CELL AND TISSUE CULTURE TECHNIQUES FOR CEREAL CROP IMPROVEMENT

Proceedings of a workshop cosponsored by
The Institute of Genetics, Academia Sinica
and
The International Rice Research Institute

Science Press, Beijing, China
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Workshop on Potentials of Cell and Tissue Culture Techniques in the Improvement of Cereal Crops Beijing, 1981

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—2.0t/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

CONTENTS

REGULATION OF MORPHOGENESIS

CROP IMPROVEMENT THROUGH TISSUE CULTURE

SCREENING MUTANTS THROUGH TISSUE CULTURE

PROGRESS IN ANTHER AND POLLEN CULTURE TECHNIQUES

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, 91190 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	Protoplast	Regeneration
	Pollen-yielding plants (%)	Protoplasm division (%)	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						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 a1 (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—

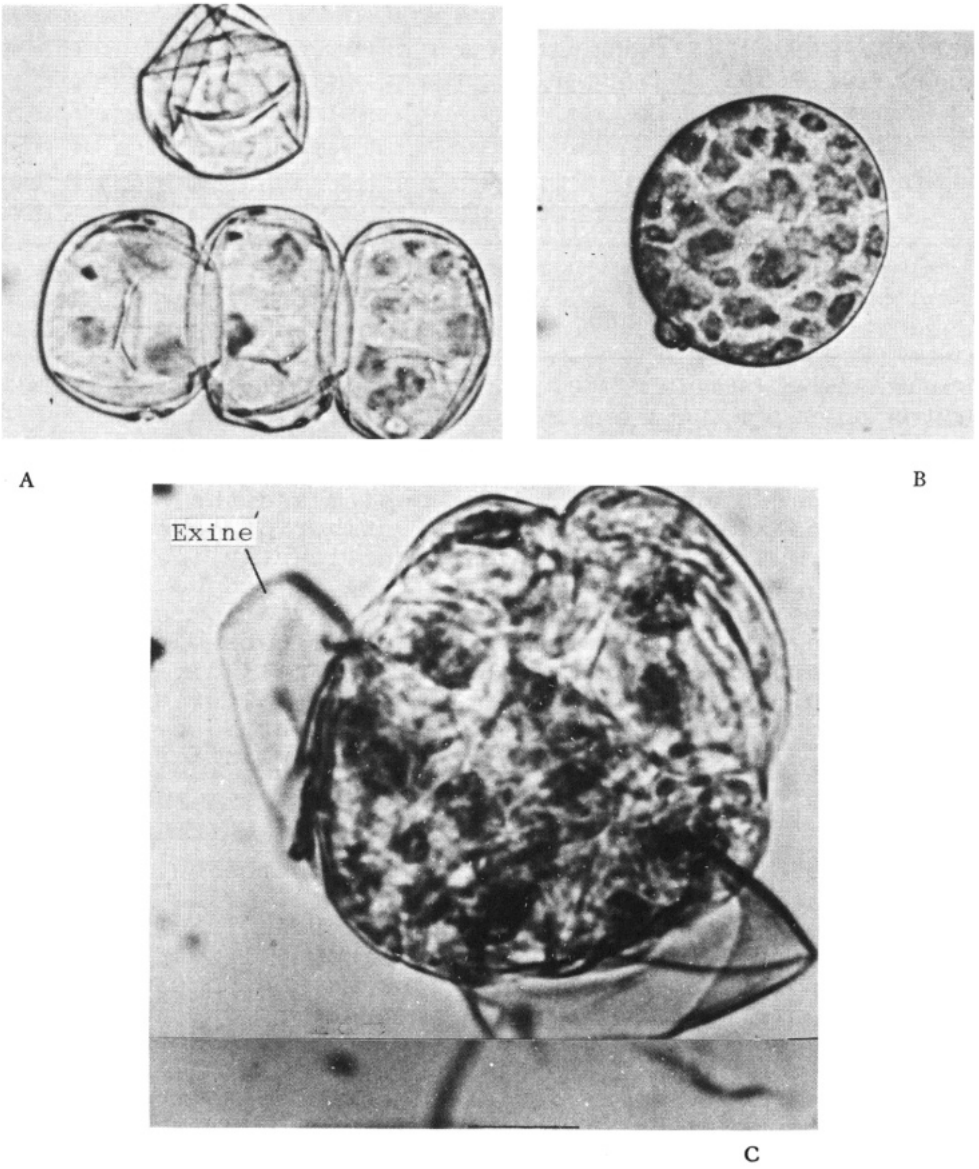


Fig. 1. Induction toward androgenesis of Pennisetum americanum var. Massue. A = 10 days in culture; B = 15 days in culture; C = 18 days in culture.

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)

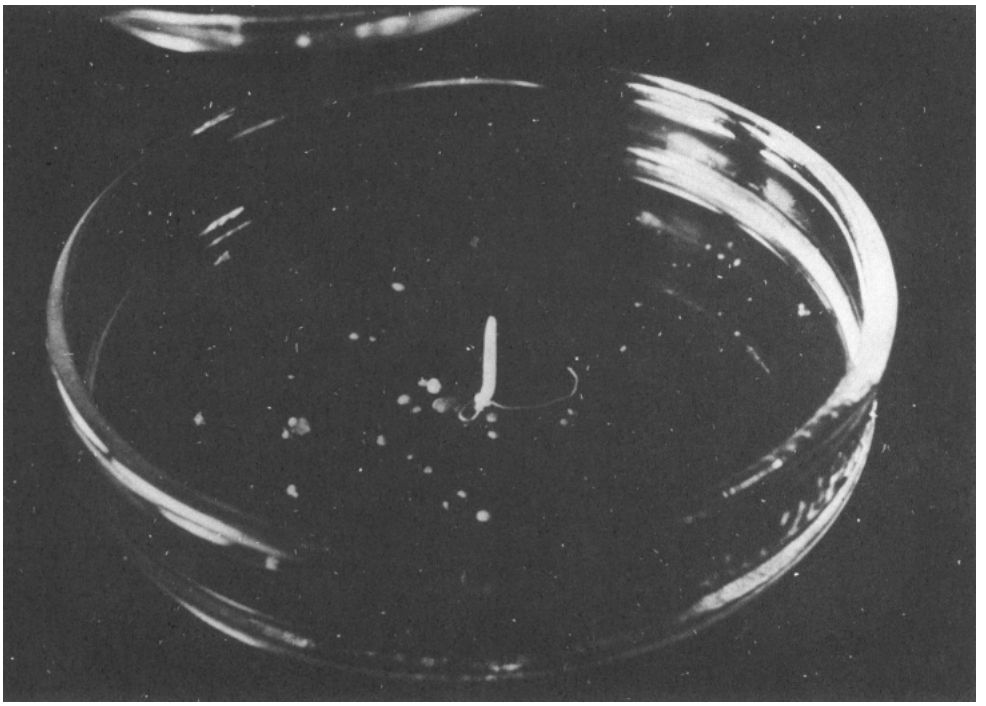
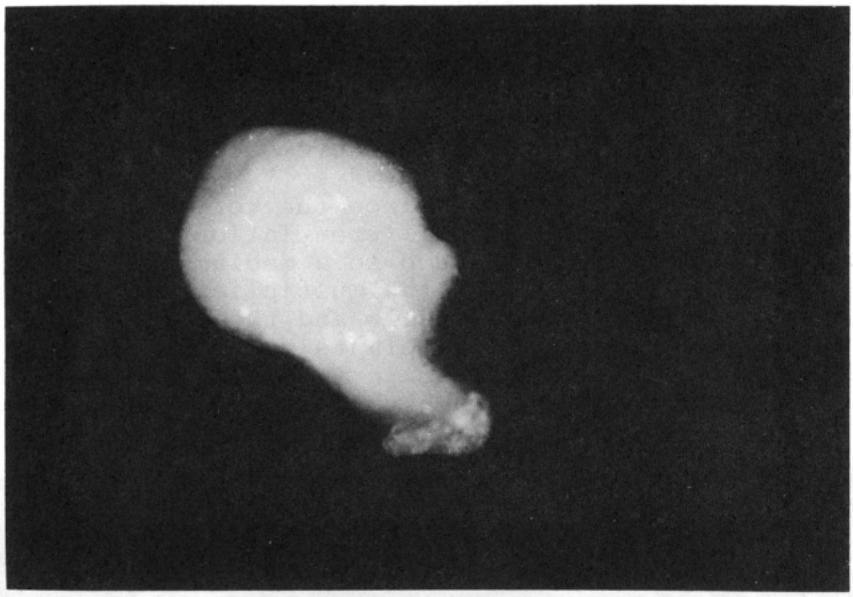
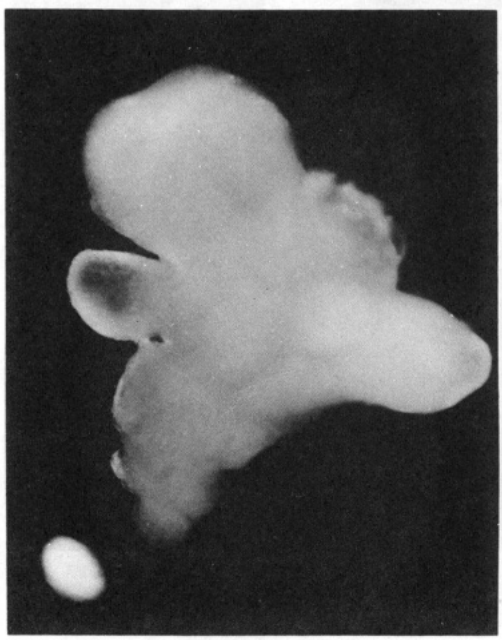


Fig. 2. Plantlet developed from proembryo of Pennisetum americanum var. Massue.

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





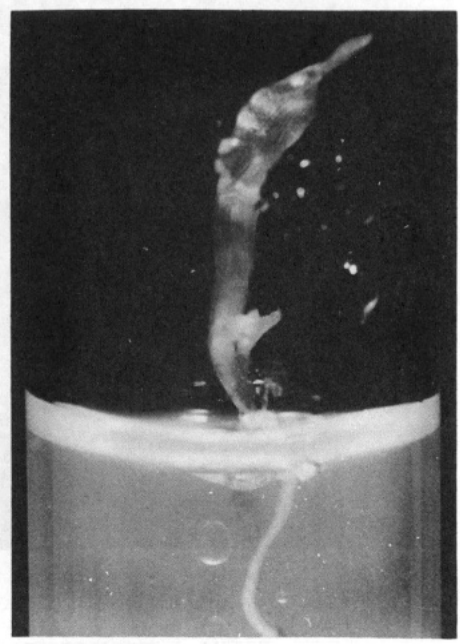


Fig. 3. Process of pollen embryogenesis from A = globular shape to B = heartshape to C = haploid plant of maize.

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 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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When available, names of scientists in China are given in the traditional Chinese order: surname followed by full given name with no comma. Given names are hyphenated, with only the first syllable capitalized. Initials of given names normally are not used.

ANTHER AND POLLEN CULTURE OF RICE IN CHINA

Chen Ying

The first successful induction of haploids from anther culture in an important food crop, Oryza sativa, (Niizeki and Oono 1968) created great interest among scientists in China. Research on rice anther culture started in 1970. Green plants derived from microspores were obtained the same year (2nd Division, 3rd Laboratory, Institute of Genetics, Academia Sinica, 1973, 1974). Since then, research has been done on culture techniques, androgenesis, and cytology and genetics of pollen plants and on their application in breeding. Some mechanical problems also have been studied.

The induction frequency of pollen plants, both in O. sativa subsp. keng (japonica) and subsp. hsien (indica), has been increased since the early 1970s so that it is now possible to generate many pollen plants for use in rice breeding programs. Through anther culture, 7 improved varieties have been developed and released in China, with production on 10,000—100,000 ha. Rice breeding by anther culture has been shown to be effective in accelerating the process of plant improvement.

This report summarizes advances in research on anther and pollen culture of rice in China since 1977.

IMPROVEMENT OF ANTHER CULTURE TECHNIQUES

Factors affecting the yields of anther culture have been investigated extensively. Many aspects of cultural techniques have been improved and induction frequency has been greatly enhanced.

Cold pretreatment of panicles

The favorable effect of cold pretreatment of flower buds or inflorescences on induction frequency has been reported for many species — tobacco (Nitsch 1974), Datura (Nitsch and Norreel 1973), barley (Sunderland 1978)) and rice (Genovesi and Magill 1979).

In anther culture of hsien rice, the frequency of green plantlet induction has been rather low. But when panicles were pretreated at 9—11°C for 14 days, induction frequency in 4 materials averaged 7.6% (number of green plantlets/100 anthers inoculated). Shan—You No. 2 pretreated

Institute of Genetics, Academia Sinica, Beijing, China.

Table 1. The effect of cold pretreatment on green plantlet formation[a].

Medium	Pretreatment at 9—11°C (days)	Anthers inoculated (No.)	Callus produced No.	Callus produced %	Differentiation: Callus transferred (No.)	Differentiation: Green plantlets No.	Differentiation: Green plantlets %	Green plantlets/100 anthers inoculated (No./100)
N_6	0(ck)	760	26	3.4	9	1	11.0	0.37
	14	5240	819	15.6	367	94	25.6	3.98
	17	760	182	24.0	93	31	33.5	8.1
	20	720	289	40.0	84	26	30.9	12.3
L_8	14	2080	872	41.9	195	89	45.6	19.0
	17	1000	361	36.1	84	34	40.5	14.6
	20	920	531	57.7	111	60	54.0	31.2

a) Material used was Shan—You No. 2 (Oryza sativa subsp. hsien) (Zhou Xiong—tao et al 1981).

Table 2. The effect of days of cold pretreatment on frequency of callus induction.

Pretreatment (days)	Anthers inoculated (No.)	Callus yield: 0—5 days pretreatment No.	0—5 days pretreatment %	6—10 days pretreatment No.	6—10 days pretreatment %	Total No.	Total %
0	450	9	2	46	10	55	12
5	450	96	21	132	29	228	51
10	450	125	28	138	31	263	58
20	150	267	178	30	20	297	198
30	300	260	87	196	65	456	152

for 20 days reached an induction frequency of 31.2% (Table 1) As many as 86 microspores within 1 anther developed into

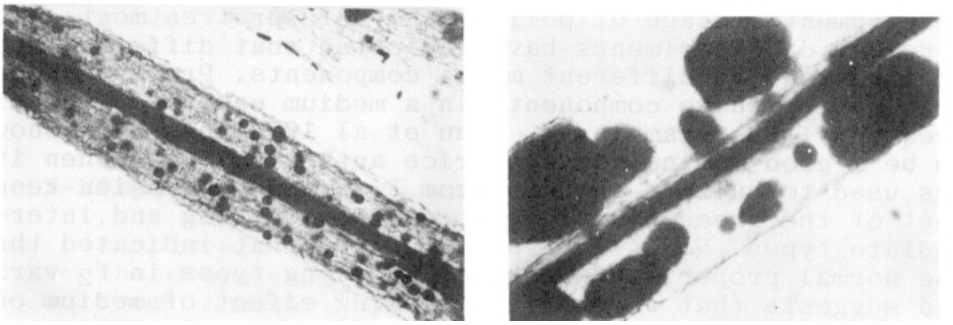

Fig. 1. 86 Microspores developed into multicellular pollen (A) and many of them developed into calli (B) with in an anther.

multicellular pollen (Fig. 1) (Zhou et al 1981). In serial culture experiments on keng rice, the best results were obtained from pretreatment at 10°C for 20 days. The induc— tion frequency of callus was 15 times more than in the con— trol (Table 2) (Chen et al 1980).

