PLENARY LECTURE

FREEZE-PRESERVATION OF PLANT CELL AND TISSUE CULTURES - PROGRESS AND PROSPECTS

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INTRODUCTION

The freeze-preservation of plant cells and tissue cultures is rather a recent development, but had aroused much international interest because of its potential use for the long-term preservation of germplasm (1-4). The survey of literature has shown that cells belonging to diverse groups of plants such as legumes, cereals, trees, ornamentals and tubers (Table I) have been frozen, successfully revived, and induced to regenerate entire plants.

The technology of cryopreservation involves the bringing of cultures to a state of non-division and inactive metabolism by subjecting them to ultra-low temperatures in the presence of cryoprotectants. This is somewhat like the preservation of semen, but has abundant potentials.

The cell and callus cultures on repeated and periodical transfer are known to undergo genetic erosions (5), and also have a tendency to show a gradual loss in their morphogenetic potential. Various methods, such as mineral oil overlay (6) and the use of minimal media (7) have not been of much help. Thus one of the important significant uses of cryogenic methods would be the prevention of genetic changes, and the maintenance of the genetic stability of the germplasm in cultures.

PROTOCOL FOR CRYOPRESERVATION

The freeze-storage of cell and tissue cultures, with certain modifications depending on the system, basically involves the following steps:

1. Establishing sterile culture of tissues, organs, and cell suspensions.
2. Treatment with suitable cryoprotectants.
3. Freezing cultures in liquid nitrogen by slow-regulated cooling, pre-freezing, or by sudden cooling.
4. Storage of frozen cultures in liquid nitrogen (-196°C).

5. Rewarming or thawing of the cultures.
6. Removal of the cryoprotectant by washing, if necessary.
7. Determination of the cryoability of the cultures.
8. Reculturing of the retrieved cells and tissues to induce growth and morphogenesis.

PROGRESS OF WORK DURING THE LAST DECADE

Since the first report in 1968 by Quatrano (8) on the revival of frozen cell cultures of flax, tremendous progress has been made. During the last five years, callus cell suspensions (Fig. 1), somatic embryos and endosperm (Fig. 2,3 - Table II), pollen embryos (Fig. 3 - Table III) and meristems (Fig. 4,5 - Table IV) have been employed for the cryopreservation studies, and the literature is summarized in Table I.

The capacity to withstand freezing, the extent of survival, and the eventual resumption of growth depends on a number of factors, some of which are outlined below, and discussed in the subsequent pages:

1. Physiological stage, age and nature of the culture to be preserved.
2. Density of the culture in the case of cell suspension.
3. Concentration and nature of the cryoprotective agent.
4. Method and the rate of freezing.
5. Storage temperature.
6. Method of thawing.
7. Method for the determination of viability.

From the survey of the literature (Table I), it emerges that for the successful revival of the cells, the following are some of the guidelines which may be taken into consideration.

1. Actively growing and periodically transferred cell suspensions containing small clumps of cells should be used (9).

2. If single cells are to be used, the cell suspension should be filtered through a fine mesh to collect small, highly cytoplasmic, and non-vacuolated cells as they can withstand freezing better than the large thick-walled, and highly vacuolated cells (9, 10).

3. For the maintenance of clones, especially those of the vegetativ propagated crops, meristems should be used.

4. For the preservation of plantlets and whole organs, partial dehyd before freezing would be advisable (11,12).

5. Before freezing, the cells or tissue may be (pre-) grown for a co of days on a medium containing a low concentration of cryoprotect (5% DMSO for instance).

6. A mixture of various cryoprotectants at low concentrations rathe than a single cryoprotectant at a high concentration should be us

7. During treatment with the cryoprotectants, the cultures should b maintained in ice to avoid their deleterious effects.

8. It should be borne in mind that any data based on staining metho alone can be misleading; thus every effort should be made to determine the cryoability of the cells, taking growth as the impo parameter.

9. To avoid any deterioration of the germplasm, the cultures should stored at -196°C.

10. The cultures thawed at 35-40°C are expected to yield higher surv

11. Lag phase is due to the cryodamage; it results in the depletion viable cells, and eventually, selection pressure. This is high undesirable in respect of the maintenance of clones. Thus the maximum recovery of the cells is of the utmost importance.

12. The cultures are normally frozen along with the cryoprotectant solution, and washed repeatedly. However, higher viability of t carrot plantlets has recently been achieved by using the dry-fre method (12), i.e., by wrapping them up in an aluminium foil inste of pooling them in an ampuole containing cryoprotectant. By usi this method the need for repeated washing is also avoided.

