CELL AND TISSUE CULTURE TECHNIQUES FOR CEREAL CROP IMPROVEMENT

Proceedings of a workshop cosponsored by
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and
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FOREWORD

Scientists do not expect improved crop varieties to remain stable forever. Breeders must provide farmers with a continual supply of new varieties to replace older varieties not adapted to changing ecological conditions, to open adverse environments to crop production, and to stabilize yields in farmers' fields.

Cell and tissue culture are the newest of the innovative breeding techniques being applied to meet this accelerating need for improved cereal crop varieties. Tissue culture techniques can shorten the time and can lessen the labor and space requirements needed to produce a new variety. The innovative techniques also can provide a means of seed germination in genetic crosses not possible in conventional varietal development programs.

The Workshop on Potentials of Cell and Tissue Culture Techniques in the Improvement of Cereals was held 19–23 October 1981 in Beijing, China. Cosponsors were the Institute of Genetics, Academia Sinica, and the International Rice Research Institute. Scientists from basic and applied research areas met to identify potential areas in which cell and tissue culture could aid in varietal development of cereals, particularly of rice.

China has used tissue culture techniques to develop a number of new rice varieties in the past decade. IRRI began to apply tissue culture to rice breeding in 1979. Significant progress also has been made in the laboratories of many nations.

Participating scientists have been innovative in developing tissue culture methodologies and in applying them to the creation of new plant variants, to overcoming the incompatibilities inherent in distant hybridization, to genetic manipulation, to plant molecular genetics, and to cryopreservation. Their exchange of knowledge, experience, and understanding will lead, we hope, to the collaborative work needed to move varietal improvement programs further, more efficiently.

This proceedings volume includes reports of progress made by scientists from a dozen countries and the recommendations of 50 participants. Dr. Hu Han and Dr. M.D. Pathak, IRRI director for research and training, coordinated the workshop. Dr. F. Javier Zapata, associate plant physiologist, IRRI Tissue Culture Facility, was the technical editor. The papers were edited by Dr. LaRue Pollard, visiting science editor, assisted by Ms. Emerita P. Cervantes, editorial assistant, IRRI, in collaboration with Mr. Lee Xian-wen, editor, Institute of Genetics, Academia Sinica.

Hu Han,
director, Institute of Genetics, Academia Sinica
M.R. Vega,
acting director-general, IRRI

OPENING REMARKS FOR ACADEMIA SINICA

Dear colleagues—ladies and gentlemen:

I have the honor to declare open the workshop on Potentials of Cell and Tissue Culture Techniques in the Improvement of Cereals.

On behalf of the Division of Biological Sciences of the Academia Sinica, I would like to take this opportunity to convey my warmest welcome to all of my friends who attend this workshop. As you may know, this workshop is cosponsored by the International Rice Research Institute (IRRI) and the Institute of Genetics, Academia Sinica. Dr. Nyle C. Brady and Dr. M.D. Pathak, as well as many other distinguished scientists, have given their active support and cooperation to make this workshop a reality; for this I express my sincere gratitude.

The aim of this workshop is to review the recent progress and the newer achievements in cell and tissue culture techniques in order to explore the potentiality of this new technique in the improvement of cereal plants—a very important issue relevant to human welfare and economic development of all countries. I am sure that the workshop will make a valuable contribution toward reaching this goal.

The scientists participating in this meeting are from 12 different countries—Australia, China, Denmark, France, Federal Republic of Germany, India, Japan, Peru, Philippines, Switzerland, Great Britain, and the United States of America. No doubt the discussions among the scientists will be lively and full of color. I sincerely hope that the workshop will promote mutual understanding and friendship among all the scientists, from all countries represented.

It is my anticipation that the workshop will be a great success! Thank you.