It was observed that different genotypes, such as keng rice, hsien rice, and keng—hsien hybrids, required different temperatures and pretreatment durations. Zhao et al (1980) reported that cold treatment at 6-8°C for 6 days was optimal for keng rice. Three days was optimal for hsien rice. However, when the pretreatment duration exceeded a certain limit, the induction frequency in keng rice and in hsien rice decreased markedly. The callus aged and lost its ability to regenerate into plantlets.

The part cold treatment plays in improving anther culture has been discussed in many reports (Nitsch and Norreel 1973, Sunderland and Roberts 1977, Sunderland 1978, Duncan and Heberle—Bors 1976, Takahashi 1975). Zhao et al (1980) found that during cold pretreatment, the intensity of breath and the consumption of nutrients in anthers decreased by examining the breath intensity of anthers incubated under different temperature levels. As a result, the lives of the anther wall cells were prolonged the degeneration of microspores was delayed, and the pro— portion of cultures that survived was enhanced. We found that microspores not only initiated androgenesis, but also divided several times during the course of the cold pretrea— ment of panicles (Chen et al 1981b).

Media

Certain responses for rice anther culture occur in many basic media (Miller's, 1/2 MS, modified LS, modified White's, and N_6). The effects of medium components on callus induc— tion frequency is less obvious than the effects of genotype, developmental stage of pollen, and cold pretreatment. But a number of experiments have indicated that different sub— species require different media components. Proper concen— trations of these components in a medium enhanced induction frequency. For example, N_6 (Chu et al 1978) has been shown to be a good medium for keng rice anther culture. When it was used to culture anthers from F_1 hybrids of hsien—keng, most of the green plantlets obtained were keng and inter— mediate types. No hsien type occurred. That indicated that the normal proportions of hsien and keng types in F_2 varied and suggests that a certain screening effect of medium on microspores might occur in culture (Shen et al 1978).

To screen media suitable for hsien rice and hsien/ keng hybrids, media Beh 5 (Huang et al 1978) and SK 3 (Chen et al 1978a) were developed by mathematical test. They had a better effect on induction for both hsien rices and hsien/keng hybrids. The NH_4 content, in milligram equivalent (me) per liter, of 3 media are 7 me/liter for Ng, 3 me/liter for Heh 5, and 4.76 me/liter for SK 3. The suitable level of NH4 for the hsien/keng hybrid is intermediate, between those for hsien rice and keng rice.

Yang et al (1980) established a general medium by regulating the content of major elements (using $NH_4H_2PO_4$ instead of (NH_4) SO_4 and KH_2PO_4 in N_6 medium) and supple— menting with a complex of initiation factors (NAA/2, 4—D/ kinetin = 4/1/1—3 mg/liter). Better induction results were obtained for hsien rice anther culture with this medium.

Biochemical analysis of amino acids for anthers in which the microspores are at the mid—late uninucleate stage was done by Zhou et al (1981). In general, alanine content in the anthers of hsien rice was lower than in keng and hybrid rice. Alanine content and induction frequen— cy seemed to be related. When 2—4 mg Dl—alanine/liter was added to the medium for culturing hsien rice anthers, the induction frequency was enhanced more than twice that on the original medium.

The potato medium, in which 20% potato extract was used as the main constituent to substitute for the majority of inorganic and organic compounds of a synthetic medium, had better effects on callus induction and especially on green plantlet induction from anthers and pollen (Chen et al 1978b, 1981a). To examine the available constituents of the potato medium, the extract was separated and purified by the Dextran Gel. G50 method (You et al 1980). The effects of various fractions were determined by plant tissue cul— ture methods. The results indicated that different fractions had different effects on cultures. Some of them promoted cell division and accelerated proliferation, others showed

inhibiting effects. Purification and examination of extracts with positive effects are still being conducted.

Anther float culture

Liquid media have shown better results in the anther culture of some species, such as tobacco (Wernicke and Khohlenbach 1976), datura (Tyagi et al 1979), wheat (Wei 1980), and barley (Xu et al 1981). Anther float culture also was used to increase the induction frequency of keng rice (Chen et a1 1979). Recently, success in hsien rice was achieved (Zhu De—yao et al 1980).

In our work, we found that the induction frequency of callus could be increased greatly by float culture (the maximum mean number of callus per anther has been as high as 8). But the differentiation frequency of callus was far

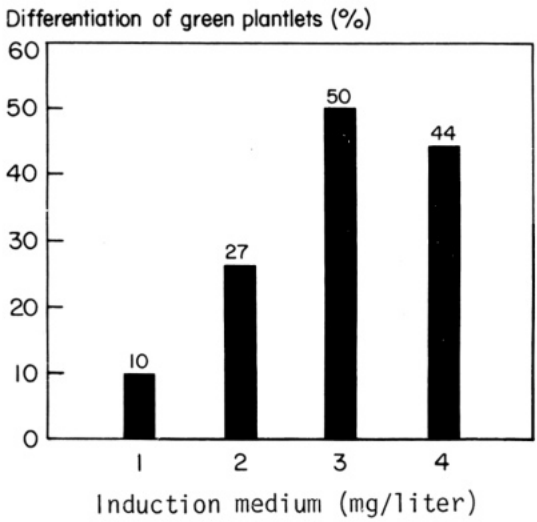

Fig. 2. Effect of potato medium on differentiation of callus in float culture. Induction Media (mg/liter):
1 = N_6 + 2,4—D 2 + LH 500 + 6% sucrose
2 = N_6 + Ser 50 + Arg 50 + Gln 50 + Asp 50 + 2,4—D 2 + 3% sucrose
3 = 20% potato extract + KNO_3 1500 + $Ca(NO_3)_2 \cdot 4H_2O$ 100 + Fe—EDTA 40 + 2,4—D 2 + 3% sucrose
4 = N_6 + 2,4—D 2 + LH 500 + 6% Sucrose + 0.58% agar
Differentiation medium (mg/liter):
MS + IAA 2 + K 1 + 3% sucrose + 0.58% agar

lower than when a solid medium (agar) was used. When several media were tested for increasing differentiation in float

culture, induction media were more influential than differentiation media in determining the differentiation ability of callus. The best results in callus differentiation was obtained with the potato medium sterilized by filtration (Fig. 2). Differentiation of green plantlets was as high as 50% (Tian and Chen Ying 1981).

Pollen plantlets induced directly from anthers

Microscopes of wheat and rice can be induced to plantlets directly through the embryoid without developing a callus in anther culture (Ouyang et al 1973, Chu et al 1976). However, a rather low response has made it unsuitable for plant improvement. To raise the frequency of plantlets regenerated directly from pollen, Lin et al (1980) carried out a series of studies on the coordination of proportions of auxin and kinetin. When 2,4—D was decreased to 0—0.1 mg/liter and kinetin and NAA were increased to 2—3 mg/liter in the induction medium, microspores could develop and differentiate into plantlets directly instead of by the development of calli from cultured anthers. Microscopic observation indicated that these regenerated plantlets might have developed both from embryoids and from calli. The induction frequency of green plantlets was 4% in keng rice and less than 1% in the hybrid of hsien—keng.

More recently, a similar result was obtained through anther float culture (Fig. 3) (Chen et al 1982). Although the frequency of plantlets regenerated directly from microspores is still very low, I believe it is possible to raise the response by further improving the method.

CULTURE OF ISOLATED POLLEN

Since 1974, when Nitsch made her report on pollen culture at the Guelph Conference, many workers have thought that pollen culture might substitute for anther culture. But so far, except for several species of Solanaceae, only limited success has been achieved in rice (Chen et al 1979, 1980, 1981a). The obstruction could be attributed to too little understanding of factors influencing microspore in vitro culture for induction of development.

Intact green plantlets were obtained from pollen culture in 1979 (Fig. 4) (Chen et al 1980). Recently, we have been investigating the preparation of isolated pollen, media, androgenesis, and the factors affecting microspore initiation and development. We found that few cultured microspores could initiate androgenesis when they were isolated from fresh anthers and placed onto the culture medium. When the panicles were treated at temperatures of about 10°C for 10—15 days and the anthers were precultured on the medium for 3—4 days before the isolation of pollen,

the amount of pollen with embryogenesis ability increased. Some pollen could develop and differentiate into green plantlets in subsequent cultures.

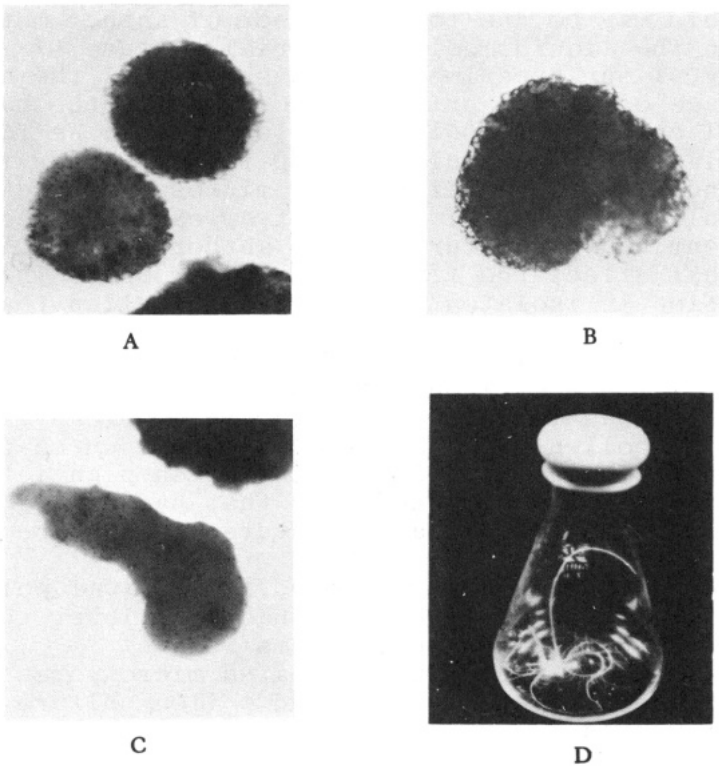

Fig. 3. Pollen plantlets induced directly by anther float culture: globular embryo (A), heart-shaped embryo (B), germinative embryoids (C) and then plantlets (D).

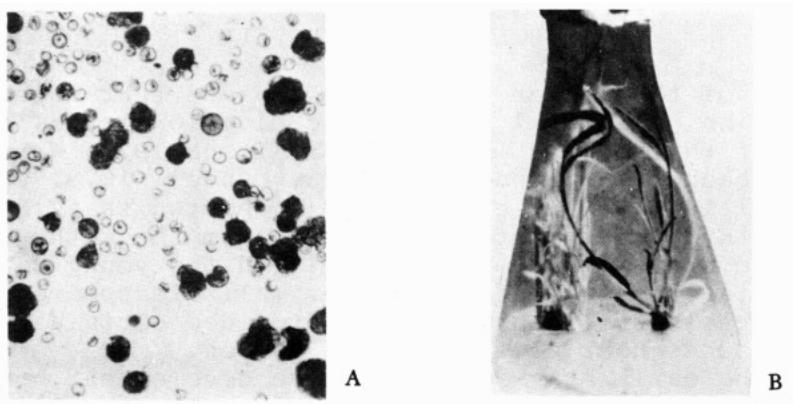

Fig. 4. Many small calli(A) and intact plantlets (B) obtained from isolated pollen cultures.

For the preparation of isolated pollen grains, shed culture (Sunderland and Roberts 1977) or serial culture (Tyagi et al 1979) was found to be much better than mechanical methods (Nitsch and Norreel 1973, Zheng et a1 1978) for induction of pollen callus and pollen—derived plants. This may be due to the effect of anther metabolites present in the liquid medium of serial culture.

Many of our experiments have shown that the develop—mental stage of pollen prior to isolation is the basis for successful culture of isolated pollen grains. We found that only initiated pollen could develop further under in vitro culture and that the initiation of microspores had to take place within the anthers. So far it appears that cold pretreatment and preculture of the anther are the main factors that affect the frequency of microspore initiation and induction in isolated pollen grains. Pollen that had undergone preculture for 5 days could form a multicellular mass and callus. During this period, part of the pollen within the anthers divided twice or more.

It also was found that the initial sporophytic divi—sion of rice pollen might occur during the course of cold pretreatment of panicles. Callus and green plantlets were obtained only from pollen cultures that had undergone cold pretreatment. The role of a preculture might be replaced by cold pretreatment.

Although success was achieved in isolated pollen cul—ture in rice, induction frequency has been lower than that in anther culture. Two main problems remain:

- Efforts to induce uninucleated microspores to initi—ate development in isolated pollen culture have not succeeded.
- The differentiation frequency of callus produced from pollen culture is low in general and the proportion of albinos among the plantlets differ—entiated is relatively high.

ROLE OF ANTHER WALL

The tissue of the anther wall plays an important role in the induction of initial sporophytic division in pollen development (Nitsch and Norreel 1973, Nitsch 1974, Wernike and Kohlenbach 1976, Sunderland 1978, Dunwell 1978, and Tyagi et al 1979). Sung et al (1979) reported that in rice anther culture, metabolites that came from the anther wall entered the anther chamber and enveloped the young callus. After the tapetum cell of the anther wall disappeared, remaining cells still survived and provided certain metabo—lites for subsequent development of pollen.

At the early stage of microspore development, the microspores and the cells of the anther wall were found to be connected by a tubelike structure (Fig. 5). When the

microspores developed to the globular embryo stage, a sus—pensor—like or multilayered cell bar also adhered to the anther wall (Fig.6)(Chen et al 1982). In the culture of isolated rice pollen, experimental results indicated that

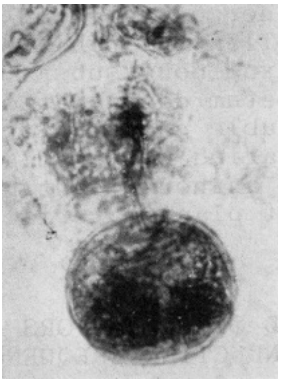

Fig. 5. Microspores and cells of the anther wall connected by tubelike structures.

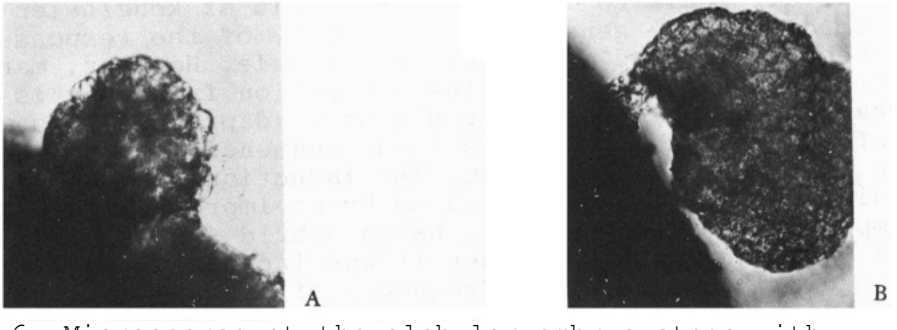

Fig. 6. Microspores at the globular embryo stage with suspensor—like (A) or multilayered cell bar (B) adhering to anther wall.

only initiated pollen could develop further in vitro. In addition, the initiation of microspore dedifferentiation had to be within the anther. Certain metabolic products of anther tissue were necessary for further sustained division and proliferation of pollen (Chen et al 1981a). In anther float culture, the callus derived from the pollen released by anthers showed a loose structure with little ability to differentiate into plantlets. The callus from pollen developed within the anther were fine and compact in structure (Chen et al 1980). This also was considered to be related to anther wall factors.