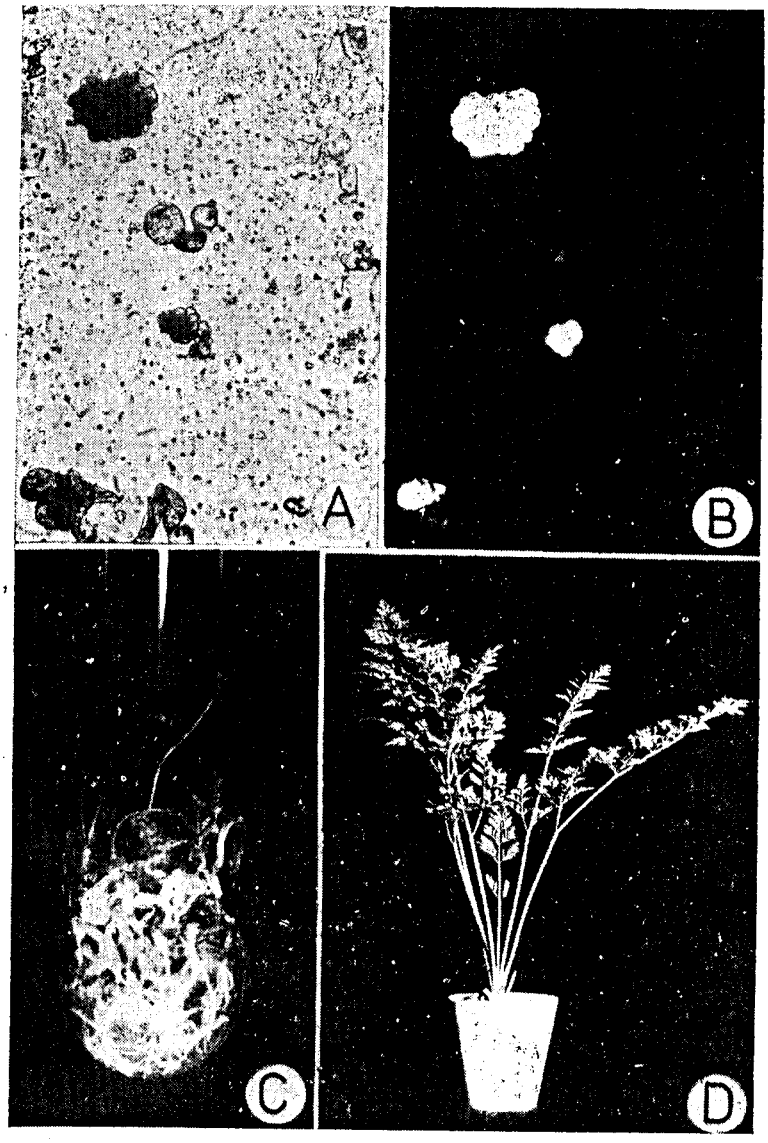
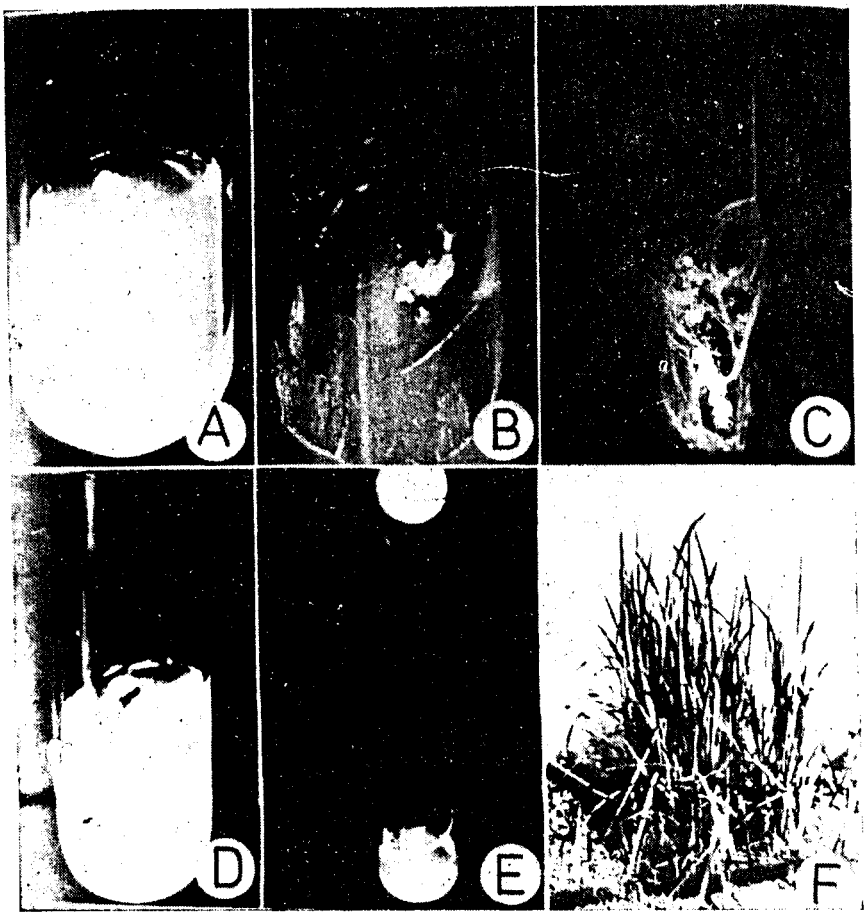


Fig. 1. A–D. Regeneration of carrot plants from cell suspensions subjected to liquid nitrogen (cryoprotectant 7% DMSO, cooling rate 2°C/min, thawed at 37°C). *Fig. A–B.* 10-day-old cell suspension containing free cells and actively growing clumps of cells, in tungsten light *(Fig. A)* and in ultraviolet light *(Fig. B)*; note the complete survival of a clump while the free cells are dead. *Fig. C.* Differentiation of plantlets on a 2, 4-D-free medium, 9 weeks after culture (including 3 weeks of lag period) of frozen cell suspensions. *Fig. D.* Same, 6 weeks after transfer to a pot. (After Bajaj 1976b).