Zhang Zhi-yi
Vice-Director, Division of Biological Sciences
Academia Sinica
Beijing, China

OPENING REMARKS FOR THE INTERNATIONAL RICE RESEARCH INSTITUTE

Prof. Zhang, Dr. Hu Han, members of the staff of Academia Sinica, fellow scientists:

It is my real pleasure to welcome all of you to this important workshop jointly organized by the Institute of Genetics, Academia Sinica, and the International Rice Research Institute. Chinese scientists have made remarkable progress in plant improvement and in the creation of new plants through the use of cell and tissue culture. Many scientists in China are pursuing this project vigorously.

IRRI is dedicated to helping improve rice production on a global basis, with particular reference to Asia where 90% of the world's rice is grown and consumed. In most developing tropical and subtropical countries, rice is the staple diet of the population and the basic source of national economies. In these countries, most farmers have limited land areas, limited sources of inputs, and limited modern rice production technology. As a consequence, rice yields have been very low-about 1.5–2.0 t/ha. In some developed countries, rice yields average in excess of 5t/ha.

IRRI was established with the objective of helping small farmers produce more rice, which will not only help improve their individual economic conditions but will also help improve national economies. This objective has been the guiding principle in developing our research programs, which are sharply focused on helping small farmers produce more rice per crop and more crops per hectare per year. In this effort, we work in close collaboration with national rice scientists to evaluate the suitability of research findings to various agroclimatic and socioeconomic conditions and to implement the appropriate disseminations of seed and production technology to farmers.

Through the collaborative work of the interdisciplinary team of scientists in the Genetic Evaluation and Utilization (GEU) program, IRRI has been developing high yielding and good grain quality rice varieties adapted to various physical and biological constraints. The work on tissue culture, initiated about two years ago to help meet this objective, is categorized as an innovative breeding technique.

IRRI has been extremely pleased to collaborate with Chinese scientists on several projects of rice improvement and production. It is with our common objective of helping farmers in developing countries that we have joined hands now in bringing together some of the world's leading scientists on plant cell and tissue culture to review the existing information on this subjects and how it can be used for improving the rice plant. This also may lead to the development of appropriate collaborative projects among various scientists. It is with this consideration that the final day of this meeting has been set aside for overall discussion and the development of future research projects.

M.D. Pathak
Director, Research and Training
The International Rice Research Institute
Los Baños, Philippines

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C. Nitsch

Plant breeding by doubled-haploid in vitro culture has shown its value. It not only shortens the time needed to create a new variety, it also can elicit new information on the quality of the selection process at the pure line level. It offers the possibility of measuring the improvement made in a new genotype at the homozygous stage. The time gained in fixing a new variety as well as the ability to introduce new and often different types of variability also has to be considered. The time to fix a variety can be reduced to a minimum when homozygous plants are obtained at the first generation by androgenesis and when immature embryo culture is used after fertilization to shorten seed production.

RESPONSE TO ANDROGENESIS

The chances of obtaining haploid plants by anther or pollen culture depend on:

- pollen viability.
- plant vigor at the homozygous stage (allogamous varieties have less chance than autogamous).
- haploid plant reaction to chromosome doubling agents.

Pollen viability

Species vary widely in the viability of their pollen. Maize

Table 1. Effect of low temperature on pollen viability of Datura and Nicotiana.

Cold treatment	Survival of pollen grains(%)	
	At time 0	After 5 days of culture
Datura		
No cold	100	62
48 h at 3°C	98	92
Nicotiana		
No cold	92	45
48 h at 5°C	93	68

Genetique et Physiologie du Developpment des Plantes, 9119(Gif-sur-Yvette, France.

pollen is particularly fragile and does not survive the stress of in vitro culture without special care. The cold treatment given flowers to induce androgenesis also has an effect on pollen survival in culture. Table 1 shows the effect of low temperature on survival of Datura and Nicotiana pollen (Nitsch and Norreel 1972).

Plant vigor at the homozygous stage

Homozygous vigor can be used to predict the success of androgenesis. Lethal genes in the genome can interfere with the possibility of growing haploid plants. In varieties with lethal genes, it is necessary to modify the genome before working for haploid production. Plant material for androgenesis needs to be prepared to introduce in the hybrid not only the characters desired for the selection program but also the characters needed to adapt the plant for in vitro requirements.

