets. The prospects of the cryopreservation of embryos for the conservation and multiplication of germplasm and the possibility of the establishment of 'Germplasm Banks' are discussed.

Key words: Cryopreservation – Embryo culture – Haploids – Wheat – Rice – Germplasm bank

Introduction

During the last decade considerable progress has taken place in the field of cryobiology, and entire plants have been regenerated from cells, tissues and organs freeze-preserved for various length of time (Bajaj 1983). Literature concerning the cryopreservation of embryos is rather scanty, however much interest has generated because of the far-reaching implications for the long-term conservation of the germplasms of recalcitrant, rare and endangered species of plants. In addition, this techniques has a number of potential uses, especially in view of the great importance of haploid cultures in genetics, induction of mutations and breeding (Hu Han 1983), it is necessary that at least during the course of experiment the cultures should maintain their genetic (haploid) stability.

The present communication deals with the survival of pollen embryos and zygotic embryos of wheat and rice frozen in liquid nitrogen. The prospects for the cryopreservation of embryos, and the possibility of the establishment of germplasm banks are discussed.

Materials and methods

The spikes of *Triticum aestivum* (cv. 'Kalyansona') and (*Oryza sativa* cv. 'Basmati 370') were removed 15–19 days after pollination (DAP) and the immature embryos were aseptically excised. Twenty to twenty-five of them were pooled in vials containing 1 ml of Murashige and Skoog's basal medium (1962) with 10% dimethyl sulfoxide and 4% sucrose as the cryoprotectant, or on a filter paper soaked in the cryoprotectant solution. The filter paper along with the embryos was wrapped in aluminium foil. They were subjected to a sudden immersion in liquid nitrogen (LN), and stored. Likewise, the young anthers of wheat (Bajaj 1977) and rice (Bajaj 1980) were excised and cultured on their respective media. After five to six weeks, the androgenic anthers were cut into segments and treated with the cryoprotectant solution (containing 7% DMSO, 5% glycerol and 5% sucrose). The segments from 50 anthers were either wrapped in aluminium foil (Withers 1979), or were put in an ampoule containing 1 ml of the cryoprotectant solution. From some anthers a suspension of pollen embryos was prepared. The cultures were frozen by two methods, (i) subjected to the vapours of LN and then immersed gradually, (ii) rapid freezing by quick immersion in LN.

The materials were then thawed in a beaker containing water at 35°–40 °C. The zygotic embryos were cultured on agar solidified MS + 2,4-D (0.2 mg/l), whereas segments of the retrieved anthers of wheat and rice were recultured on their respective media.

The survival of the frozen embryos was judged by their ability to (i) increase in size, (ii) turn green, (iii) proliferate to form callus, and (iv) develop root/shoots/plantlets.

Results

The data on the extent of survival of zygotic and pollen embryos frozen to –196 °C, and their subsequent

Table 1. Survival of pollen embryos and segments of the androgenic anthers of *Triticum aestivum* and *Oryza sativa* frozen and stored at −196 °C for two months

Crop	No. of anther segments frozen	No. of anther segments resumed growth	% survival	No. of pollen embryos frozen	No. of pollen embryos survived	% survival
Wheat	155	8	5	112	21	19
Rice	209	13	6	171	36	21

Table 2. Survival of immature zygotic embryos of wheat and rice frozen in liquid nitrogen in the presence of DMSO (10%) + sucrose (4%)

Species	Control				Frozen			
	Exp. no.	No. of unfrozen embryos cultured	No. of growing cultures	% growing (control)	No. of embryos frozen	No. of embryos revived	% survival	Revival % of control
1. *Triticum aestivum* (cv 'Kalyansona')	1	31	28	90.3	37	14	37.8	41.8
	2	43	39	90.9	52	23	44.2	48.7
2. *Oryza sativa* (cv 'B370')	1	34	29	85.3	38	18	47.3	55.4
	2	53	47	88.8	47	22	46.7	52.5

growth responses are presented in Tables 1 and 2, and Figs. 1–5.