Figs. 2 A–F. Induction of growth and morphogenesis in excised endosperm and embryos of rice frozen in liquid nitrogen. *Fig. A.* Frozen-thawed segments of endosperm 4 weeks after culture on MS + 2, 4-D (2mg/l); note the initiation of callus. *Fig. B. C* Differentiation of endosperm callus into a shoot and plantlets after transfer to MS+IAA (4mg/l) + kinetin (2mg/l). *Fig. D.* An embryo (excised from a frozen seed) 3 weeks after culture; note the formation of a root but the growth of the shoot apex is suppressed. *Fig. E.* A normal plant obtained from a frozen embryo. *Fig. F.* Transfer of test-tube plants to soil (Bajaj – unpublished).

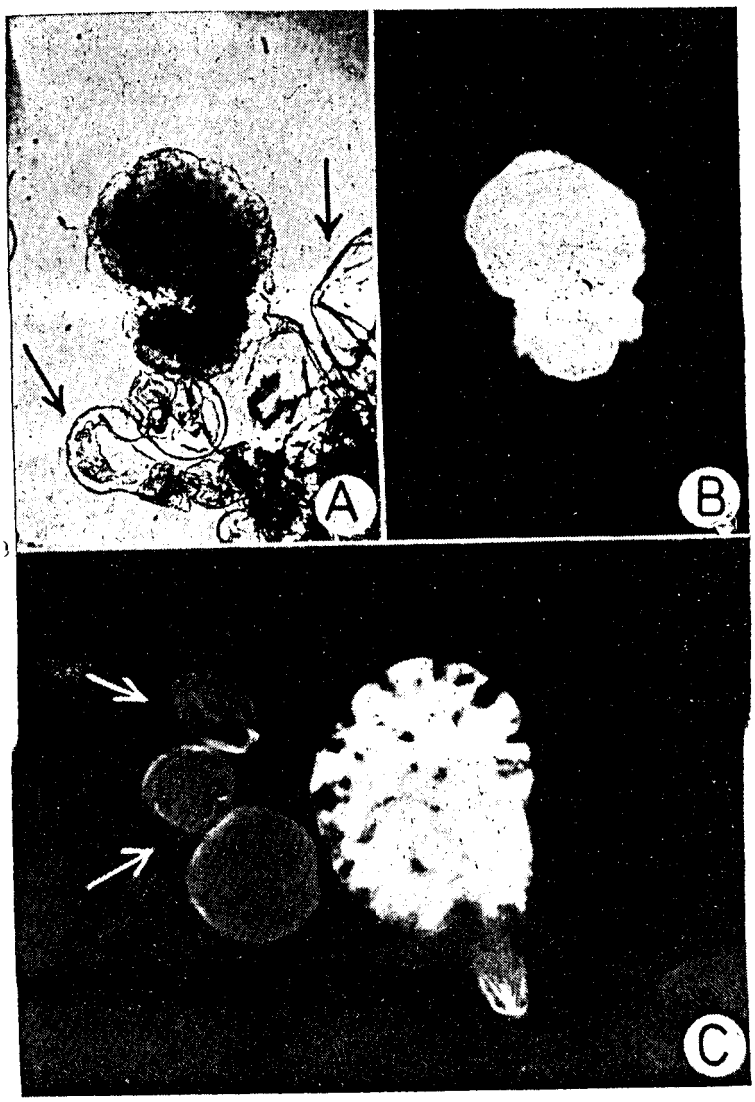


Fig. 3. A-C. Survival of embryos in liquid nitrogen. *Fig. A.* A somatic embryo of carrot along with cell suspension frozen at the rate of 2°C/minutes, and stored for one year at -196°C (Photographed in tungsten light). *Fig. B.* After staining with fluorescein diacetate; note the survival of the embryo, and death of callus cells (shown with arrows – From Bajaj 1976b). *Fig. C.* Survival of pollen-embryo of *Atropa belladonna* subjected to-196°C in the presence of 5% DMSO; note the aborted pollen (marked with arrows). After Bajaj 1977a.