In other words, a breeder has to think of the breeding process in a new way if androgenesis is to be used for plant improvement. The physiologist can help by working out the environmental conditions that will adapt the material to in vitro culture and that will overcome the epigenetic phenomenon.

By following such an idea, new hybrids in Nicotiana have been created. N. alata, a self-incompatible variety, has been studied intensively to understand the mechanism of incompatibility. Tissue culture techniques such as pollen and protoplast culture have improved the capacity to regenerate plants derived from hybrid calli and opened a new approach to understanding this mechanism. N. alata responds poorly to haploid production — the haploid plant does not grow well. We have been able to dilute the lethal genes by a cross with N. sylvestris. The cross, impossible in nature, has been achieved by putting the very young zygote in vitro 2 days after fusion of the gametes in vivo. The hybrid thus produced has been tested for viability using three techniques of pollen and protoplast culture as

Table 2. Response of N. sylvestris/N. alata hybrid to in vitro techniques.

	Androgenesis Pollen-yielding plants (%)	Protoplast Protoplasm division (%)	Regeneration Callus giving plants (%)
N. sylvestris	8	0.5	98
N. alata	0.05	40	2
Hybrid (s/a)	2	90	85

well as for its plant regeneration ability (Table 2).

The hybrid has 42 chromosomes (N. sylvestris has 24,

N. alata 18), good potential for pollen culture, a surprisingly high rate of division in protoplast culture, and an excellent rate of plant regeneration, although it is self-incompatible. From the same haploid plant, we produced one plant by callus culture that was self-compatible and another that was self-incompatible. This system opens a new way to study the incompatibility gene in N. alata and is an example of how plant breeders and physiologists must work together to build the material that has the best chances for experimentation.

Doubling chromosome number

Doubling the chromosome numbers of pollen-derived plants is difficult. Colchicine, the chemical most commonly used as a doubling agent, is not successful in all cases. Because colchicine acts on mitosis, the apex of the young haploid plant or the pollen itself at the time of the first pollen mitosis are good parts to treat. A method that gives a good percentage of doubling in barley is to submerge the apical part of the haploid plant still growing in test tube in a solution of colchicine (Jensen 1974). In pollen-derived plants, the highest percentage of diploid plants was produced when the anther was treated by colchicine at the time of the first pollen mitosis (Nitsch 1977). Doubling the chromosome number at the unicellular level has the advantage of uniformity. There will be no chimera in the plant and the diploid tissue grows better than the haploid. Forcing the chemical into the cell under vacuum increases the number of diploids as much as 70% in Nicotiana pollen plants. Despite the high rate of pollen mortality caused by the chemical, this technique has filled semi-industrial requirements.

Another difficulty found in maize comes from the delay between the appearance of the male inflorescence and the silk. The pollen can be frozen until the right time for self-fertilization to obtain homozygous lines.

ADAPTING ANDROGENESIS TO SPECIES

To adapt the androgenesis technique to produce isogenic lines, assuming that the right synthetic hybridization has been done, it is necessary to:

- Detect the exact stage of pollen development.
- Promote the pollen for maximum survival in culture.
- Prevent the inhibitory effects of compounds leaking out of the tissue.
- Develop the embryo toward a plant.

Stage of pollen development

The stage at which a microspore can change from the normal evolution of gametogenesis to androgenesis is fixed, but varies from one species to another and between varieties in the same species. Generally, it is at the time of the first pollen mitosis. In Solanaceae, it is just before or shortly after. In cereals, it seems to be before mitosis.

Nuclei of maize microspores were stained using the Feulgen reaction. This showed that this change occurred at the end of the first DNA replication after mitosis. The morphological aspect of the pollen may be a guideline to identify this stage (Guo et al 1978). Careful observation of the microspores that develop as embryos and plants shows that for some hybrids in the cultures that produced embryos, a majority of the microspores had one central nucleus. For other hybrids, the best results were obtained if the nucleus was at the site of mitosis, at the opposite end from the pore (Table 3). The observation was on an aliquot of

Table 3. Morphological aspects of microspore population yielding embryos.