To demonstrate the mechanism of initiation of dedif—ferentiation in the microspore during anther culture of

rice and wheat, Liang et a1 (1980) and Zhong et a1 (1981) studied changes in anther wall tissue and their effects on dedifferentiation of the microspore. Experimental results suggest that the anther wall might provide some essential nutritional substances to pollen cells for their dedif— ferentiation during anther culture and play a role as metabolite pools for pollen through absorption, storage, and transformation of exogenous substances from the culture medium. The time of tapetum degeneration and the electro— phoretic pattern of soluble proteins from anther wall tissue on polyacrylamide gel varied with media and materials cul— tured. The responses of anthers might be due partially to the effects of different plant materials and different media on the anther wall.

GENOTYPE AND OTHER FACTORS INFLUENCING INDUCTION FREQUENCY

The response of microspores to anther culture is determined to a great extent by the genotype of the materials. In gen— eral, keng rice often gives good induction results. Hsien rice is difficult to induce and a hybrid of keng/hsien is intermediate. The genetic heterogeneity of the response has been considered a formidable obstacle. However, many experiments have indicated that induction frequency is not unchangeable. Some varieties had wide adaptability to medium constituents and higher induction frequencies whereas others were sensitive to the medium. The induction frequency of sensitive varieties was enhanced by an improved medium. For example, Shan—you No. 2 is a hsien hybrid showing high induc— tion frequency. However, when it was incubated on a differ— ent medium, the induction frequency of green plantlets va— ried from 12.3 to 31.2% (Table 1).

Some researchers have indicated that the proportion of albinos among plantlets derived from microspores was gene— tically determined(Oono 1975, Chen and Lin 1976). The proportion of albinos was related to genotype, but it also was related to certain constituents of the medium and es— pecially to incubating temperature. A high concentration (such as more than 10 mg/liter) of 2,4-D or NAA used as the initiation factor enhanced the frequency of albino plantlets. Higher temperature was never favorable for differentiation of green plantlets. When inoculation temperature exceeded 30°C, the majority of plantlets differentiated from callus were albinos (2nd Division, 3rd Laboratory, Institute of Genetics, Academia Sinica 1974, Wang et a1 1977).

CONCLUSIONS

Researchers in several laboratories in China regularly gen—

Table 3. Callus induction and green plantlets from rice anther culture [a].

Year	Combination (No.)	Anthers inoculated (No.)	Callus induced (%)		Green plantlets differentiated (%)		Green plantlets/ 100 anthers inoculated (No./.100)
			Av	Max	Av	Max	
1977	16	5682	6.40	20.20	13.71	22.42	0.88
1978	16	9571	36.48	72.30	19.06	51.61	6.94
1979	32	36719	44.70	76.80	22.88	71.10	10.23

[a] Material used was Oryza sativa subsp. keng (Ge Mei—fen et al 1981).

erate green pollen plantlets in large quantities with the comprehensive application of selected techniques to in vitro anther culture. For instance, Zhang et al (1981) inoculated 65,000 anthers derived from 14 hybridization combinations of keng rice. They obtained an average callus induction frequency of 16.93% and a differentiation of green plantlets frequency of 37.3%. Five to ten green plantlets from a hundred inoculated anthers, an average of 6.3%, can be obtained.

Ge et al (1981) reported that the induction frequency of green plantlets averaged 10.2% from anthers of 32 keng rice hybrids inoculated in 1979. Table 3 shows the con— tinuous improvement in induction frequency in the last 3 years.

The induction frequency of green plantlets in hsien rice was lower than 1% for a long period. Zeng et al (1980) has succeeded in increasing the induction frequency to 1.9% by improving the medium. Zhou et al (1981) increased the induction frequency in hsien rice to 7.9% by using cold treatment and an improved medium.

We are confident that these improved anther culture techniques can be applied to crop improvement.

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RICE ANTHER CULTURE AT IRRI

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Developments in plant tissue culture techniques offer possibilities of introducing into plants variability that could be utilized for crop improvement. Haploids, with their unique genomic constitution, have potential for accelerating the production of homozygous new varieties.

The production of rice haploids and subsequent homozygous diploid plants by in vitro anther culture has advanced dramatically in the last 15 years. But the application of this technique to the improvement of rice varieties has been hampered by the difficulty of inducing morphogenesis, either directly from cultured microspores or indirectly from callus derived from microspores.

Also, the callus produced frequently loses its plant regeneration ability with time in culture, making studies on selection of cell mutants difficult when longer periods of in vitro culture are required. Another difficulty is that, when cultured in vitro, all rice varieties do not respond equally in producing callus and in regenerating plants. This could be due to genetic or environmental characteristics of different varieties (Niizeki and Oono 1968, Guha—Mukherjee 1973).

Despite these difficulties, scientists in China have succeeded in generating new rice varieties from pollen—derived plants of F_1 sexual crosses between japonica and indica subspecies (Hu 1978). In some cases, these plants combined characteristics of each parent which were better than the parent cultures, such as high tillering capacity and desirable panicle and grain characteristics (Chen and Li 1978, Hu and Hao 1980).

Rice anther culture at the International Rice Research Institute (IRRI) began in September 1979. Its main purpose is to evaluate aspects of the application of rice anther culture to varietal improvement.

The immediate objective is to identify rice varieties with anther culture capacity (ability to regenerate callus and plants). For this purpose, a wide diversity of rice germplasm from IRRI has been screened:

- to study the stage of pollen development that triggers embryogenesis processes,

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- to study the effect of cold shock in improving the efficiency of pollen grains going through the em—bryogenic process,
- to compare media as well as different media components
- to determine the best environmental conditions for optimum callus and green plant regeneration, and
- to study possible variability among plants regenerated from anthers.

From this initial research, rice varieties were clas—sified as high, medium, and low callus and green plant producers. These classifications led to long—term objectives:

- to study the sexual inheritance of high, medium, and low callus and green plant production,
- to study long—term calli for plant regeneration to screen for resistance to stress factors, and
- to culture anthers from F_1 plants of specific crosses to establish F_2 populations composed of true—breeding individuals.

This paper describes initial findings from studies of rice varieties, both japonica and indica subspecies, and sexual hybrids obtained from the germplasm bank at IRRI (Table 1). Varieties were screened for their capacity to produce callus as well as to regenerate plants. Taipei 309 was selected as a model system. Unless otherwise stated, current studies have been carried out only on this rice subspecies.

Factors such as stage of microspore development, phys—ical pretreatment of the anther before culture, and genotype of the anther donor plant have been measured. A comparison between anther responses using a liquid medium or one solid—ified with agar also was done.

The rice varieties tested were classified into high, medium, and low callus and plant regeneration producers (Table 2). Media and such media components as growth reg—ulators were varied. Natural complexes such as coconut milk and yeast extract also were added to media.

A preliminary study evaluating the agronomic character—istics of regenerated plants has been initiated.

RESPONSE TO ANTHER CULTURE

Stage of pollen development

The pollen stage has been found critical for the successful triggering of proembryo initiation and haploid plants from pollen grains. Guha—Mukherjee (1973) studied 20 rice vari—eties. She pointed out that yound rice anthers at the tetrad or earlier stage failed to develop and that only anthers containing uninucleate microspores showed differentiation into pollen embryoids. According to Chen (1977), the maximum

response of Tainan 5 occurred at the mid—uninucleate stage. The calli recovered from differentiating pollen grains at early and mid—uninucleate stages showed an excellent capac— ity to regenerate green plants, with a micimum number of albino plants. Calli arising from microspores in the late uninucleate stage appeared less capable of plant regenera— tion. When pollen was at first mitosis, only albino plants were recovered.

Niizeki and Oono (1968) cultured anthers containing mature pollen grains 1 or 2 days before heading. Callus formation occurred in anthers of some rice varieties, although their efficiency was very low.

The best pollen development stage for producing cal— lus in rice was studied by Sun (1978). In anthers inocu— lated at the binucleate stage, most pollen grains died. In anthers inoculated at the early, mid—, and late uninu— cleate stages, the rates of viable pollen grains were relatively higher.

At IRRI, we used 500 anthers of selected rice varie— ties containing pollen at different stages of development.

Table 1. Indica and japonica rice varieties tested for their callus and plant regeneration capacity.

IR5	IR10781-75-3-2	Leb Mue Nahng III
IR8	IR11248-131-3-2-2	Leng Kwang
IR20	IR13146-41-3	M7
IR24	IR13240-39-3	Mahsuri
IR26	IR13292-5-3	Mingolo
IR28	IR13419-113-1	Moosa Tarum
IR30	IR13426-19-2	MR10
IR32	Anlong Phnom	Nong Baek
IR34	B2266B—CW—16—2—1	Pai—kan—tao
IR36	Beak Ganges	Patnai 23
IR38	BKN BR1031-7-5-4	Pelita I—1
IR40	BRRI Sail	Peta
IR42	Cul. 854	RP1064-2-2
IR44	Cul. 956	Sathra 278
IR46	Cula	Silewah
IR520-126	D66	SML Temerin
IR2797-125-3-2-2-2	Double Dwarf 1	Suduwee
IR4215-27-3-2-2	Double Dwarf 3	Sukamandi
IR4422-98-3-6-1	Dudmona Barisail	Sungail
IR4432-52-6-4	Dumsiah 81	Taichung 65
IR4683-54-2	ESD 7—1	Tainan 5
IR5657-33-2	Giza 170	Taipei 309
IR5853-118-5	Goda Heenati	Tatsumi mochi
IR8073-65-6-1	Gowa 38—13	Tetep
IR9129-209-2-2-2-1	IET 5656	TN1
IR9264-324-1	Kuatik Putih	Zenith
IR9764-45-2-2	Kurkaruppan	

Those plated on culture medium between the mid—uninucleate and the early binucleate stages showed better response than those plated at early or late stages of development. This confirmed earlier results. We concluded that for successful induction of androgenetic development, rice anthers should be cultured between the mid—uninucleate and early binucleate pollen stages.

A correlation was found between the morphological features of the panicle and the stage of the pollen grains. Panicles were collected from the field when the distance between the auricle of the flag leaf and the subtending leaf was 4—8 cm, depending on the variety. At this flag leaf distance, pollen grains were between the mid—uninu—cleate and the early binucleate stages. Anthers containing pollen at a later stage than the binucleate were less cap—able of being developed, possibly because the accumulation of starch grains in the late stages which hampers pollen cell division.

Table 2. High, medium, and low callus and green plant producing rice varie—ties.

High	Medium	Low
Taipei 309	IR30	IR20
Minehikari	IR40	IR38
BG90—2	Moosa Tarum	IR8
Taipei 177	Taichung 65	IR36
Nong Baek	Pelita I—1	IR28
	Mingolo	BR51—91
		Sathra 278

Effect of cold shock

While pollen stage is critical for callus and plant produc—tion, total callus production has been too low to be of any significant use in basic or applied research. To overcome this difficulty, physical treatments such as cold shock have been used (Nitsch 1974, Sunderland and Roberts 1979).

O. sativa Dungham shali anthers cold—shocked at 6°C for 5—daysreached callus induction efficiencies of 22.2% of the number of treated anthers plated, compared to 10.5% when anthers were not treated (Chaleff et al 1975). Anthers cold—treated at 10°C for 4 to 8 days produced efficiencies up to 10%, compared with 2% in nontreated anthers (Hu et al 1978). The rate of call-us and green plant production of Norin 21 was 68% when the panicles were treated for 14 days at 13°C, compared to 18% without treatment (Genovesi and Magill 1979). Chaleff and Stolarz (1981) studied factors that in—fluence the frequency of callus formation in Minehikari. The frequency of callus production reached 41% when panicles were

maintained at 7°C for 3 days or more, compared with 31% when anthers were not treated.

In our experiments, the best response was observed when panicles containing anthers were cold—shocked for 8 days at 8°C(Table 3). Callus production increased 50%. Although efficiency also was high after 14 days of treatment, the number of albino plants increased.

Table 3. Effect of cold shock on Taipei 309 anthers.

Duration of cold shock (days)	Anthers			
	Plated (No.)	Producing callus		With multiple callus (No.)
		No.	%	
0	377	20	5.3	19
2	345	18	5.2	15
4	346	14	4.0	11
8	348	36	10.3	23
12	325	16	4.9	7
14	337	30	8.9	17
16	325	17	5.2	13
18	329	25	7.6	10

When anthers were plated on either semisolid or liquid media, the response of cold—shocked anthers was higher than that of anthers plated on solid media and the efficiency in number of anthers producing multiple calli increased.

One problem in obtaining multiple callus (calli derived from different pollen grains) in a semisolid medium is that, if we were working with hybrid pollen grains, the callus produced would mix and we would obtain chimeric plants. To control that possibility, we used a liquid medium in which the anthers float freely. Floating anthers plated directly on the culture medium were cold—shocked for 8 days at 8°C. The effect on their efficiency in producing callus were obvious immediately. Proembryos derived from individual pollen grains developed, burst, and produced small pieces of callus which in turn dropped into the liquid medium, allowing the development of other pollen grains.

From this experience, we can say that efficiency is reduced when semisolid medium is used, possibly because of impurities in the agar itself which are harmful to the pollen. The leaking of inhibitors produced by the anther sac as well as by dead pollen grains could also stop further pollen development. In a liquid medium, these effects are minimized because compounds that inhibit further development are diluted.

Cold treatment alters pollen grains four ways:

- It triggers the pollen mother cell to produce two

identical nuclei instead of one vegetative and one generative nucleus.

- It maintains a higher percentage of viable pollen, slowing down the senescence of somatic tissues.
- It triggers the production of specific proembryo inducers; and
- It synchronizes the cells.

The result is a greatly increased number of pollen grains that can produce proembryos.

CULTURE MEDIA

Callus production efficiency

Among the many factors that affect the ability of pollen grains to produce calli and subsequently plants is the culture medium. Research groups have been involved in the search for a suitable medium to such an extent that most of the known media have been used, either as first formulated or with slight variations. Most researchers agree that the source and amount of total nitrogen, as well as the kind of amino acids and the use of complex compounds, are important. Growth regulators such as 2,4-D have been important in some studies of callus production but often were found not necessary for plant regeneration.

Niizeki and Oono (1968) used the basic semisolid Blaydes medium (1966), which is Miller's (1963) containing high concentrations of indole-3-acetic acid (IAA), 2,4-D, and kinetin (K). Calli were regenerated after 4 to 8 weeks in culture at a 0.57% efficiency. A few green plants as well as albinos were recovered when the calli were cultured in Blaydes medium containing high concentrations of IAA and K.

Chu (1978), studying factors that affect callus and plant production from rice anthers, developed the N-6 medium He found that rice anthers were sensitive to the total nitrogen concentration. A low nitrogen concentration (490 mg/liter) was beneficial for callus production whereas a higher concentration was inhibitory. The callus induction frequency of the medium was 16.29% and green plant production based on anther number was 57%.

Chaleff and Stolarz (1981) used a modified Murashige and Skoog medium (1962) supplemented with NAA to produce shoots and roots from rice anther callus. However, only 35% of the plated anthers produced callus.

It appears that the total amount of nitrogen affects the growth of pollen cllus, possibly by inhibiting the enzymes in the Kreb's cycle.

We decided to use basic Gamborg's (1968) medium, which contains 26.72 mM total nitrogen compared to 60 mM nitrogen in Murashige-Skoog and 35 mM nitrogen in N-6.

The modified semisolid or liquid Gamborg's medium J-19

used contained 20g sucrose/liter, 160 mg inositol/liter, 1 mg Kinetin/liter, and 1 mg NAA/liter with 5.6 pH. Medium E-24 contained 20g sucrose/liter, 160 mg inositol/liter, 0.5 mg benzylaminopurine (BAP)/liter, 0.5 mg NAA/ liter, and 1 mg 2,4—D/liter, with 5.6PH. Semisolid media were gelled with 8g agar/liter.