FREEZE PRESERVATION OF POTATO GERMPLASM

Fig. 4. A–O. Tuberization of the potato plants regenerated from frozen meristems. *Fig. A–C.* Various types of ampoules and vials used for freezing. *Figs. D–F.* Containers for freezing and storing ampoules. *Fig. G.* A liquid nitrogen cylinder (VEB, German Democratic Republic). *Fig. H. I.* Frozen-thawed meristems 4 and 10 weeks after culture on agar medium, and on filter-paper wick (liquid medium) respectively. *Fig. J.* Eleven-week -old culture of retrived meristem undergoing proliferation to form callus. *Fig. K.* Same after next 4 weeks on MS + IAA + BAP showing initiation of shoot primordia from callus. *Fig. L.* A plantlet obtained from meristem freeze-preserved for 15 days at -196°C; note the formation of a tiny tuber. *Fig. M–O.* Transfer of plants from test-tube to the pot and field, and the production of tubers (From

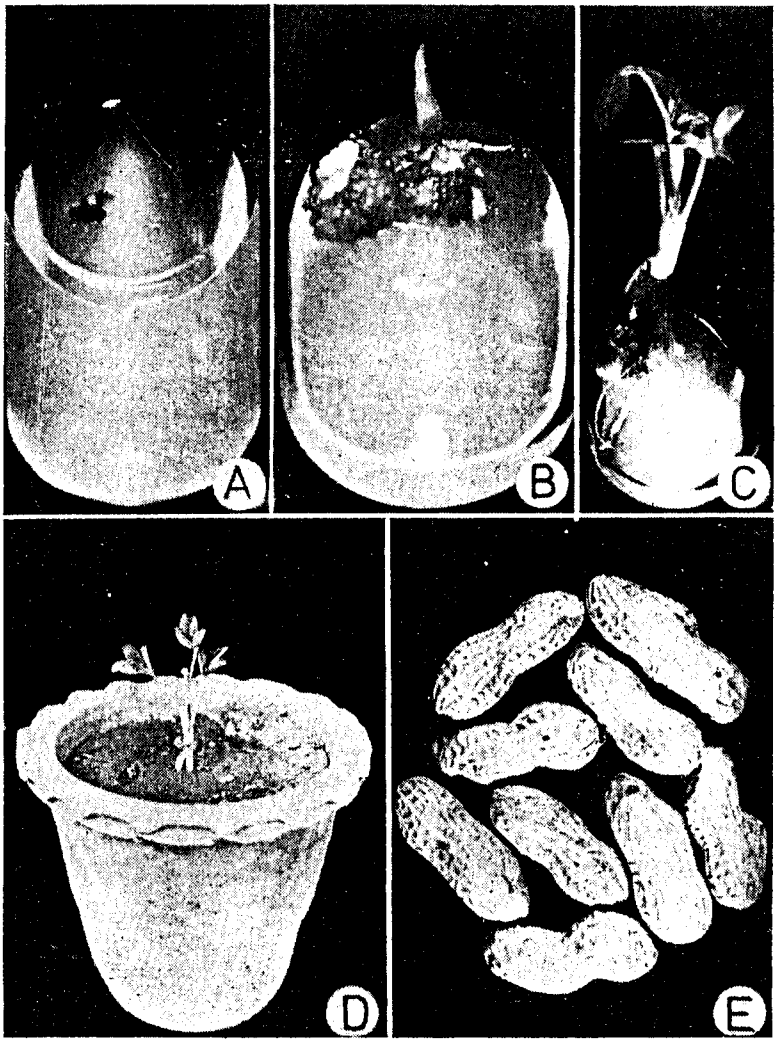


Fig. 5. A–E. Various stages in the regeneration of plants of *Arachis hypogaea* from freeze-preserved meristems. *Fig. A–C.* Frozen-thawed meristems after 6,9 and 12 weeks of culture respectively; note the initaial callusing of the meristem in *Fig. A. Fig. D.* A test-tube plant transferred to soil. *Fig. E.* Normal seeds obtained from such plants (After Bajaj 1979c).

ADVANTAGES OF FREEZE-PRESERVATION OF MERISTEMS OVER CALLUS CULTURES

Although the actively growing cell suspensions can withstand freezing much better than the relatively old and vacuolated cells, yet the possibility of genetic changes is not ruled out. Thus, for long-term preservations, excised meristems should be preferred to cell cultures for the following reasons:

1. In some cases, it is difficult to regenerate entire plants from the callus, whereas the meristematic tips are relatively easy to grow.
2. They are genetically more stable. On the other hand, the callus cultures are unstable, and on periodical subculturing over an extended period show chromosomal aberration, and changes in nuclear and ploidy levels.
3. It is quicker method for vegetative propagation.
4. Excised meristems yield pathogen-free plants.
5. Meristems taken especially from haploid plants, would ensure their maintenance.
6. The meristematic cells are small, densely cytoplasmic and thinwalled, thus a higher survival would be expected.
7. Whereas cell cultures require a slow and controlled rate of cooling, the meristems, on the contrary, can withstand sudden freezing, thus elaborate and expensive cryostats are not needed for the preservation of meristems.