Hybrid	Microspores (%)					
Lai Pin Pai	3	56	34	0	0	7
Ching Huang 13	0	20	63	11	2	4
W23Nj/BMS	29	65	4	1	1	0
IHO/BMS	0	59	32	4	5	0
AHO//LPP/G113	0	65	20	10	4	1
BMS//LPP/G113	0	46	50	2	2	0
After 7 days at 14°C	 N pore	 N pore	 N pore	 Mit pore	 2N pore	Dead

the pollen population put in culture for each plant (500 to 800 grains).

Increased pollen survival

Improving survival of pollen in in vitro culture increases the chances of inducing androgenesis. The cold shock given flowers before plating the anthers to induce the pollen toward androgenesis also affects the viability of the pollen in culture.

Maize pollen is extremely vulnerable and dies easily if subjected to drastic environmental changes. The cold shock which enhances androgenesis in maize is 12-14°C. The increased longevity caused by weekly increasing the temperature at which the cultures are grown, from the regular 1-week induction period at 14°C to 19°C to 23°C to the final 27°C, also has increased the number of anthers yielding embryos as much as 10% in some hybrids.

On the other hand, some substances (such as cytokinins)

are known to delay senescence. We investigated the effect

Table 4. Effect of temperature on survival of pollen in culture of Pennisetum americanum var. massue.

Temperature[a]	% Survival			
	Basal alone	Basal medium with 0.01 mg Zeatin/liter		
	10 days	10 days	15 days	21 days[b]
19°C	39	72	60	1.5
23°C	3	46	39	2
27°C	0	13	0	0

[a] Plant induced 7 days at 14°C. [b] After 21 days, proembryos>15 cells survived.

of Zeatin and Kinetin on pollen longevity in culture (Table 4). When Zeatin was added to the medium and the cultures were kept between 19 and 23°C, the number of pollen grains and the formation of proembryos significantly increased in Pennisetum americanum var. Massue (Fig. 1). However, Keeping the cultures at low temperatures does not allow enough cell division in the pollen. Therefore, it is necessary to adapt the temperature to increase the rate of cell division for good growth. At 21 days, proembryos from cultures at 19°C had 10-15 cells and those at 23°C had about 20 cells. After 10 days at 27°C, they had developed into plantlets (Fig. 2).

Inhibitory substances

The release of inhibitory substances into the culture medium is another constraint of in vitro culture. Cells that have been injured by the dissection of the organ from the plant frequently give off some toxic substances. In many cases these are polyphenols, but they also may be some healing substances of the hormonal type produced by the wounded tissue. Anagnostakis (1974) counteracted this inhibitory effect on tobacco anthers and Guo et al (1978) in maize anther culture by adding activated charcoal to the culture medium. It is also possible to minimize the effect of inhibitory substances by floating the anthers or the pollen on liquid medium for the first 24-48 hours after plating. The liquid medium then can be pipetted out and replaced by fresh medium. This method keeps a well-defined medium. Because the action of charcoal is not specific, its use creates an uncertainty about media composition.

Proembryo development

Development of the proembryo to a plant is the next step. The medium used for induction of the pollen toward andro-