Pollen embryos

Earlier experiments on freshly excised anthers yielded unsatisfactory results (Bajaj 1978) as the whole anthers became soft and spongy. The present work therefore was concentrated on segments of the five to six week old cultures of anthers undergoing pollen embryogenesis. The results obtained with wheat and rice are comparable (Table 1). These segments of the androgenic anthers showed a viability of 5–6%, whereas 19–21% of the pollen embryos survived freezing. The retrieved cultures underwent a lag period of two to four weeks, thereafter, proliferation was initiated to form callus (Fig. 1). The callus on transfer to a hormone-free medium, or to a medium supplemented with IAA + BA + CH differentiated shoots/plantlets (Fig. 2). The pattern of morphogenesis appeared to be disturbed in some cultures, as malformed and stunted shoots and abnormal-looking plantlets were formed.

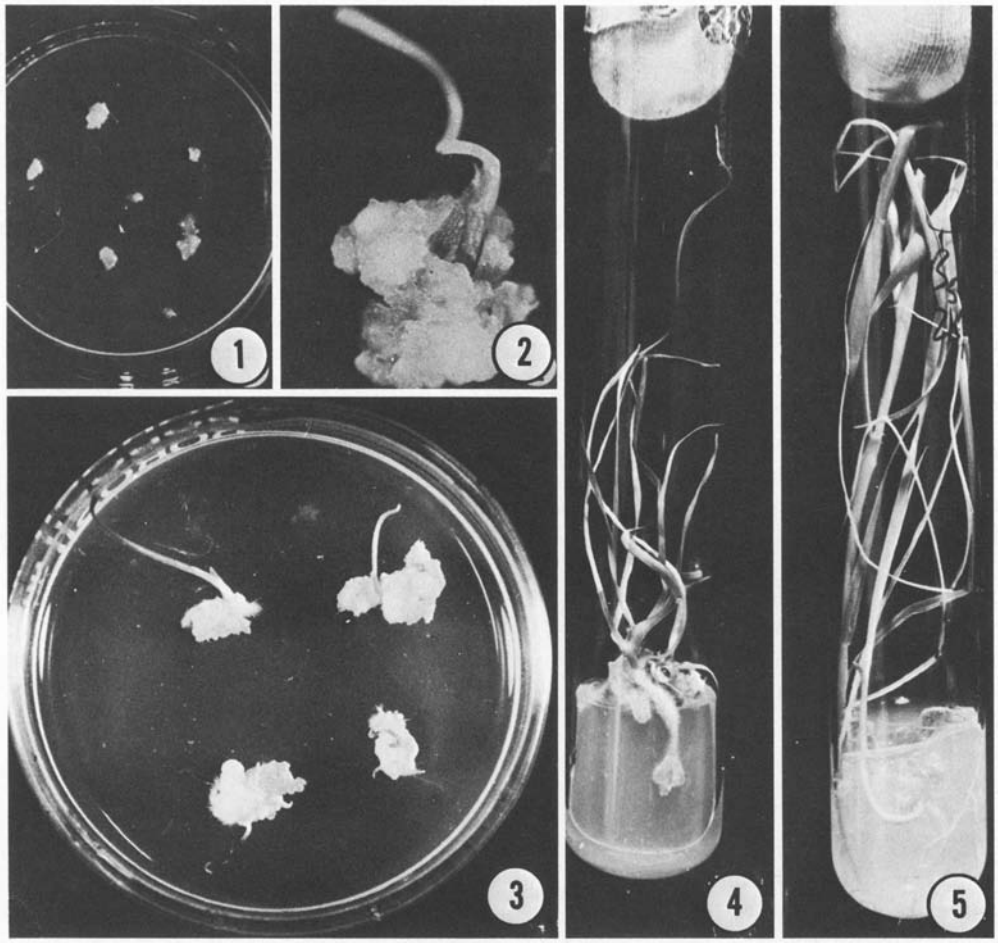
Zygotic embryos

The revival of immature zygotic embryos varied from 41.8–48.7 and 52.5–55.4% in wheat and rice, respectively (Table 2). The control embryos started to elongate or initiated proliferation within three days; the frozen embryos underwent a lag period of about two weeks, after which they showed signs of growth. These embryos then elongated and proliferated to form a mass of callus (Fig. 3), and developed shoots and plantlets (Figs. 4 and 5). The callus on transfer to MS + IAA (1 mg/l) + BA (5 mg/l) + CH (500 mg/l) underwent differentiation to form multiple shoots (Fig. 4). The callus was loose, friable, and in shake cultures occasionally underwent somatic embryogenesis. The zygotic embryos and the callus-derived plants developed further and matured.