PROSPECTS OF THE FREEZE-STORAGE

The freeze-preservation of plant cell and tissue cultures offers a number of potential uses, some of which are shown in figure 6 and are enumerated below:

1. Preservation of genetic uniformity.
2. Conservation of rare and important germplasm.

3. Maintenance of unstable (haploid) cultures.
4. Storage of disease-free material.
5. Retention of morphogenetic potential.
6. Avoidance of periodical subculturing.
7. Economising on manpower, media, and storage space.
8. Possibility of prevention of aging.
9. International exchange of material.
10. Induction of cold acclimation.
11. Regeneration of frost-resistant (mutant) plants.
12. Pollen storage for longevity and incompatible crossing (13).

The depletion of the germplasm pools and the shrinkage of the naturally occurring genetic resources of crops has caused a great concern, and thus an urgent need is felt to resort to means other than conventional ones to increase the available germplasm stocks. In this respect, callus tissue cultures are a rich source of genetic variability. In addition, haploid and homozygous plants, mutants, aneuploids, somatic hybrids, cybri and other novel germplasms which do not exist in nature can be obtained by various in vitro means (Fig. 7). Some of the in vitro induced genetic alterations which may not be of immediate use, but may be frozen and preserved, and later, when the need arises, they can be revived and incorporated into the plant improvement programmes.

From the applied point of view, the cryopreservation of germplasm o the vegetatively propagated crops will be of immense value. For instance, in the case of potato, to maintain its germplasm, the 'seeds' (tubers) have to be grown and multiplied in the nurseries every year. As an alternative to this, attempts have been made to store callus tissue and meristem cultures. However, for long-term preservation, one of the main drawbacks of callus cultures is that they undergo genetic erosions, and therefore, the clones cannot be maintained. Although this drawback

can be surmounted by culturing the excised meristems, yet they need periodical transfers to the fresh medium, require extra space, manpower, and there is also the risk of contaminations during transfer. Thus the regeneration of potato plants from meristems (Fig. 4, Table III) freeze-preserved (14-16) for up to 14 months demonstrates the potential practical use of cryogenic methods in this crop.

To conclude, it has been established that plant cells can be frozen preserved and revived. However, the main task ahead is to increase the rate of viability, so that there is no selection pressure. This can be achieved through a better and understanding of the system under study by manipulating various factors influencing the cryoability of cells, and by refinement of techniques. Emphasis should especially be laid on the vegetatively propagated crops, for which, at present, no method exists for their longterm preservation.

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TABLE I. FREEZE-PRESERVATION OF PLANT CELL, TISSUE, AND ORGAN CULTURE

Model plants	Culture	Results Survival	References
Carrot	Cell suspension Somatic embryos	Survival upto 70%	17, 18 9,12,19,20
Atropa	Cell suspension Pollen-embryos	20-40% upto 31%	21 1,22
Datura	Cell suspension	40%	9
Tobacco	Cell suspension Androgenic anthers Pollen-embryos	11% 6.6% 36%	9 23 22
Vegetatively Propagated Crops			
Sweet Potato	Cell suspension	Occasional	17
Cassava	Meristems	21% shoots	24
Potato	Meristems	18-26% plants	14-16, 25
Sugarcane	Cell suspension	60%	26,27
Strawberry	Meristem	60-80% plants	28
Cereal and Grasses			
Bromus	Cell suspension	38%	29
Rice	Cell suspension Anthers Endosperm Embryos	60-65% Androgenesis Plantlets	30 31 32
Wheat	Callus	29%	32
Legumes			
Soybean	Cell suspension	52%	9
Gram	Shoot tip	Plants	33
Groundnut	Shoot tip	Plants	33
Pea	Shoot tip	Plants	34
Ornamentals			
Chrysanthemum	Callus	8%	35,36
Carnation	Shoot tip	15-33% plants	37
Petunia	Callusing anthers	5% callused further	23
Primula	Androgenic anthers	Callus & Plants	38
Trees			
Prunus	Callus	Increased hardiness	39
Poplar	Callus	Cold acclimation	40
Sycamore	Cell suspension	20-30%	41

TABLE II EFFECT OF SUDDEN FREEZING (-196°C) ON THE SEEDS, EXCISED EMBRYOS AND ENDOSPERM OF RICE PRESERVED FOR 3 WEEKS IN LIQUID NITROGEN*

	Control	Frozen	
		Growth response (survival)	Growth (percentage of control)
Seeds	98% germination	96% germination	98
Dehusked seeds	94% germination	82% germination	87
Excised embryo with a portion of endosperm	86% grew	71% embryos callused and developed shoots	83
Segments of mature endosperm	16% callused	11% proliferated to form callus	68

* The seeds were germinated on moist filter papers in a petri-dish, whereas dehusked seeds, excised endosperm and the embryos were cultured on MS + 2,4-D 2 mg/l. Data based on 350 seeds, 92 dehusked seeds, and 360 cultures of embryos and endosperm (Ref. No. 32).