Twenty to thirty days after the anthers were exposed to light (1000 lux) in either a semisolid or liquid medium, some anther sacs split longitudinally and multiple proembryo-like structures were visible (Fig. 1). Twenty-five percent of the anthers plated on J—19 medium produced highly embryogenic calli. After 40 to 50 days initial exposure to light, multiple green shoots were visible (Fig. 2). Plant regeneration efficiency was 40%. Twenty-three percent of the anthers plated on E—24 medium produced either multiple or single calli. Pieces of callus 1 cm in size were transferred to J-19 and 53% regenerated into plants.

Multiple calli derived from different pollen grains were difficult to individualize. Because the main objective of our program is to work with hybrid anthers in which individual pollen grains carry different characteristics, we decided to use our two best media in liquid form. The advantage of liquid medium, conclusively shown by many researchers (Wernicke and Kohlenbach 1976, Sunderland and Roberts 1979), are:

- Competition among the developing proembryos is alleviated. Once embryos develop, they drop into the liquid medium and allow the growth of other pollen grains;
- In many instances, the deleterious compounds produced by killed pollen diffuse into the liquid medium, diminishing their harmful effects; and
- Liquid medium facilitates the exchange and penetration of nutrients.

These advantages should bring about an increase in the total efficiency of developing proembryos.

Table 4. Callus production of Taipei 309 anthers plated on semisolid and liquid media.

Medium		Anthers					
		Plated (no.)	Producing callus		With multiple callus		Callus Production	
			No.	%	No.	%	No.	%
J-19	Liquid	507	-	-	-	-	1644	324.3
J-19	Semisolid	515	127	24.7	120	23.3	-	-
E—24	Liquid	177	-	-	-	-	1201	678.5
E—24	Semisolid	384	87	22.7	87	22.7	-	-

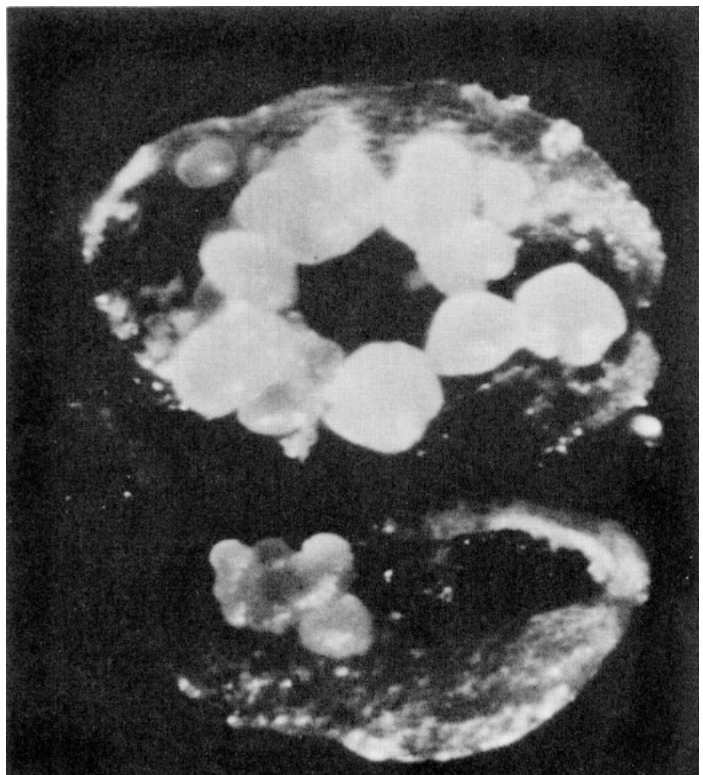


Fig. 1 Longitudinally split anther sacs showing multiple proembryo—like struc—tures.

Fig. 2. Multiple green shoots developed after 40—50 days exposure to light.

There were striking differences in the response of rice anthers cultured in liquid media and those cultured in semi—solid media (Table 4). More than 1,000 pieces of callus were recovered from 177 anthers plated in E—24medium, an effi—ciency of 675.5% of the anthers plated compared with only 22.7% when the same medium was solidified with agar. Effi—ciency also was improved with J—19medium, where 1,644 callus were obtained out of 507 anthers plated, with an ef—ficiency of 324.3% compared to only 25% when the same medium was solidified with agar (Fig. 3). Proembryos were not only de—rived from small pieces of callus but also developed into more advanced forms of embryogenic processes. These proem—bryos further developed into plants which developed to maturity.

When pieces of callus about 1 mm in size were picked out of the liquid medium and placed on semisolid J—19 or semisolid Murashige—Skoog medium (1962) containing 3% sucrose and 1 mg NAA/liter and 1 mg Kinetin/liter as growth regula—tors (N—19),many turned brown and died. But, when they were placed individually on top of a paper bridge wetted with N—19,small pieces of callus developed and differentia—ted into shoots.

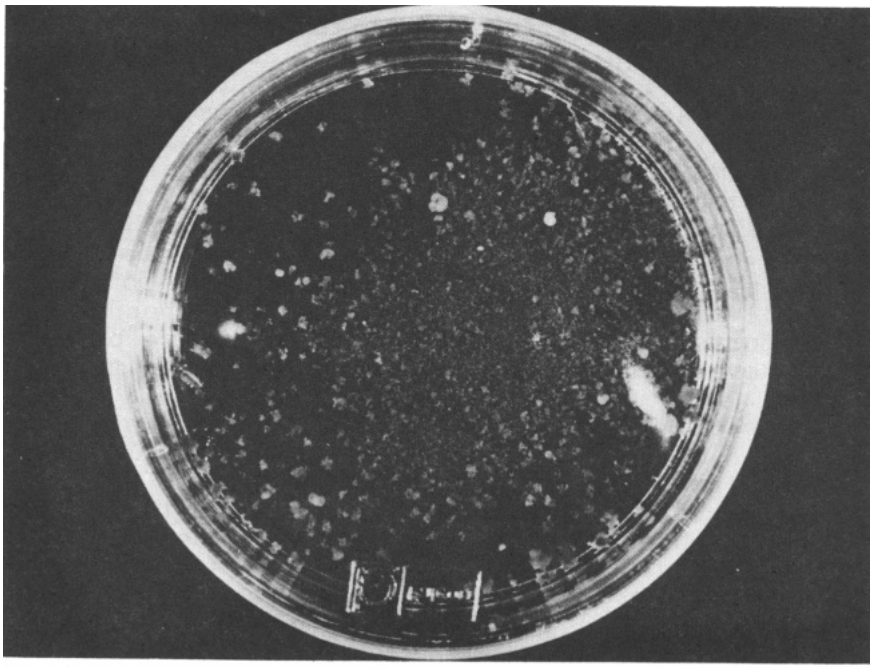
Coconut water as a growth regulator

To increase further the efficiency of callus produced from anthers, we experimented with medium J—19 supplemented with 4—16%coconut water. The efficiency of callus—producing anthers was low compared with the medium without coconut water (Table 5).

Table 5. Effect of coconut water on callus production (Taipei 309 anthers, J-19 medium).

Coconut water (%)	Anthers plated (No.)	Calli produced (No.)	Induction frequency (%)
0	507	1644	324.3
4	197	563	285.3
8	109	299	274.3
12	160	396	247.5
16	136	352	258.8

Nitsch and Godard (1978) observed that growth regula—tors were required when tobacco and Datura free pollen cul—ture cells enclosed in the exine were dividing. When the formed proembryos were free from the exine, amino acids were necessary for further development. This was not the case in our floating—anther method, suggesting that the tapetum



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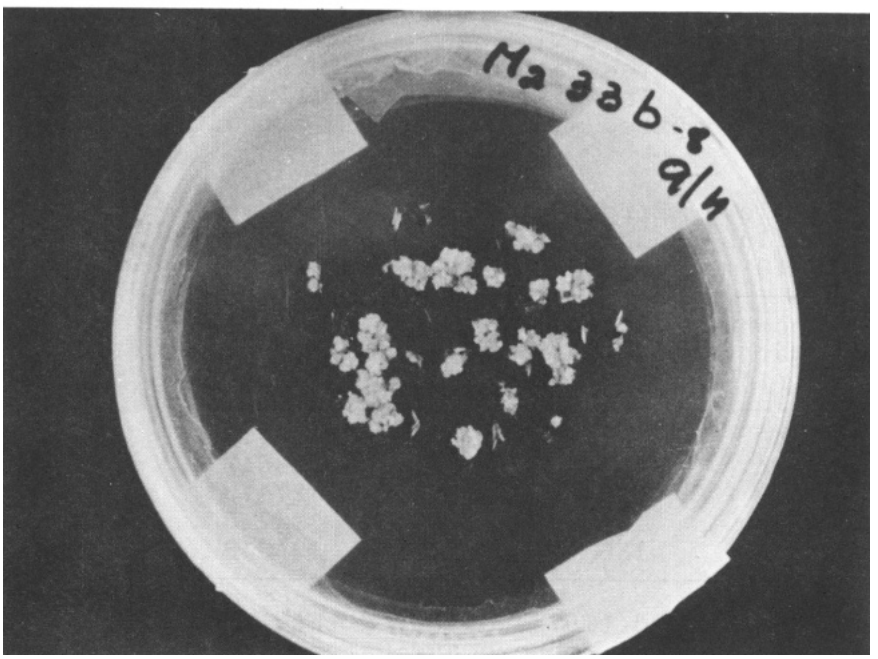

Fig. 3 A. Many proembryos developing in liquid medium. B. Few individual growing units (calli) derived from plated anthers.

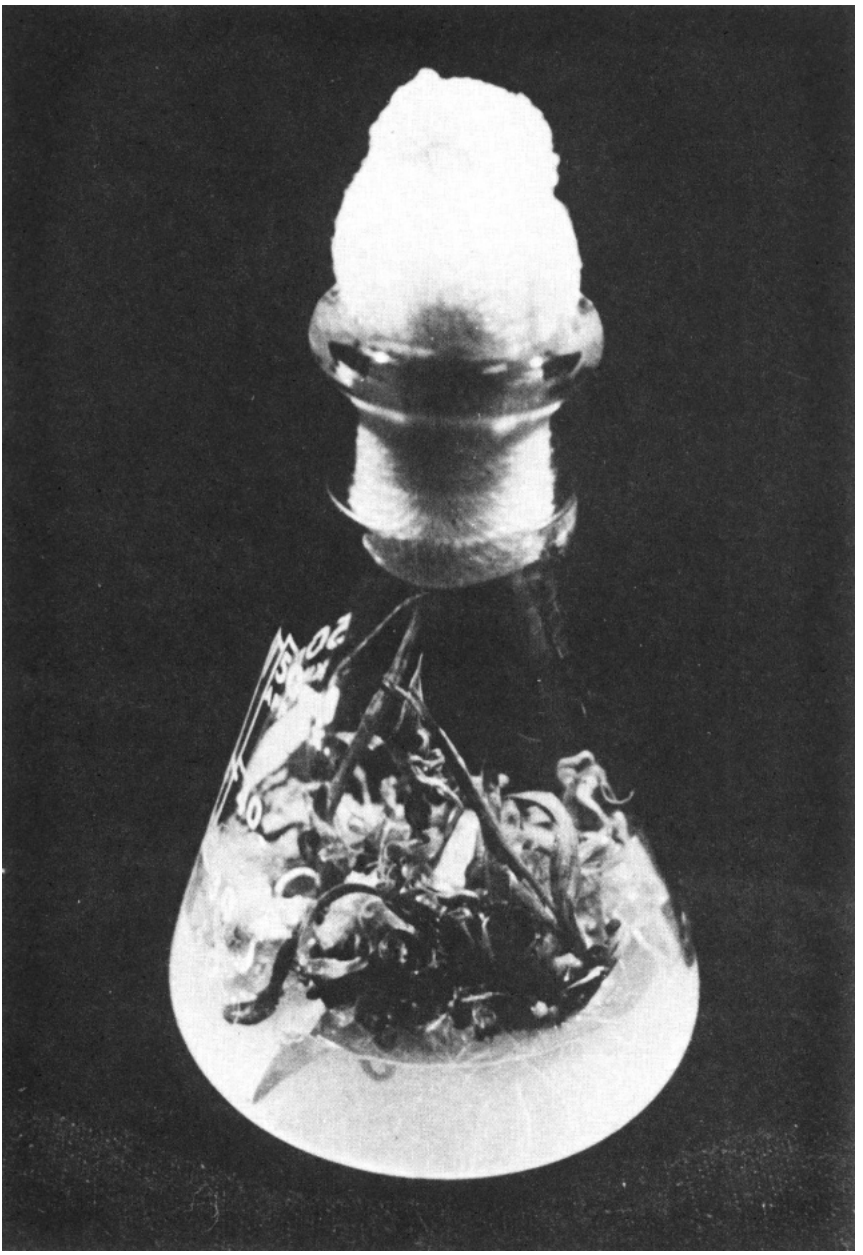

Fig. 4. Multiple shoots developed from calli.

tissues were active in producing the necessary factors from the degenerating tapetal cells or that the total concentrations of amino acids in the coconut water were more than optimum for a positive response. Wernicke and Kohlenback (1976) have suggested that one factor could be glutamine.

Plant regeneration efficiency

If anther culture techniques are to be useful in a rice breeding program, it is necessary to induce reproducible plant regeneration. Although many researchers (Niizeki and Oono 1968, Guha—Mukherjee -1973, Hu 1978) have succeeded in regenerating plants from anther callus, efficiency has been low. Chaleff and Stolarz (1981) did get high plant regeneration from callus derived from anthers of japonica variety Minehikari.

Using Taipei 309 as the model system, we found a high number of androgenic pollen grains which later were transformed into callus. The next step was to learn how to trigger plant morphogenesis from the calli obtained.

Many media were tested with callus derived from J-19 and E—24liquid media. Callus chosen at random, including J—19 and N—19, were plated in different regenerative media. Individual pieces of callus derived from individual pollen grains were placed on top of a filter paper wetted with a medium. In J-19 and N—19, some pollen—derived callus regenerated plants but others did not. The buds of those that did not regenerate plants were transferred to the same medium solidified with 0.8% agar. Then calli developed further and produced multiple shoots (Fig. 4). In some instances, we recovered up to 11 plants derived from 1 pollen grain. This could have resulted from the cloning of individual haploid genotypes to produce twin plants. Studies on phenotypic characteristics are under way.

In other instances, green and albino plants regenerated from the same piece of callus, possibly because of a mutation. When calli derived from J-19 were placed on semisolid J-19 or N-19 containing 10g yeast extract (J—19Y_1, N-19Y_1)/liter the efficiency of plant regeneration was reduced. Efficiencies up to 10.1% were reached in J-19 (Table 6).

When calli derived from E—24were plated on J—19or J-19 Y_1, plant regeneration efficiency was nearly 4 times more than that from calli derived from J—19. When calli produced from E—24were plated on N—19or N-19 Y_1, green plant production was more than 100 times higher than from J-19—derived calli.

The explanation could be that, in addition to requiring a more complete medium (in this case Murashige—Skoog), the medium in which the callus was formed (in this case E—24) is important. E—24contains the growth regulator 2, 4—D, which induces cell proliferation. But a condition that favors

growth usually suppresses differentiation. However, 2,4-D accelerates shoot production in rice by increasing cytokin in synthesis (Inoue et al 1979, Yao and Krikorian 1981).

Table 6. Plant regeneration from rice variety Taipei 309 anthers on semisolid media derived from liquid media.