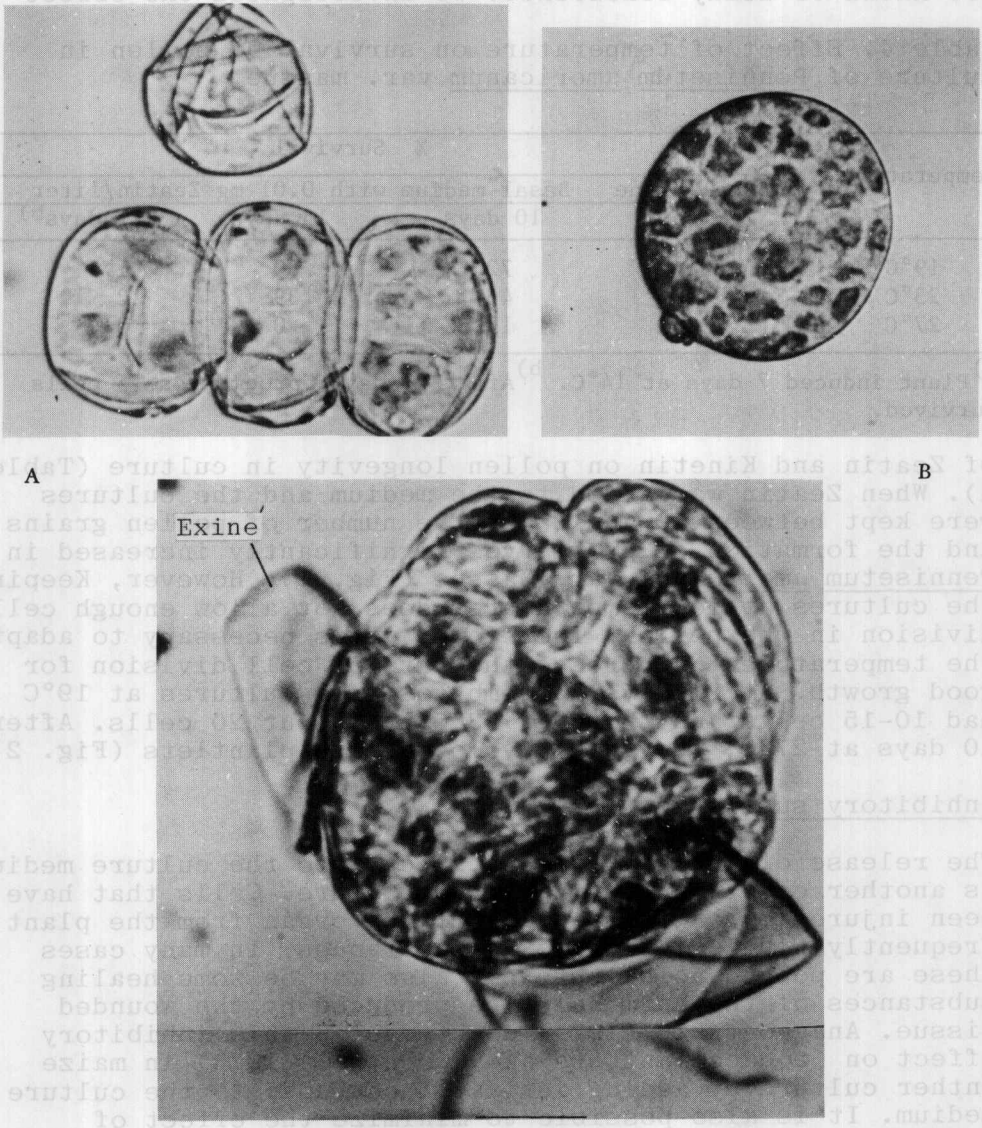


Fig. 1. Induction toward androgenesis of Pennisetum americanum var. Massue. A = 10 days in culture; B = 15 days in culture; C = 18 days in culture.

genesis might have a carryover effect on embryo development. In maize and millet, the number of plantlets produced by anther culture could be increased by lowering the hormonal and the sugar concentration of the induction medium (Nitsch et al 1980). The proembryos originating from anthers grown on high sucrose (12%) and auxins developed more callus and produced fewer plantlets.