TABLE III RESPONSE OF NICOTIANA TABACUM ANTHERS (4 WEEKS AFTER CULTURE) SUBJECTED TO VARIOUS TREATMENTS AND RECULTURED ON AGAR-SOLIDIFIED MEDIUM*

Experiment	No. of Anthers Cultured	No. of Growing Anthers	% Survival	Total Number of plantlets	No. of Plants Per Anther
Untreated (Control)	85	69	81.1	890	12.8
Treated with 7% DMSO	70	54	77.1	530	9.8
Treated with 7% DMSO and warmed at 37°C for 10 min.	66	45	68.1	390	8.6
Treated with DMSO, cooled at the rate of 2°C/min, subjected to -196°C and thawed at 37°C.	130	2	1.5	7	3.5

* Ref. No. 23

TABLE IV. EFFECT OF VARIOUS CRYOPROTECTANTS ON SURVIVAL OF MERISTEMS FROM TUBER SPROUTS AND AXILLARY BUDS OF TWO CULTIVARS OF POTATO SUBJECTED TO -196°C.

Experiment*	Cryoprotectant	cv. 'Kufri Chandramukhi'		cv. 'Kufri Sindhuri'	
		Tuber sprout (3-5 mm)	Axillary bud (1-2 mm)	Tuber sprout (3-5 mm)	Axillary bud (1-2 mm)
		Survival % of the control			
I	Glycerol 15%	9	16	11	18
	Sucrose 15%	7	12	9	14
	Glycerol 10% + Sucrose 5%	9	15	10	18
	Sucrose 10% + Glycerol 5%	8	11	9	12
II*	DMSO 10%	11	19	12	21
	DMSO 5% + Glycerol 5% + Sucrose 5%	13	23	14	26

* In experiment II, the cultures were freeze-preserved for 31 days in liquid nitrogen (Ref. No. 15)

DISCUSSION

K.S. JAGADISH CHANDRA : In what way the 'freeze storage' of Meristematic parts/callus cultures is more advantageous compared to controlled radiation 'treatment' of plant parts like the seeds and fruits?

Y.P.S. BAJAJ : Plant parts 'freeze stored' can be prolonged fairly to a longer time than the 'radiation treated' ones.

M.S. RANGASWAMY : I wonder whether you have attempted mineral oil overlay preservation of tissue cultures vis-a-vis Freeze preservation especially in terms of economics of the techniques.

Y.P.S. BAJAJ : I am very much familiar with this work. The use of mineral oil overlay has worked quite well for the preservation of lower organisms, however, for tissue cultures, it has proved to be of little significance. By using this method, although one can slow down the rate of growth, and also delay periodical subculturing, yet for long-term storage of the clonal material, the possibility of the genetic alterations is not ruled out. As a matter of fact better outcome would be expected from cultures grown on minimal media combined with storage at 5-10°C than mineral oil method. Thus for long-term conservation of germplasm, cryopreservation would seem to be the answer.

R.D. IYER : What are the possibilties of storing bulky tissues like embryos? This is of significance to large seeded plants like coconut. which h only one meristem and we would have to think of sto ng only embryos for ger plasm conservation since coconut requires a large area for planting to establish such a 'Bank' ?.

Y.P.S. BAJAJ :Our attempts with freeze-preservation of young and small embryos, like pollen-embryos and the callus-derived somatic embryos have been successful. It is however, observed that as the embryo differentiates and matures, the rate of survival decreases, and also only portions of the retrieved embryo show signs of growth. However, by subjecting embryos to partial dehydration prior to free the rate of survival can be increased. Now, in the case coconut, where the embryo is exceptionally large it sho possible to freeze-preserve it by this method.

N .MAHERCHANDANI: Whether there is a difference in % survival after sho term (say 6 months) and prolonged storage of twins ?