Plating medium	Regeneration medium	Calli plated (No.)	Plants regenerated			
			Green		Albino	
			No.	%	No.	%
J19	J19	69	7	10.1	2	2.9
	$J19Y_1$	37	2	5.4	0	0
	N19	41	0	0	2	4.9
	$N19Y_1$	36	1	2.8	3	8.3
E-24	J19	18	7	38.9	1	5.6
	$J19Y_1$	14	4	28.6	3	21.4
	N19	26	3	11.6	7	26.9
	$N19Y_1$	14	58	414.3	33	235.8

If that is the case, rice callus derived from anthers on E-24 medium with 2,4—Dcould have a higher plant regenera— tion efficiency when transferred to either J-19 or N-19 medium because of the balance of auxin—cytokinin necessary for shoot induction. Using this method, we have produced callus from 48 varieties and sexual hybrids (Table 7). We have recovered more than 1,000 green plants from 20 va— rieties, including japonica and indica varieties, and about 100 plants from F_1 sexual crosses (Table 8) which include F_1 sexual hybrids of high and low callus and plant regen— eration producers as well as other hybrids with economic importance.

ALBINO PLANTS

Besides the green plants recovered, production of albino plants was high. It seems that albinism is a widespread phenomenon in Gramineae plants obtained through in vitro techniques. Sun et al (1979) demonstrated that albino polle plantlets in rice have lost the capacity to synthesize fraction I protein due to an alteration of the chloroplasts before the formation of the vegetative and generative cells

Table 7. Varieties and F_1 sexual crosses that have produced anther callus.

Indica	Japonica	Hybrid
Anlong Phnom	Baegogna	Baegogna/ IR48
BG90—2	Giza 170	BR51—91
ESD 7—1	Jado	IR4683—54—2/Cul.854
Hunan 72	Krosnodorski	IR5853—162—1—2—3
IR8	Kuban 3	IR54
IR20	Kwan Fu 401	Mingolo/D66
IR28	Minehikari—US	Silewah/IR8
IR30	Nong Baek	Silewah/Taichung 65
IR32	Pai Kan Tao	Silewah/Taipei 309
IR40	Sathra 278	Taipei 309/IR36
IR10781—75—3—2	Taichung 65	TP 309/IR20
IR8973—65—6—1	Tainan 5	TP 309/IR30
IR9264—364—1	Taipei 177	Tatsumi Mochi/IR48
Leb Mue Nahng III	Taipei 309	
Mahsuri	Taipei 309—AC	
Mingolo		
Mingolo—AC		
Moosa Tarum		
Pelita I		
Taichung Sen Yu 165		

We found two types of albino plants, true albinos and viridescent albinos. When viridescent albinos were sub—merged in a liquid medium (N-19 or J-24). some reverted to green color and the plants matured and set seed. This suggests the possibility that, in some cases, physiological changes rather than genetic changes had occurred.

GENETIC VARIABILITY

Preliminary studies on the variability of plants recovered from anther culture have been initiated. Variability ob—served in seed producing plants of Taipei 309 generated from anther culture include spreading panicles (Fig. 5), small round grains (Fig. 6), big grains, and tiller with three panicles (Fig. 7) and awns. Variability in plant height, panicle number, panicle length, and sterility has

Table 8. Varieties and F_1 sexual crosses in which anther culture—regenerated plants have produced seed.

Japonica	Indica	Hybrid
Baegogna	BG 90—2	IR4683—52—2/Cul. 854
Giza 170	Hunan 72	Mahsuri/IR2061—213/ IR2061—213
Jado	IR30	
KwanFu 401	IR40	Mingolo/D66
Minehikari	Leb Mue Nahng III	Silewah/Taichung 65
Nong Baek	Mingolo	Taipei 309/IR20
Paikan tao	Mingolo AC—2	Taipei 309/IR30
Taichung 65	Moosa tarum	Taipei 309/IR36
Tainan 5	Taichung Sen Yu 195	
Taipei 177		
Taipei 309		
Taipei 309 AC—2		

Table 9. Some agronomic differences observed in Taipei 309 tissue culture—generated plants grown in the phytotron (21/29°C, 95% RH).

	Plants (No.)	Plant height (cm)	Panicle (No.)	Panicle length (cm)	Sterility (%)
Haploid	107				
Range		34—121	2—50	8.1—29	100
AV		76.6	14.9	14.6	100
Diploid	82				
Range		46—125	1—29	11.1—29	0—50
Av		90.7	7.9	19.5	3.6
Seed plant	6	134	14	25.7	0

been shown (Table 9).

This variability could be due to environmental stress during in vitro growth or to genetic changes. Experiments to determine whether variability is genetic or physiological are under way.

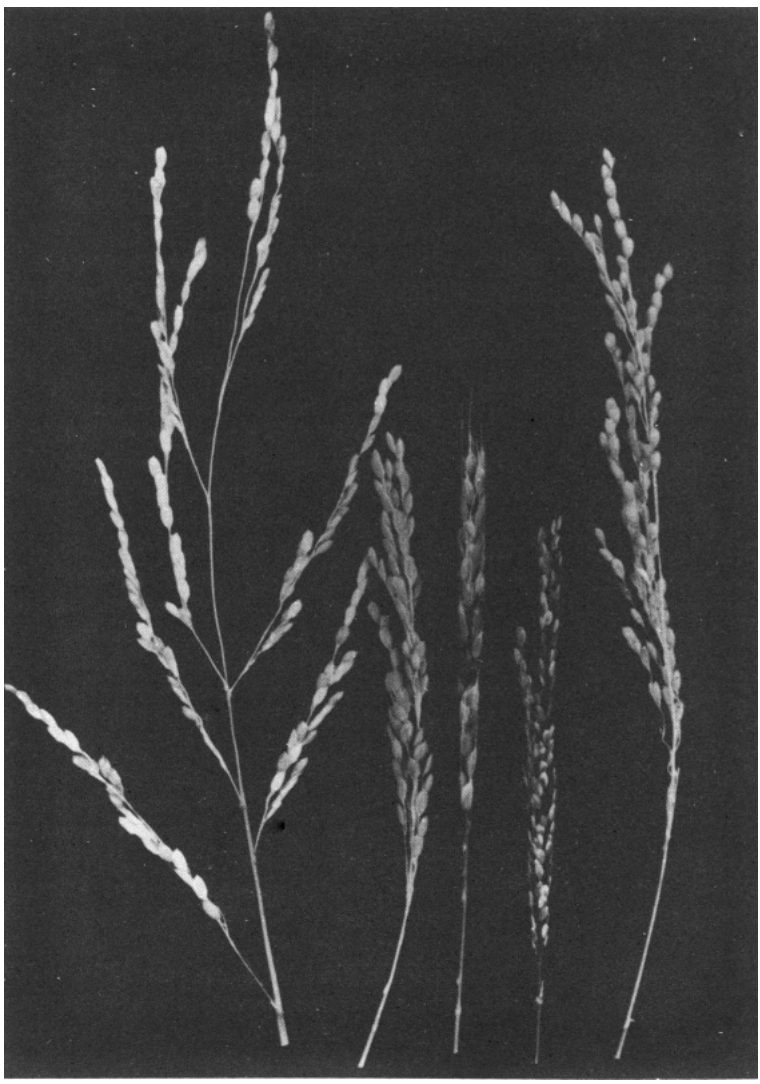

Fig. 5. Spreading panicle and other pan— icle types among Taipei 309 anther culture—generatedplants.

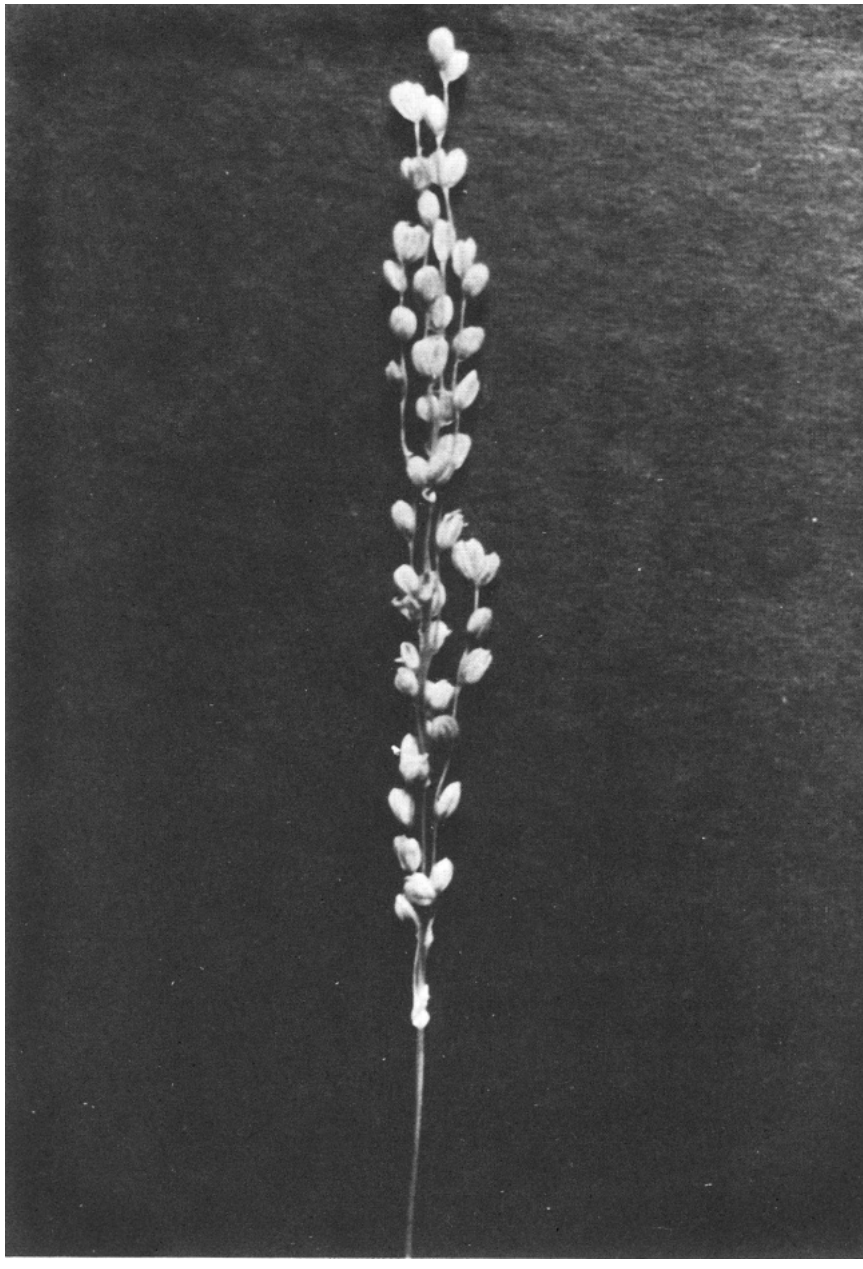

Fig. 6. Round grains from one of the Taipei 309 anther culture—generated plants.

Fig. 7. Plants with a single tiller giving rise to three panicles.

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MICROSCOPIC OBSERVATION OF HEVEA BRASILIENSIS CULTURES

Cheng Zheng—hua

Pollen plants have been obtained from more than 110 species of cereals (Chu 198l), vegetables (Wang et al 1977, Chung et a1 1977), economical crops, and trees (Chang 1977, Chen et a1 1980). Rut anther culture techniques for many species have not yet been developed. The development of a pollen grain into an embryoid and then a plantlet is a complex pro— cess, with factors and complicated interactions between fac— tors affecting its success.

Hevea brasiliensis Muell. —Arg. is a perennial tree belonging to the Euphorbiaceae. Production of pollen plants by anther culture takes 6 — 7 months, 4 times longer than for cereals and Solanaceae.

The Institute of Genetics of Academia Sinica, in co— operation with the Boating Institute of Tropical Crops and the Hainan Institute of the Rubber Tree, obtained intact pollen plantlets of rubber trees in February — March1977 (CAS — GRI — HRS et al 1977). The yield of pollen embryoids and plantlets (Fig. 1) was increased markedly by optimizing the composition of the medium and cultural conditions. The his— tological and cytological observations of cultures we made have played an important role in the establishment of anther culture techniques for Hevea (Chen et al 1978, Chen et al 1979). This method also helped in the development of anther culture techniques for sugarcane (Chen et al 1980) and the enhancement of induction frequency of pollen embryoids of rape (Chen and Chen 1980).

This paper reports the systematic histological and cytological investigations that helped establish anther culture techniques, using Hevea as an example.

Some calli were obtained in 1974, 1 year after beginning our research on the anther culture of rubber tree. A large— scale inoculation using MS medium supplemented with 2 mg kinetin/liter, 2 mg 2,4—D/liter, 3% sucrose, and 0.7% agar— agar resulted in a great number of calli (Chen et a1 1981).

Microscopical investigations of cultures showed that the somatic tissues of anthers proliferated vigorously, with a frequency of callusing anthers as high as 80 — 90%.But the microspores could not develop.

We thought that it might be necessary to depress the callus formation of somatic anther tissues to promote the development of pollen grains into callus or embryoid. More

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than 30,000 anthers were inoculated on media with compositions that could depress somatic tissue. Histological investigations 25 days after inoculation showed that the somatic tissues senesced without any proliferation. The microspores also could not develop into calli or embryoids on such media.

We designed new media with compositions that could promote the development of somatic tissue to a certain degree, but not its overgrowth, and at the same time could promote the development of pollen grains. On one such medium, the frequency of anther callus formed was 40—60% and 10—20% of the microspores developed into multicellular masses (Fig. 2). Histological investigations of callusing anthers showed that the pollen grains developing into multicellular masses were in close contact with the somatic cells around them. More pollen in close contact with somatic cells developed into multicellular masses than did those not in close contact (Table 1).

Table 1. Role of somatic cells in the development of microspores.

Site of pollen	Pollen observed (No.)	Multicellular masses		Undeveloping and exhausted pollen grains	
		No.	%	No.	%
In close contact with somatic cells	129	60	47	69	53
Not in close contact with somatic cells	227	12	5	215	95

Histological observations showed that, as a rule, environmental factors exerted their influence on microspores by way of calli of the anther wall. Callus formation of the anther wall seems to be a prerequisite for formation of pollen embryoids. But only those somatic calli that were in a particular state of metabolism had a positive effect on the development of pollen grains into embryoids.

We must distinguish between the callus formation of somatic tissues and the start and development of microspore into embryoids. At the same time, we must see the close relationship between them. Histological investigations of anthers revealed the close dialectical relationship between somatic cells and microspores in the process of Hevea anther culture.

Defining the right moment to transfer the callusing anthers to a differentiation medium is another example. We found that more multicellular masses derived from pollen cracked the pollen wall after 30 days of culture. Small embryoids began to form in the callusing anthers. Systematic microscopic observation of callusing anthers cultured on the dedifferentiation medium for different periods of time showed

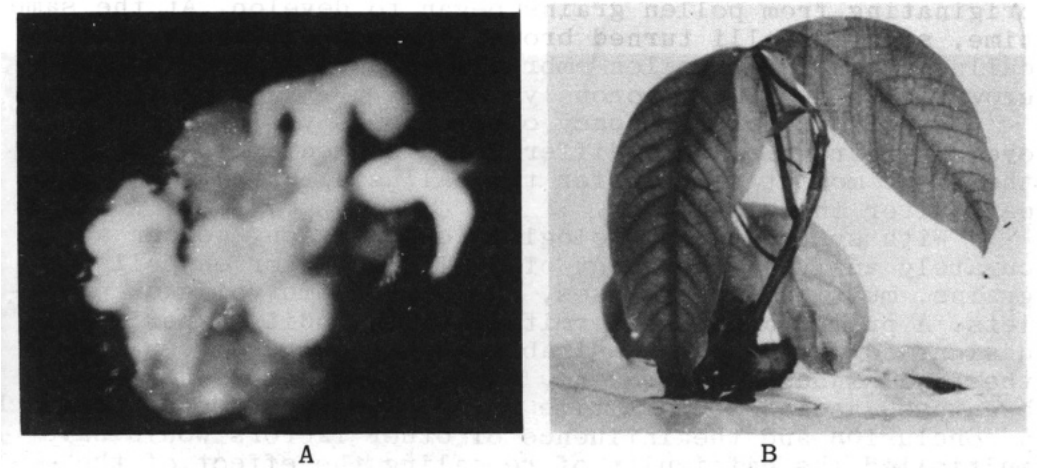

Fig. 1.Pollen embryoid (A) and plantlet (B) regenerated by anther culture from Hevea brasiliensis Muell. —Arg.