In contrast to the pollen embryos, the zygotic embryos showed higher viability, a shorter lag phase and normal shoots. The long lag phase, formation of malformed shoots and low viability reflect the extent of cryodamage caused to the system. However one could overcome these by refining the technique and by manipulation of various affecting cryoability.

Discussion

The successful revival and the regeneration of plants from pollen embryos and from zygotic embryos freeze-preserved at −196 °C points to the possibility of long-term storage and conservation of rare and desirable



Figs. 1–5. Regeneration of shoots and plantlets from pollen embryos and zygotic embryos of wheat and rice frozen to −196 °C. **1** Callus masses obtained from frozen segments of the androgenic anthers of wheat; **2** Same, showing the differentiation of a shoot; **3** Formation of callus and shoots from immature embryos of wheat frozen in liquid nitrogen and cultured on MS + 2, 4-D (0.5 mg/l) for six weeks; **4** Regeneration of multiple shoots from retrieved wheat embryos 10 weeks after culture; **5** Rice plants from frozen embryos 10 weeks after culture

germplasm. The prospects for the cryopreservation of embryos, and the possibility of the establishment of Germplasm Banks are discussed below.

1 Prospects for the cryopreservation of embryos

Recalcitrant seeds. The storage of seeds is the customary method for the conservation and the international exchange of germplasm. However, in a number of plant species, the seeds are sensitive to humidity and temperature (recalcitrant), and thus can not be preserved for long periods due to the degeneration of the embryos. In such cases the germplasm could possibly be conserved through the cryopreservation of excised embryos.

Substitute for seeds and mass propagation. The somatic embryos are being looked upon as 'seeds' in plants which do not set seeds. Since they can be produced in large numbers from the cell suspensions, it would be rewarding to freeze and use them for large scale multiplication of a desirable plant in which the seeds are either not set or are produced in less quantities. This is an area which can be commercially exploited.

Wide hybridization. In the incompatible crosses where generally the hybrid embryos abort at early stages, the immature embryos can be excised, cryopreserved, and cultured when the need arises.

Haploid germplasm. Haploid cell cultures are known to be genetically unstable (Sacristán 1971) and within a short period of time they revert to the diploid state. In such cases cryopreservation of pollen embryos or their callus would be rewarding.

2 Establishment of germplasm banks

International efforts are being made to develop innovative methods for the conservation of germplasm. In this regard various in vitro methods combined with the technology of cryopreservation have attracted considerable attention for the conservation of germplasm (Bajaj 1979, 1983) which is elite, rare and threatened with extinction. These approaches would be especially rewarding for the storage of germplasm stocks of the vegetatively propagated crops, the clones of which have to be maintained through annual propagation in nurseries and this involves the hazards of pests and pathogens. At present there is no satisfactory method for the long-term conservation of germplasm of such crops. The immediate application of the cryopreservation of embryos would be for plants with 'recalcitrant seeds', such as those of coffee, cocao, coconut, castanea etc. Such seeds are short-lived and can not be stored due to early degeneration of the embryos. It would be rewarding to freeze them not only for the conservation of germplasm, but also for large scale propagation. Thus, it is highly desirable that this approach for conservation as well as for the international exchange of germplasm should be seriously considered, and that "Germplasm Banks" be established at a few selected laboratories. International institutes such as IRRI (Los Banos), CIAT (Colombia), CIMMYT (Mexico), CIP (Peru), etc., could be entrusted with the job of storage, maintenance, distribution and exchange of disease-free, rare and elite germplasm of their respective crops.

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