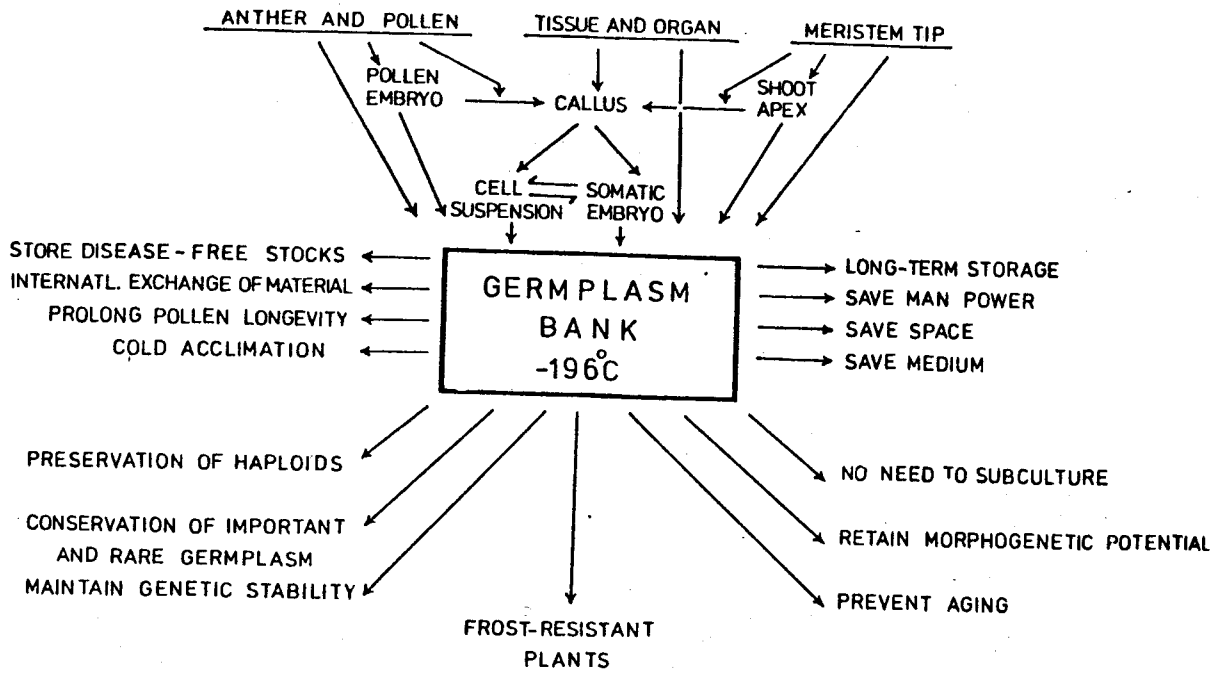
Y.P.S. BAJAJ : If the technology of freezing is not faulty, i.e., the material under study is in a good physiological state, is frozen under optimal conditions of cryoprotectants a the rate of cooling etc., then no difference in the twin cultures should be expected. However, if the specimen frozen and stored under sub-optimal conditions, then so cryodamage is expected which would result in relatively low survival on prolonged storage.

V.S. JAISWAL : Does the ability of cryoprotectant vary from tissue to tissue ?

Y.P.S. BAJAJ : Yes. As physical state of tissue will be different.

A.D. KARVE : Among factors affecting revival of frozen cells, you have not mentioned the genotype. Do all varieties of a given species behave in the same manner or does the omission of this factor only mean that you have not tested different genotypes ?

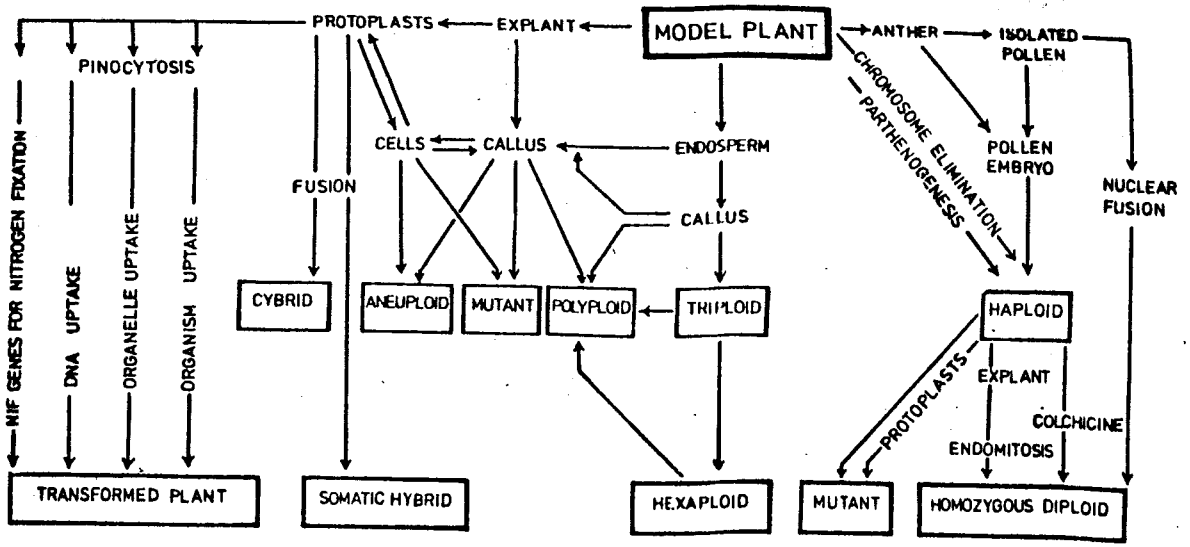
Y.P.S. BAJAJ : Genotype is found to have some effect, but we have not tested different genotypes.