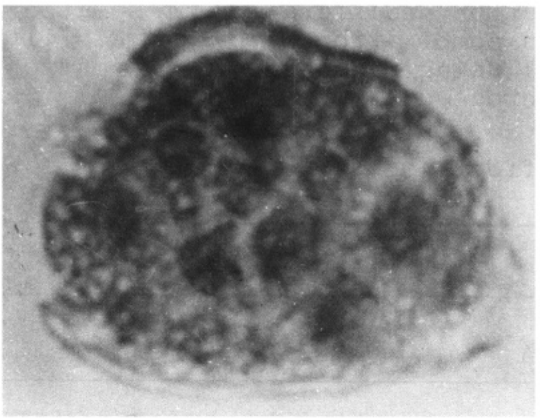

Fig. 2. Multicellular mass developed from close contact of pollen with somatic cells during anther culture of Hevea brasiliensis.

that there is a cycle of prosperity and decline of somatic tissues. In 10 callusing anthers cultured for 25 days, 80% of the mitotic metaphase plates revealed a diploid chromosome number of 2n=36. This demonstrated the vigorous growth of calli originating from somatic tissues.

But in a sample of 142 metaphase from 10 callusing anthers cultured for 50 days,only 12.17% had 36 chromosomes. The majority (67.5%) had 18 chromosomes. Of the rest, 15.5% had 19 to 27 chromosomes and 4.3% had 28 to 35 chromosomes.

It became clear after 50 days culture on the dedif— ferentiation medium that the medium had become unfavorable for proliferation of somatic tissues and calli and embryoid

originating from pollen grains began to develop. At the same time, somatic calli turned brown. In such outwardly senile callusing anthers, pollen embryoids and calli started to grow and to develop vigorously.

The induction frequency of embryoids visible to the eye after transfer to a differentiation medium showed that the right moment to transfer the callusing anthers was 50 days after inoculation.

With systematic histological observation, we can accurately analyze the effect of a single factor on pollen grains, multicellular masses, calli, and embryoids or plantlets. A prompt analysis permitted us to modify experimental designs to obtain more desirable anther culture results. If the final induction frequency of embryoids or plantlets had been used as index, more time would have been needed to reach a conclusion and the influence of other factors would have multiplied the difficulty of revealing the effect of the factor under study.

For example, in the study of the effect of a—napthalene acetic acid (NAA) on dedifferentiation of pollen grains we found a positive effect on proliferation of somatic tissues when the acid was used in combination with 1 mg KIN/liter and 1 mg 2,4—D/liter. Callusing frequency and formation of multicellular masses increased.

Table 2. Effects of NAA on formation of multicellular masses.

Treatment	Pollen observed (No,)	Multicellular masses No.	Multicellular masses %
With NAA	730	149	20.4
Without NAA	795	84	10.6

In the studies of multicellular masses in callusing anthers by the smear method (Table 2), we added NAA to the medium used for inoculation and obtained more multicellular masses than in the past.

Histological investigations on callusing anther smears were also conducted in a study of the effect of low—temperature treatment before inoculation on the development of microspores. The results showed that low—temperature treatment of the inflorescence before inoculation affected the development of callus from pollen grains but not that from somatic tissues (Table 3) (Chen et al 1981).

Microscopical observations of cultures are easy and quick ways to draw conclusions about the effect of a single factor on anther culture.

The process of anther culture changes constantly. Systematic observations of the histological structure of callusing anthers and of the chromosome number of their cell

Table 3. Effect of low-temperature pretreatment on induction of multicellular masses.

Treatment	Pollen grains observed (No.)	Multicellular masses		Multinuclear pollen		Undeveloping pollen grains		Exhausted pollen grains	
		No.	%	No.	%	No.	%	No.	%
Without storage	703	149	21.2	63	9.0	221	31.4	270	38.4
About 11°C, 24 hours	610	42	6.9	35	5.7	333	54.6	200	32.8
26-29°C, 24hours	677	122	18.0	71	10.5	215	31.8	269	39.7

Table 4. Chromosome number of metaphases of embryoid cells and plantlet root-tip cells.

Cultures	Cultures observed (No.)	Metaphases (No.)	Chromosome counts							
			9	18	20	24	27	32	36	45
Embryoid	46	238	9	190	0	0	36	1	1	1
Plantlet root-tips	18	145	6	47	1	1	85	1	3	1

reveal that change.

The determination of chromosome numbers in embryoids, test plantlets, and transplanted pollen plants was carried out systematically. A successive increase in chromosome number in the developing pollen embryoids, plantlets, and transplanted pollen plants was found.

Table 4 shows that with the culture technique employed, embryoids arose from pollen cells. Among 238 metaphase plates from 46 embryoids analyzed,79.8% had 18 chromosomes and 15.1% had 27 chromosomes. A few cells had 9 chromosomes and a few had more than 27 chromosomes (Fig. 3).

Root tips from 18 plantlets were studied. Of 145 mitotic metaphases analyzed, 32.4% of the cells had 18 chromosomes, 58.6% had 27 chromosomes and 4% had 9 chromosomes. Three plates revealed aneuploid numbers of 20, 24, and 30 chromo—somes and 2 cells had 36 and 45 chromosomes, respectively. Chromosome number tended to increase during culture from embryoids into plantlets.

Table 5. Chromosome number of metaphases of transplanted plants.

Plants (No.)	Plants height (cm)	Leaves (No.)	Metaphases (No.)	Chromosome count 9—17	18—27	28—36	36
8	50	8	221	38	172	6	5
1	160	10	69	1	26	41	1

Metaphases of leaf cells were studied after transplant—ing in the field (Table 5). In plants less than 50 cm tall, leaf cells revealed a considerable variation in chromosome number. Most cells had between 18 and 27 chromosomes. A larger tree, 160 cm tall, had many leaf cells with 28—36 chromosomes. This may indicate a successive increase in chromosome numbers in the meristematic cells.

Our observations show that the increase in chromosome number continues even in the bud—grafted stocks of pollen plants. Therefore, it seems more reliable to examine the chromosome number of embryoid cells (but not of pollen plantlets or transplanted plants) for judgment on the origin of plantlets obtained by anther culture.

The consistent occurrence of a few dividing cells with 9 chromosomes in our preparations of calli, embryoids, and root—tips support the amphidiploid nature of the rubber tree. Deregulation of the chromosome number of the polyhaploid to the true haploid chromosome number is an intriguing problem requiring further study. The mechanism of the increase in chromosome number leading to aneuploid, triploid, and te—traploid cells in embryoids of Hevea arising from microspores needs further elucidation. This chromosomal variation might be useful in breeding work.

Histological and cytological investigations of cultures

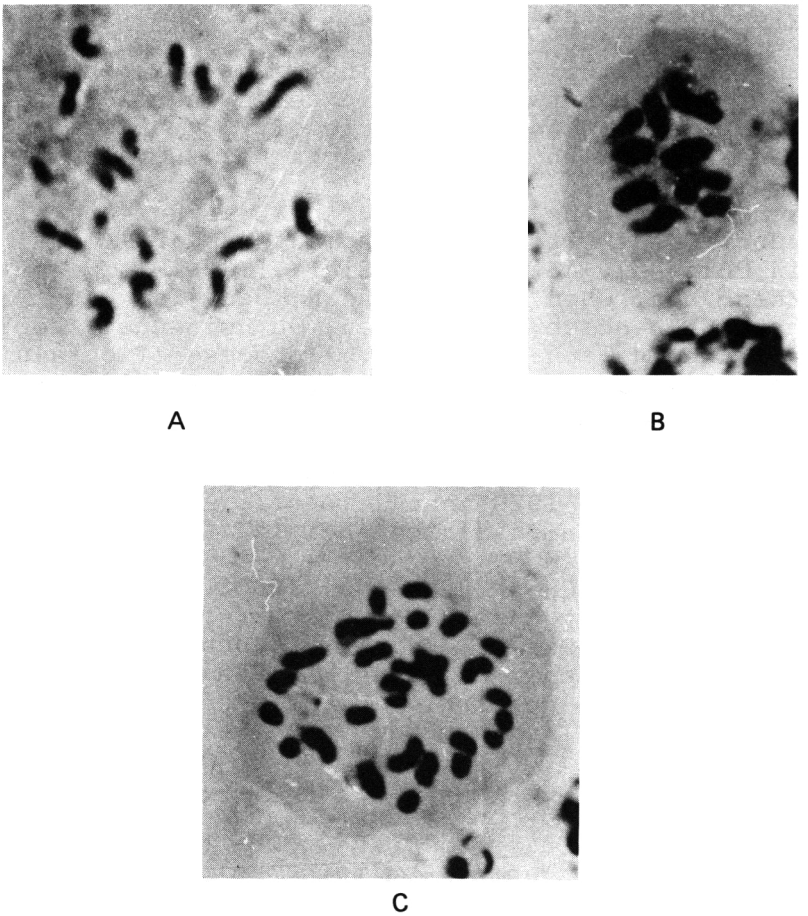

Fig. 3. Embryoids with 18 chromosomes (A), 9 chromosomes(B), and 27 chromosomes (C) arising from pollen cells of Hevea brasiliensis.

in the anther culture process help reveal the dynamics of culture and probe deeply into the essence of different phe—nomena. With systematic microscopical observations of cul—tures, we can analyze the effect of a single factor in a rather short time, permitting modification of experimental design and accelerating work on culture techniques.

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When available, names of scientists in China are given in the traditional Chinese order: surname followed by full give name with no comma. Given names are hyphenated, with only the first syllable capitalized. Initials of given names normally are not used.

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PRODUCING HAPLOID PLANTS BY CHROMOSOME ELIMINATION

C. John Jensen

Haploid production as a potential plant breeding tool has been discussed at two international symposia during the last 10 years (Kasha 1974a, Davies and Hopwood 1980). Direct practical application of haploids in cereal crop breeding has also been shown by a number of researchers (Choo et a1 1979; Clapham 1977; Collins and Genovesi 1981; De Paepe et a1 1977; Fouilloux 1980; Griffing 1975; Ho et a1 1980; Kasha and Reinbergs 1975, 1980; Nitzsche and Wenzel 1977; Pickering 1980a; Reinbergs et a1 1975, 1978; Snape 1976; Snape and Simpson 1981; Song et a1 1978).

But if haploidy is to be successful as a technique in genetics and in breeding programs, the ability to produce a large number of haploids from many different genotypes of crop plants is needed. Efforts are being made to improve existing haploid production techniques (Collins and Genovesi 1981, Jensen 1977, Nitzsche and Wenzel 1977) and new methods are appearing which must be tested (Hagberg and Hagberg 1980; Noeum 1976, 1979).

In barley (a self-pollinating diploid cereal), the four routes to haploidy are anther or microspore culture, chromosome elimination, haploid initiator gene, and ovule or ovary culture. Although anther or pollen culture has been described as the technique with the most potential for obtaining large numbers of haploids, that assumption is based on results of systems using a few so—called model plants, such as tobacco or other members of the Solanaceae (Nitzsche and Wenzel 1977). When it comes to such agronomi—cally important crop plants as wheat, rice, maize, and barley, the use of anther culture techniques in practical plant breeding programs is constrained by low culture response, nonrandomized representation of gametes, and relatively high frequencies of chlorophyll—defective haploids, aneuploids, and heterozygous plants.

Kasha's (1974b) review of haploid formation referred to chromosome elimination — the loss of chromosomes or chromosome segments in either somatic or meiotic cells over a series of cell division — and to somatic reduction —

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the loss of genomes or chromosome groups in a single meiotic division (for detailed haploid terminology, see Fossard 1974).

For genetic and breeding purposes, chromosome elimination provides the fastest way to complete homozygosity from a breeding generation. It simplifies selection procedures, allows, effective selection of quantitative characters, and allows the study of gene action.

The route to haploids via chromosome elimination is based on the crossing of two distantly related species. Crossing wide species is probably the oldest known method of artificially inducing haploids in higher plants (Jensen 1982). The chromosomes of one crossing partner are eliminated after fertilization and during embryo formation.

Although the term chromosome elimination is sometimes used to mean chromosome segregation and somatic reduction of chromosomes, here the term refers to the happenings after fertilization and zygote and embryo formation. This method of inducing haploids also could be mistaken for female parthenogenesis. But in that case, there is no proper zygote formation from fusion of male and female gametes.

MECHANISMS OF CHROMOSOME ELIMINATION

Various theories have emerged to explain the chromosome elimination process leading to haploidy from Hordeum vulgare × Hordeum bulbosum crosses (Davies 1974, Barclay 1976). The most extensive description comes from Bennett et a1 (1976), who pointed to a feasible connection of nucleolar expression and chromosome elimination in hybrids. They favor the explanation that the chromosome elimination is caused by the failure of the chromosome to initiate or to complete either congregation at metaphase or migration to the poles at anaphase. They found that the rate of chromosome elimination in the embryo increased at the third day of embryo formation, which coincides with demand for protein synthesis. Ho and Kasha (1975) examined the genetic control mechanism of elimination in the hybrid combination and found that barley chromosomes 2 and 3 were responsible for controlling chromosome elimination.

The mechanism of the spindle organizer has been hypothesized to play a role in chromosome elimination or loss (Orton and Tai 1977; Tai 1980, pers. comm). In that model, if the parental spindle organizers are genetically similar, normal chromosome behavior will follow. If they are not similar, each spindle organizer will function independently, even within the same protoplast in the offspring. Each spindle organizer will then migrate and establish itself as a pole and attract its own chromosomes. The net result will be multipolar cell division and

the separation of different genomes into different daughter cells - in other words, the elimination of genomes.

Bennett et al (1976) and Kasha (1976) proposed that the elimination of genomes can be a one-step process brought about by multipolar cell division rather than a gradual process involving elimination of chromosomes individually by DNA degradation.

Although no conclusive explanation can yet be derived for the actual process of elimination, it seems widespread in the plant world and is seen as a major route to haploidization in nature.

Genetically determined chromosome loss and haploid formation has been described best for a few members of the Gramineae - tribe hordeae, subtribe Triticinae, genera Aegilops, Secale, and Triticum, and subtribe Elyminae, genera Elymus and Coix (Kasha 1974b; Barclay 1976; Bennet et a1 1976: Subrahmanyam 1977, 1980; Orton and Tai 1977). Outside the Gramineae, chromosome elimination has been found in Nicotiana and possibly in Crepis, Rosa, Vicia, and Paris (Bennett et al 1976).

Whatever the system involved, its application points to a stable mechanism. Yet recently monoploid and especially hybrid frequencies in H. vulgare. × H. bulbosum crosses were found to depend on the genotype of both parents (Tables 1, 2). Work is in progress to isolate

Table 1. Monoploid and hybrid formation from Hordeum bulbosum genotypes × two barley genotypes.