Auxins have been known since the very early days of tissue culture to promote dedifferentiation of the cell (Gautheret 1959). Sucrose added to a medium containing auxin has a synergistic effect for the multiplication of highly vacuolated and watery large cells and the formation of callus. Production of haploid plants via embryogenesis is higher when the pressure of auxin in the medium is limited to 1 week at the most and when the amount of sucrose does not exceed 6% (Fig. 3)

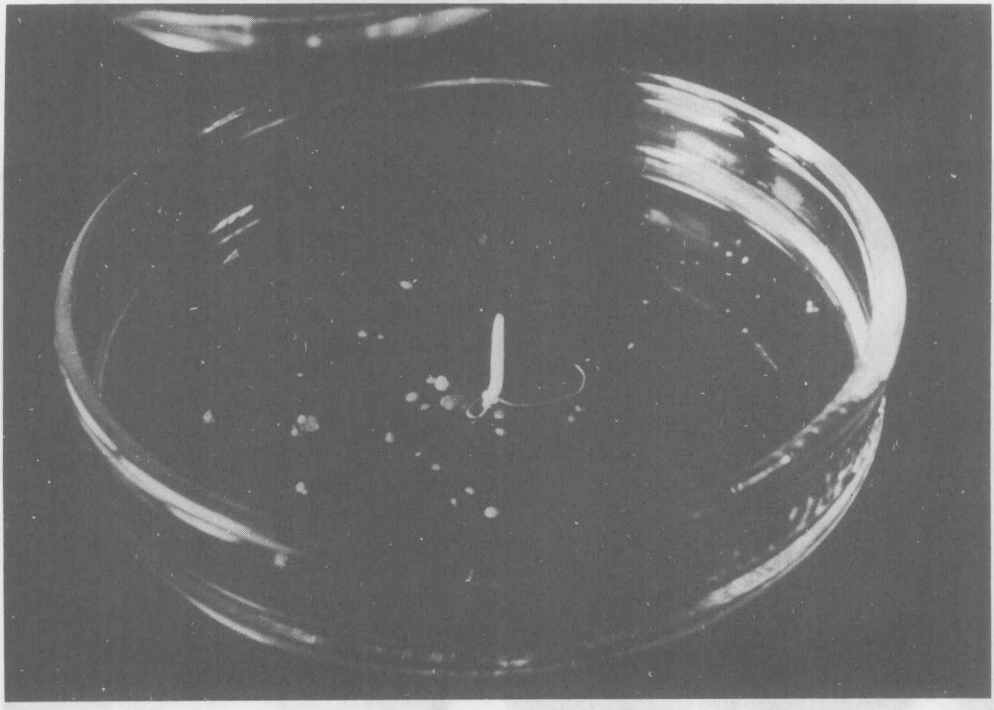
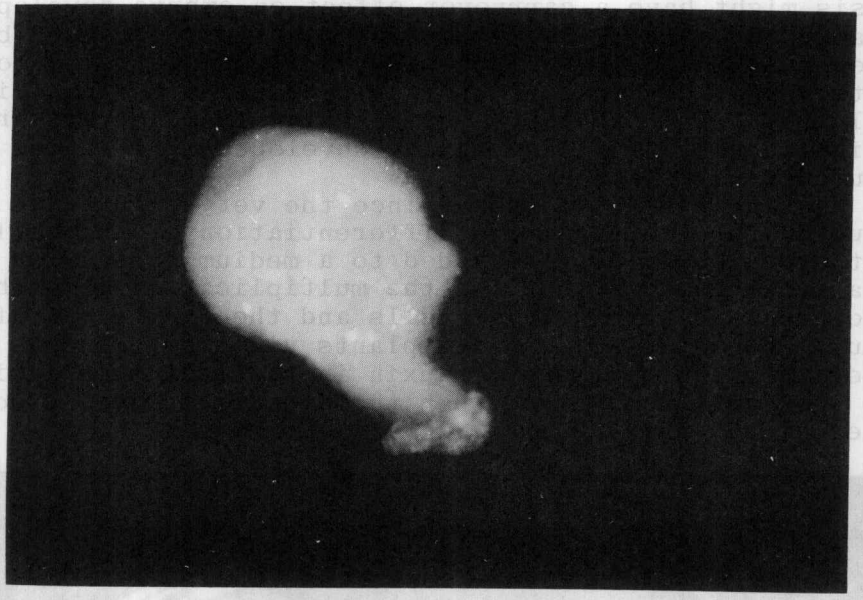
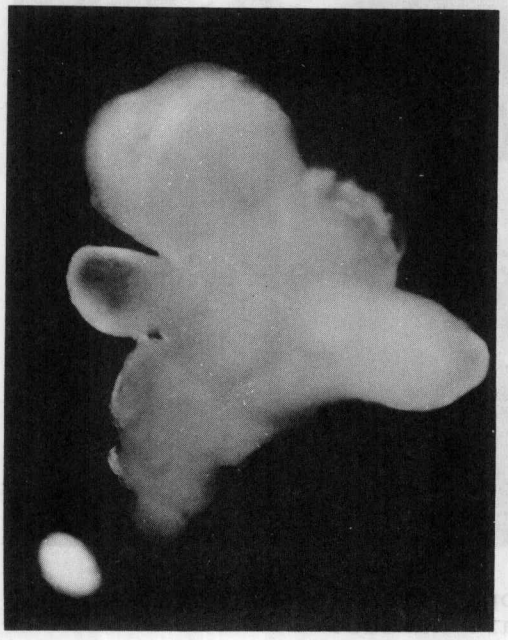