FREEZE-PRESERVATION AND ESTABLISHMENT OF GERMPLASM BANK

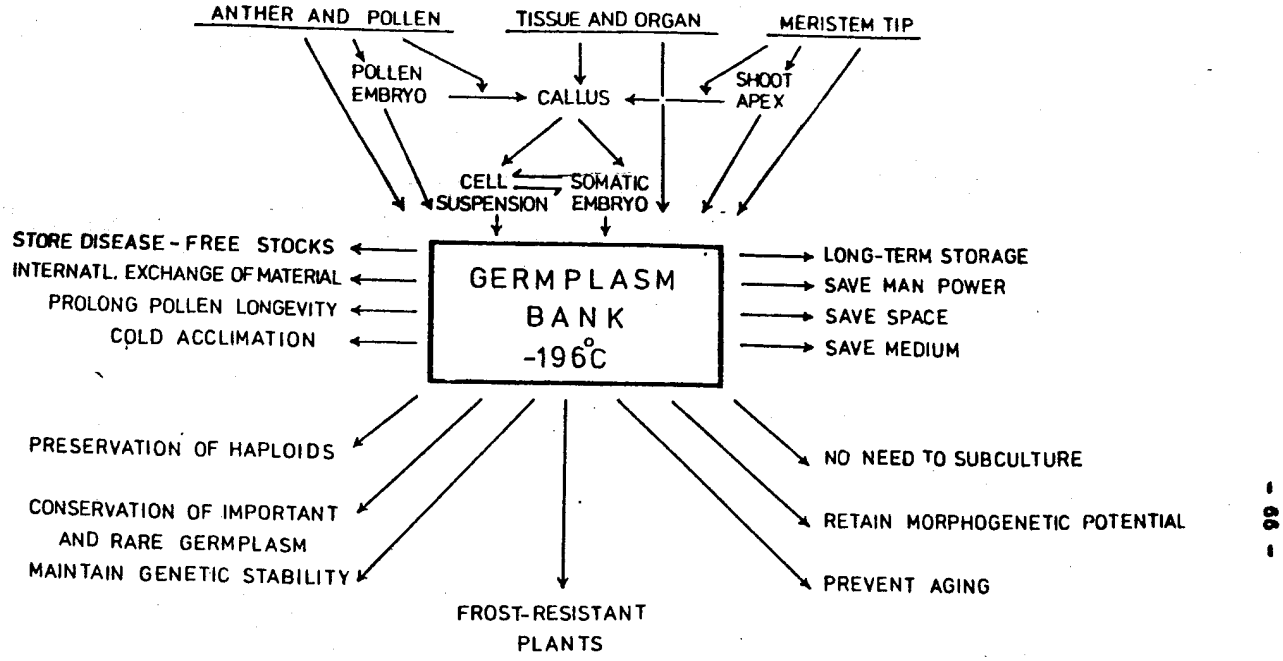
(FROM BAJAJ 1977)

Fig. 6. Diagramatic representation showing the prospects of the freeze-preservation of cells, tissues and organs, and emphasizing the possibility of the creation of "Germplasm Banks" for the long-term conservation of germplasm of rare plants, and especially, those of vegetatively propagated crops (After Bajaj



INTRODUCTION OF GENETIC VARIABILITY IN CROPS THROUGH PROTOPLAST AND CELL CULTURE

Fig. 7. Induction of genetic diversity in crops through protoplast, cell and tissue cultures. Some of the in vitro induced variables which do not exist in nature can be frozen and preserved, and later used according to the research needs (From Rajaj 1979a).



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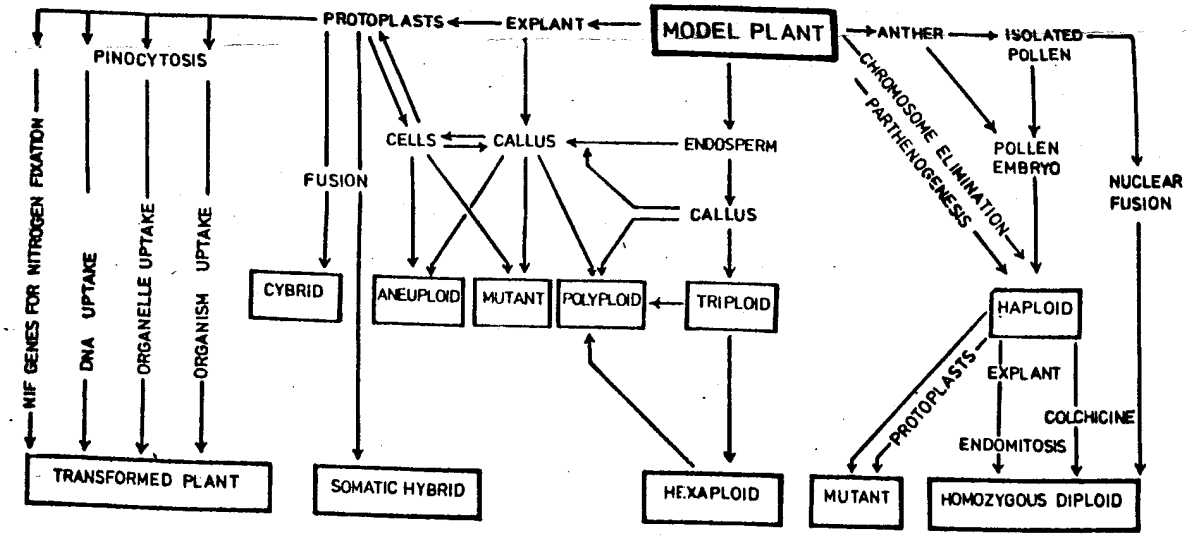

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