Barley cultivar ♀	H.bulbosum clone ♂	Fruits set (%)	Green plants regenerated		
			Monoploids No.	Monoploids %	Hybrids (%)
HP 40	B 47	71	138	61	18
VADA	B 47	23	59	23	37
HP 40	C 35	87	161	72	6
VADA	C 35	62	102	60	15

H. bulbosum genotypes that induce high frequencies of monoploids and those that give high frequencies of hybrids which can be used in gene transfer programs (Fig. 1).

Hordeum bulbosum is not necessarily the ideal partner for H. vulgare to induce chromosome elimination and monoploids of barley. There could well be a range of Hordeum

Table 2. Effect of Hordeum bulbosum genotype on frequency of monoploids and hybrids after crossing Riso mutant 1508 × HP 40 (F_1)

H. bulbosum clone	Embryos cultured (No.)	Green plant regeneration			
		Hybrids		Monoploids	
		No.	%	No.	%
87	317	9	2.8	151	47.6
219	510	64	12.5	315	61.8
263	328	25	4.6	231	70.4

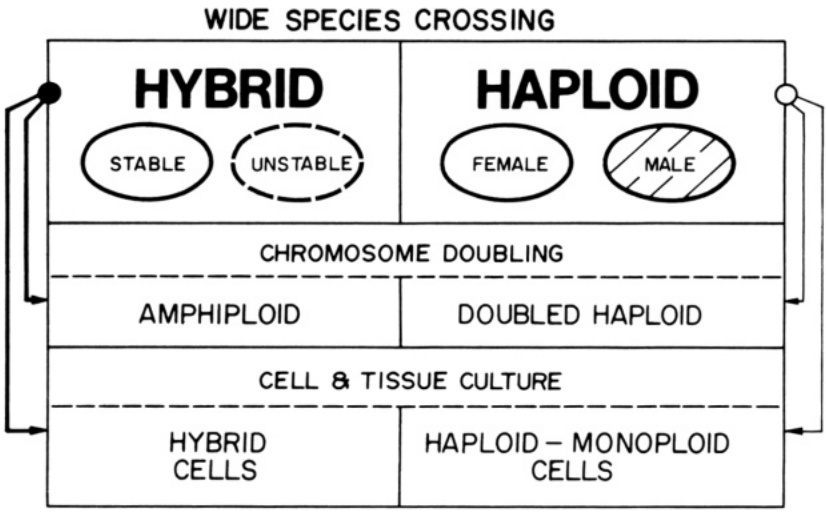

Fig. 1. Use of wide species crosses and chromosome elimination to produce hybrid and haploid plants and hybrid and haploid cell culture systems. Hybrid embryos (stable) can be turned into plants and, after chromosome doubling, constitute amphiploids.

Unstable hybrid formation may lead to complete chromosome elimination of one parent, giving haploid cells or sporophytes.

Chromosome doubling of haploid plants gives completely fertile and homozygous plants. In these systems, haploid or monoploid cells from immature embryos form useful material for cell cultures.

species that might be more efficient partners than H. bulbosum (Kasha 1974b, Jacobsen 1982). On the other hand, there seems to be a large variation in ease of chromosome elimination among different genotypes of H. bulbosum.

Tissue—specific elimination

Noda and Kasha (1981) found that chromosome elimination in triploid hybrids of H. vulgare (diploid) × H. bulbosum (tetraploid) is tissue specific. Leaf meristems close to spike primordia showed a lower rate of chromosome elimination than did the spike primordia. Thus. chimeras showing different sectors from monoploid through aneuploidy to hybrid chromosome number can occur in these hybrids. It is assumed that this chromosome elimination is due largely to disturbances in nuclear division (Orton and Tai 1977).

Chemical chromosome elimination

Attempts have been made to influence chromosome elimination by using chemicals such as cycloheximide (Wheatley and Kasha 1980) and griseofluvin (P.S. Carlson 1981, pers. comm.). However, the results so far are too inconclusive to predict the possible use of these compounds in producing hybrids. But, from the point of view of establishing research tools to examine the process of chromosome loss, these chemicals may well help.

Chromosome elimination in genotype and species crosses

The elimination of chromosomes seems to be widespread in Hordeum species (Barclay 1976, Kasha 1974a). Haploids have been produced in interspecific and intergeneric crosses (Ahokas 1970, Jacobsen 1982, Kasha 1974a, Subrahmanyam 1978) (Tables 3,4).

CHROMOSOME ELIMINATION IN THE BULBOSUM

The Bulbosum method of chromosome elimination for haploid production in barley currently fits breeding requirements. The Bulbosum method depends on the ability to hybridize and to induce high frequencies of embryos. Once embryos have been induced, it is necessary to encourage embryo development and growth in vivo and in vitro (Moss 1972, Jensen 1977). Although the technique should be improved to exclude genotypic differences, the Bulbosum method allows easy and consistent production of haploids which are cytologically stable and genetically normal.

The basis of the Bulbosum technique is an interspecific cross between Hordeum vulgare and Hordeum

Table 3. Progeny from *Hordeum* interspecific crosses.

Crosses	Florets pollinated	Seed set	Embryos cultured	Progeny plants	Genotype[a] 2n	Genotype[a] n haploid
H. brachyantherum (4x) × *H. bulbosum* (2x)	558	101	73	21	2	19
H. depressum (4x) × *H. bulbosum* (2x)	285	115	114	58	36	5
H. arizonicum (6x) × *H. bulbosum* (2x)	215	101	79	35		35
H. arizonicum (6x) × *H. bulbosum* (2x)	258	67	52	30	3	18
H. arizonicum (6x) × *H. vulgare* (2x)	311	36	35	17	4	13

[a] Includes only surviving progeny (Subrahmanyam 1978).

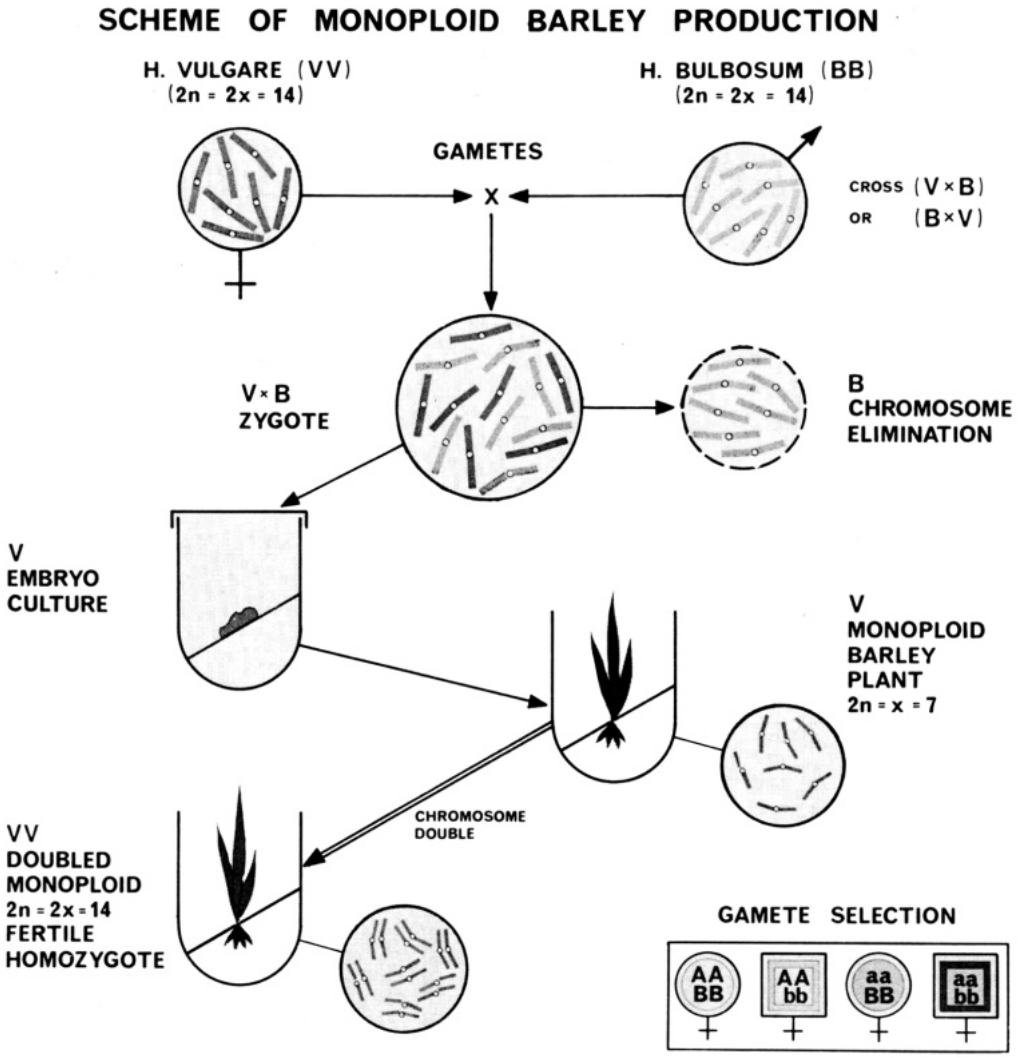

Fig. 2. Scheme of monoploid and doubled monoploid barley (Hordeum) production. Interspecific hybridization followed by elimination of H. bulbosum chromosomes. Substituting other species and ploidy level for H. vulgare and H. bulbosum gives a general approach to haploid production using a system of wide species crosses followed by chromosome elimination and embryo culture.

Table 4. Haploids produced in Hordeum species by crossing with H. bulbosum.

Parental combination				Haploid sporophyte phenotype	Somatic		Reference
Female	Ploidy level	Male	Ploidy level		Ploidy level	Chromosome No.	
H. Secalinum	4x	H. bulbosum	2x	H. secalinum	2x	14	Barclay 1976
H. secalinum	4x	H. bulbosum	4x	H. secalinum	2x	14	Barclay 1976
H. lechleri	6x	H. bulbosum	2x	H. lechleri	3x	21	Barclay 1976
H. lechleri	6x	H. bulbosum	4x	H. lechleri	3x	21	Barclay 1976
H. jubatum	4x	H. bulbosum	2x	H. jubatum	2x	14	Barclay 1976
H. jubatum	4x	H. bulbosum	4x	H. jubatum	2x	14	Barclay 1976
H. brachyantherum	4x	H. bulbosum	2x	H. brachyantherum	2x	14	Subrahmanyam 1978
H. depressum	4x	H. bulbosum	2x	H. depressum	2x	14	Subrahmanyam 1978
H. arizonicum	6x	H. bulbosum	2x	H. arizonicum	3x	21	Subrahmanyam 1978
H. parodii	6x	H. bulbosum	2x	H. parodii	3x	21	Subrahmanyam 1978
H. procerum	6x	H. bulbosum	2x	H. procerum	3x	21	Subrahmanyam 1978
H. procerum	6x	H. bulbosum	4x	H. procerum	3x	21	Subrahmanyam 1978
H. brevisubulatum	4x	H. bulbosum	2x	H. brevisubulatum	2x	14	Jacobsen 1982
H. brevisubulatum	4x	H. bulbosum	4x	H. brevisubulatum	2x	14	Jacobsen 1982
H. marinum	4x	H. bulbosum	4x	H. marinum	2x	14	Jacobsen 1982
H. lechleri	6x	H. vulgare	2x	H. lechleri	3x	21	Barclay 1976
H. arizonicum	6x	H. vulgare	2x	H. arizonicum	3x	21	Subrahmanyam 1978
H. procerum	6x	H. vulgare	2x	H. procerum	3x	21	Subrahmanyam 1978
H. marinum	2x	H. vulgare	2x	H. marinum	x	7	Jacobsen 1982
H. marinum	4x	H. bulbosum	2x	H. marinum	2x	14	Jacobsen 1982
H. marinum	4x	H. bulbosum	4x	H. marinum	2x	14	Jacobsen 1982

bulbosum. Soon after the first report of haploid induction in barley (Kao and Kasha 1969), Kasha and Kao (1970) demonstrated that crossing barley diploids with H. bulbosum diploids could be used to produce a high frequency of barley monoploids. The first report of the utilization of hybridization and somatic chromosome elimination to obtain monoploids came from crosses between tetraploid H. vulgare (2n = 4x = 28) and tetraploid H. bulbosum (2n = 4x = 28) (Kasha 1974b, Jensen 1975).

The general scheme for monoploid production is shown in Figure 2.

Three steps seem to be serious hurdles in the production of monoploids and double monoploids:

- pollen production, fertilization, and embryo induction;
- embryo culture; and
- chromosome doubling.

Growing the donor plant

The importance of growing conditions for the barley donor plant has been stressed (Jensen 1977). Barley is photoperiod sensitive and, under suboptimal conditions, short days can lead to sterility (Batch and Morgan 1974). Work on vegetative and reproductive growth phases under controlled environmental conditions shows that even in related genotypes of barley, plants express themselves differently under similar conditions (Dormling et al 1975).

Those studies point to the need for more detailed work on the optimum growing conditions of genotypes for monoploid induction and on optimum nutrition for the growing plant.

Growing donor plants in artificial conditions needs improvement. Light, temperature, and day length affect fruit development and monoploid embryo formation. Nutrition of the developing embryos in the fruit is not understood and hormone treatments have not improved embryo development.

Improvements in the production of good flowering shoots on donor plants eventually should lead to improvements in stabilized fruit set, embryo development, and monoploid regeneration (Table 5).

Growing the H. bulbosum pollen donor deserves similar attention to optimize pollen quality and quantity (Jensen 1977).

Embryo culture

One of the main difficulties with monoploids in barley (and possibly in other species) is that, even on the same spike, developing embryos vary in size and developmental stage. Liquid media favor the development and growth of monoploid embryos of varying sizes better than do solid media (Jensen 1982).

Table 5. The regeneration of barley monoploids and hybrids following crosses of barley genotypes grown at different temperatures with Hordeum bulbosum, clone C—57.

Barley genotype	Temperature regime (°C day/night)	Fruit set (% florets pollinated)	Green plants regenerated	
			Hybrids (% embryos cultured)	Monoploids (% embryos cultured)
Bomi	26/20	58	3.2	21.1
Bomi	181/13	69	5.8	39.1
HP 40	26/20	78	3.9	39.2
HP 40	18/13	82	3.3	55.3
HP 40	121/12	89	6.2	65.2

Embryos about 2 weeks old are washed with nutrient media after excision to ensure even growth. A number of media favor the growth of plants from particular develop—

Table 6. Influence of culture media and temperature on frequency of barley monoploids regenerated from barley cv. HP 40 ♀ × Hordeum bulbosum ♂ clone C 37.

Medium	Temperature (°C)	Embryos cultured (No.)	Plants regenerated		
			Monoploids No.	Monoploids %	2n hybrids (%)
B^{II}	20	139	28	20.1	8.4
J 25—8	20	122	63	51.6	5.7
B_5	20	115	26	22.6	2.0
B^{II}	26	89	29	32.6	8.2
J 25—8	26	102	41	40.2	10.4
B_5	26	93	19	20.4	3.1

mental stages (Table 6). However, for a general monoploid production series, one medium is used for all embryonic stages (Table 7).

The transfer of monoploid plants from in vitro cul— ture systems to soil and glasshouse growing conditions requires sturdy plants, a well—aerated soil (Table 81, and optimum moisture. Emphasis should be placed on treating

Table 7. Stock medium (J 25—8) for barley and Hordeum spp. embryo culture.