Fig. 2. Plantlet developed from proembryo of Pennisetum americanum var. Massue.

On the other hand, development of the embryo into a plant is enhanced by the presence of amino acids in the culture medium. Paris et al (1953) showed the effect of glutamine on the growth of zygotic embryos of Datura. We observed the same effect on the androgenetic embryos of



A



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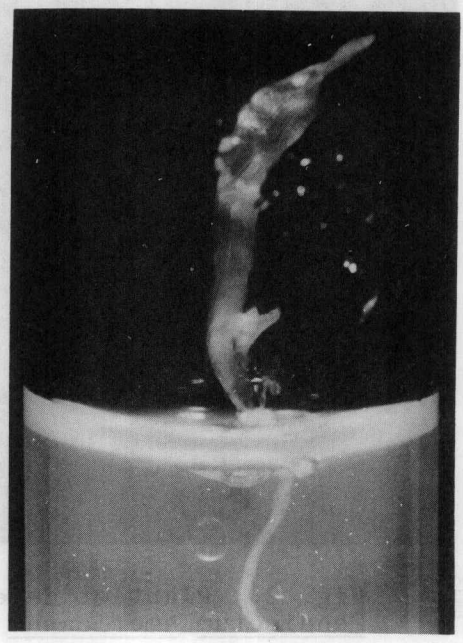

C

Fig. 3. Process of pollen embryogenesis from A = globular shape to B = heartshape to C = haploid plant of maize.

Datura, Nicotiana, maize, and millet. We also found a positive effect of L-proline in inducing embryos from maize pollen. This amino acid enhanced the development of roots

(Nitsch 1980). Casein hydrolysate is used as a source of amino acids in most laboratories. This complex has an effect on the growth of the tissue in culture. When it becomes possible to identify the specific amino acid metabolically active in the plant placed in culture, it should be possible to increase this growth response.

CONCLUSION

Despite the difficulties, most species that have been used to produce haploids through in vitro techniques respond positively. It is not too optimistic to think that androgenesis is feasible with any species, provided the technique is adapted. Better comprehension of the mechanisms involved will help fit the technique to all plants. However, adapting the plant to the method by the elaboration of specific hybrids might also be useful.

In vitro culture can allow a plant to grow at the limit of viability, thus opening a way to understanding growth and development in the plant kingdom in the same manner incubators have increased knowledge about infant life.

The use of hormones in the culture medium at a specific time might allow the creation of worthy mutants originating from callus-regenerating buds. The technique, when properly used, can be reliable for the production of normal isogenic lines. That is most important for maize breeding.

We do not think a universal method should exist for plant breeding. A choice between different techniques has to be made in terms of the cultivars. It is possible to adapt the androgenetic technique to some extent. It also is possible to introduce favorable characters into a hybrid using the F_1 for pollen culture. Several ways are open. The in vitro method is one. It shortens the time necessary for selection, produces original information on a given genotype rapidly, and creates new genotypes in vitro.

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