Concentrates [1]	Quantity
A Macronutrients	
KNO_3	2200 mg
NH_4NO_3	600 mg
$MgSO_4 \cdot 7H_2O$	310 mg
$KH_2PO_4 \cdot H_2O$	170 mg
$NAH_2PO_4 \cdot H_2O$	75 mg
$(NH_4)_2SO_4$	67 mg
$CaCl_2 \cdot 2H_2O$	295 mg
B Micronutrients	
$MnSO_4 \cdot H_2O$	5 mg
H_3BO_3	3 mg
$ZnSO_4 \cdot 7 H_2O$	5 mg
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg
$CuSO_4 \cdot 5H_2O$	0.025 mg
$CoCl_2 \cdot 6H_2O$	0.025 mg
C Vitamins	
Nicotinamide	1 mg
Thiamine HCl	10 mg
Pyridoxine	1 mg
Inositol	100 mg
D Fe - EDTA	28 mg
Ferric citrate (dissolve in about 50 ml hot water)	20 mg
E Amino acids	
L—glutamine	120 mg
L—asparagine	50 mg
L—threonine	25 mg
L—arginine	25 mg
L—proline	50 mg
Casamino acids (vitamin free)	125 mg
F Coconut milk (deproteinized)	25 ml
G Sucrose	20,000 (20 g)
Glucose	7,000 (7 g)
(Autoclave) agar (Difco, purified)	7.2 gm
active coal	4.0 gm
H L—Malic acid - 100 mg, dissolve in 50 ml H_2O adjust pH to 5.3 with NH_4OH.	

1) Procedure: Prepare stock solutions at:

(Table 7. Continued)

10	times A concentrate,
100	times B concentrate,
100	times C concentrate,
100	times D concentrate,
10	times E concentrate,
F	from store at 5°C,
G	as needed for ready medium,
H	as needed for ready medium.

Table 8. Composition of standard soil used to pot barley for growing under artificial conditions at Risø.

Mineral soil and docomile	40%
Spagnum	60%
Dry weight	320 kg/m^3
pH = 5.5 - 6.5	
Conductivity = 4.0 - 5.0	
Chemical composition	
Nitrate	55 kg/m^3
Potassium	35—55 kg/m^3
Phosphorus	20—40 kg/m^3
Magnesium	40—55 kg/m^3
Calcium	25 kg/m^3
Manganese	3.0 g/m^3
Boron	1.5 g/m^3
Copper	1.2 g/m^3
Iron	6.0 g/m^3

the material uniformly. Extra handling of plants adds to the expense of the monoploid technique. The sooner plants are established and ready for colchicine treatment, the quicker homozygous seed will be formed.

Colchicine treatment for chromosome doubling

The stage of plant development is important when using

colchicine to induce chromosome doubling (Jensen 1975). Adding hormones such as gibberellic acid (GA_3) to colchicine solution containing a wetting agent and a carrier in the form of dimethyl sulfoxide (DMSO) caused a marked increase in chromosome doubling for barley haploids.

Thiebaut et al (1979) found that adding 10 mg GA_3/liter and 0.1% colchicine with 2% DMSO to 0.3 ml Tween 20/liter gave the highest chromosome doubling of barley haploids at the 3—leaf stage. Treatment was for 5 hours at 22°C in the light. They recommend that the treatment temperature be increased from 25° to 32°C. For extra seed Per plant, they recommend adding a growth hormone such as N—6 benzyladenine (BA) at 10 ml/liter to the standard solution of 1% colchicine, 2% DMSO, 0.3 ml Tween 20/liter, and 10 mg GA_3/liter.

Our work has focused on doubling the chromosomes of monoploids of spring and winter types of barley and of haploids of various wild species of Hordeum. In general, healthy plants showing about 5 leaves or 3 to 4 small shoots at the time of treatment are submerged in colchicine solution in glass vials in the light at 20—22°C. Five hour treatments often give good doubling results.

The colchicine solution is prepared fresh using 1 g colchicine (chloroform free) dissolved in 20 ml concentrated DMSO per liter of water. Tween—20 or Tween—80 at about 0.2 to 0.5 ml/liter is added as a wetting agent.

Overwatering of colchicine—treated plants can easily cause rot and a considerable loss of plants. A close watch on pot moisture the first 2 weeks after treatment has given 90—100% survival and chromosome doubling of treated plants.

Strict safety procedures must be used in handling as colchicine can be carcenogenic. Colchicine in solution with dimethyl sulfoxide is readily absorbed by the human skin. Rubber gloves should be worn when handling colchicine solutions and safe disposal of used solutions should be standard laboratory procedure.

WHEAT HAPLOIDS

Recently, high frequencies of wheat haploids have been produced by a cross of wheat with H. bulbosum (Barclay 1975). The system requires embryo culture and experimental procedures similar to the production of barley monoploids by the Bulbosum method.

The wheat genotypes which show high seed set ability when crossed with rye also show relatively high crossability with H. bulbosum (Chapman et al 1976, Snape et al 1979, Snape and Simpson 1981, Falk and Kasha 1981). It seems that the ability of wheat to cross with H. bulbosum is genetically governed and similar to the wheat × rye

crossability which is determined by the wheat Kr_1 and Kr_2 genes located on chromosomes 5 B and 5 A (Snape et al 1979, Falk and Kasha 1981). Falk and Kasha also suggest that the H. bulbosum × wheat incompatibility might be more pronounced because of a third Kr locus, Kr_3 on chromosome 5 D (Krolow 1970).

A possible way to overcome this crossing barrier is to find compatible H. bulbosum and wheat genotypes. A large—scale search is being conducted (Snape 1981, pers. comm.). Haploids in cultivated wheat, cv. Chinese Spring, have been induced with haploid frequencies approaching those obtained in barley (Tables 9, 10). The method for

Table 9. Wheat haploids from intergeneric crosses of Triticum aestivum (cv. Chinese Spring) × Hordeum bulbosum (Barclay 1975).

Pollinator H. bulbosum	Florets pollinated (No.)	Embryos cultured (No.)	Plants regenerated (No.)	Plants	
				Florets (%)	Embryos (%)
2x	622	39	11	1.8	28
4x	435	110	59	14	54

haploid induction and treatment is the same as that for barley × H. bulbosum monoploid production.

However, unless other H. bulbosum genotypes that can induce fruit set and haploid embryos in wheat cultivars are found, application of the method will be restricted. Application is currently limited to some Australian and Chinese wheat cultivars (Table 11).

LOOKING AHEAD

The future in plant breeding of haploidy by wide species crosses and chromosome elimination obviously depends on its research priority.

The Bulbosum method has certain advantages over other forms of haploid induction. One essential characteristic is that the haploids are derived through the egg. That insures a more natural development of sporophytes than those derived from the reduced male gametophyte. Another advantage is that the relatively high embryo induction frequency permits a random sample of all possible gametes to be represented as haploids. A third advantage is that Bulbosum system techniques are familiar to most breeders (for example, hybridization and embryo culture).

Table 10. Haploids in Triticum and Aegilops x Hordeum bulbosum crosses.

Parental combination				Haploid sporophyte phenotype	Somatic		Reference
Female	Ploidy level	Male	Ploidy level		Ploidy level	Chromosome number	
Triticum aestivum cv. Chinese Spring	6x	H. bulbosum	2x	T. aestivum	3x	21	Barclay 1975
Triticum aestivum cv. Chinese Spring	6x	H. bulbosum	4x	T. aestivum	3x	21	Barclay 1975
Aegilops trinuncialis	4x	H. bulbosum	4x	Ae. trinuncialis	2x	14	Chapman et a1 1976
Aegilops crassa	6x	H. bulbosum	4x	Ae. crassa	3x	21	Shigenobu and Sakamoto 1977

Table 11. Grossability of selected wheat cultivars with tetraploid H. bulbosum clones and diploid rye variety Petkus spring (Snape et a1 1979).

Female genotype		Pollinator			
		H. bulbosum		Rye	
		Florets pollinated (No.)	set (%)	Florets pollinated (No.)	set (%)
Chinese winter wheat	TH 3929	138	35.5	85	88.2
Australian spring wheat	Gamut	90	10.1	62	58.0
Australian spring wheat	Timgalen	484	6.0	176	23.0
French winter wheat	Cappelle Desprez	58	0.0	56	1.8

On the other hand, as with most technologies based on biological phenomena, little knowledge is available which would make it easier to control haploid formation. Even in barley, where the Bulbosum system has been practiced for more than 10 years, more research is needed on improving current results. Figure 3 shows the important steps in

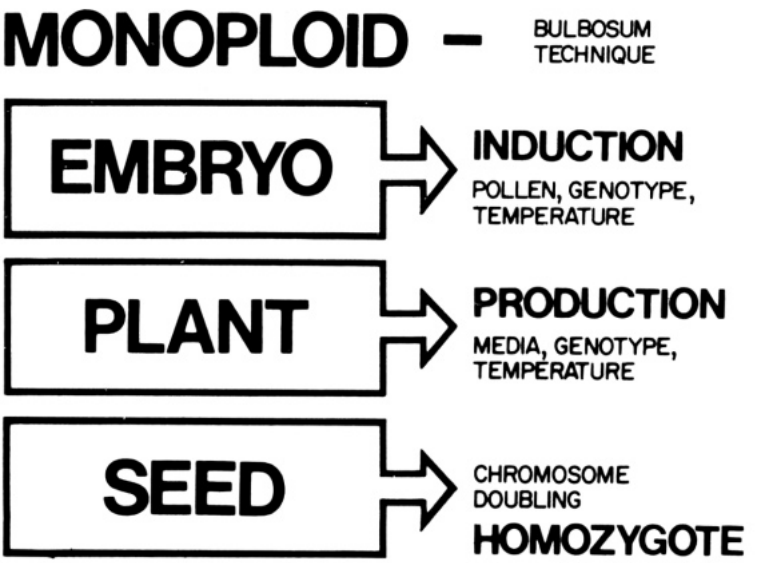

Fig. 3. Essential steps of monoploid production using wide species crosses and chromosome elimination (Bulbosum technique). The main obstacle is achieving a high frequency of embryo induction on the crossed spikes (flower heads). Embryo induction seems to depend on quality and quantity of pollen, genotype of pollen and female parent, and temperature during crossing and embryo formation. Plant production from in vivo—formed haploid or monoploid embryos is dependent on type of growth media, genotype of embryo, and growth conditions (temperature). Seed production depends on the efficiency of chromosome doubling techniques to obtain homozygous, fertile offspring.

monoploid or haploid production schemes for the Bulbosum method. Pollen production, selection of pollen donors, fertilization embryo induction, embryo culture, and chromosome doubling need further study to optimize haploid and doubled haploid production. Each step must be fully explored and tested before the method can be said to have reached haploid—producing capacity.

In addition to barley cultivars, the method will be

tried on wheat cultivars and possibly on other cereal species. On this basis, the Bulbosum method should be looked upon as a useful alternative to other haploid production methods.

The Bulbosum method also has merit in cytogenetic research. For example, the transfer of a single genome into a foreign cytoplasm (Fig. 4,5) can be easily established for H. bulbosum × H. vulgare crosses, resulting in sexual

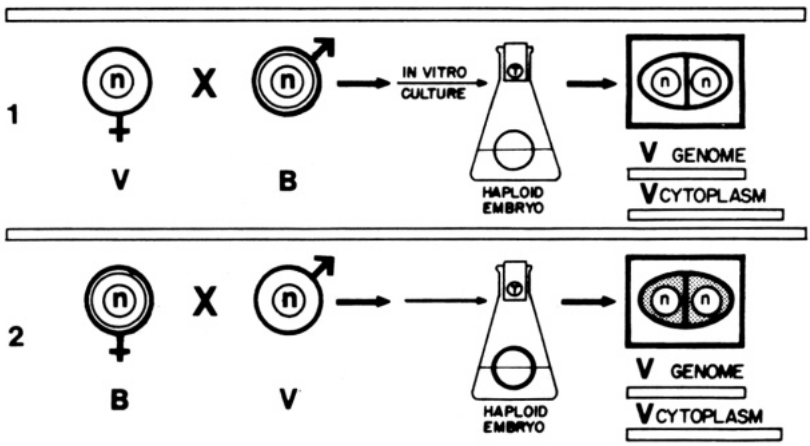

Fig. 4. Consequences of chromosome elimination in reciprocal wide crossing combinations to result in a haploid genome in alien cytoplasm. Case 1 illustrates the normal route of haploid production resulting in monoploid genome in its own cytoplasm. Here V = a single Hordeum vulgare genome and B = a H. bulbosum genome. Case 2 exemplifies the situation of a reciprocal cross (B × V) where the female egg genome B is eliminated on fusing with the pollen genome V, resulting in a single V genome in B cytoplasm.

cybrids. These cybrids can be established in a number of species using the chromosome elimination system.

Species relationships based on chromosome elimination patterns also will be of interest in cytogenetic research (Finch et al 1981).

The Bulbosum system already furnishes valuable raw material for cell and tissue culture experiments on gene transfer. In the system, immature embryos from interspecific species crosses which show the plasticity of chromosome elimination (Fig. 6) are turned into a fast-growing tissue culture. The system could be refined to incorporate specific chromosome segments into a cultivar with the help of chromosome elimination mechanisms.

Pure haploid cell lines can be established from imma-

ture haploid embryos through callus cultures. These cell lines often have a better chance of regeneration than those from other sources.

The question of chromosome elimination in somatic cells (for example, in developing embryos) is basic to many somatic hybridization studies with protoplasts. Somatic chromosome elimination after sexual fusion of compatible or incompatible genomes and chromosomes will become an important area for research. Technically, it seems easier to trace fusion products on a fixed object (the egg in an embryo sac) than on freeswimming protoplasts. The question of the origin of the mechanisms for haploidy and chromosome elimination and hybrid formation seems to be central to

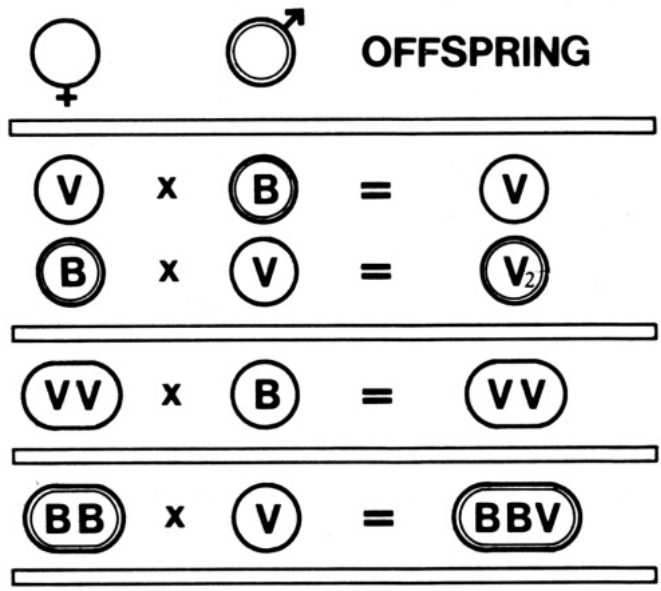

Fig. 5. The effect of single or multiple genomes — V (Hordeum vulgare) and B (H. bulbosum) — in cross combinations. The first line, V × B = V (monoploid barley), shows that hybrid formation depends on the genotypes of the female and male cross combinations. The second line, B × V = V_2 shows the production of monoploid H. vulgare (V) in H. bulbosum (B) cytoplasm. The third line, VV × B = VV, shows that using tetraploid H. vulgare as the female in a diploid H. bulbosum cross results in haploid H. vulgare plants (VV). The fourth line, BB × V = BBV, shows that using tetraploid H. bulbosum as the female with diploid H. vulgare results in stable triploid hybrids.

some evolutionary processes and its solution may indirectly

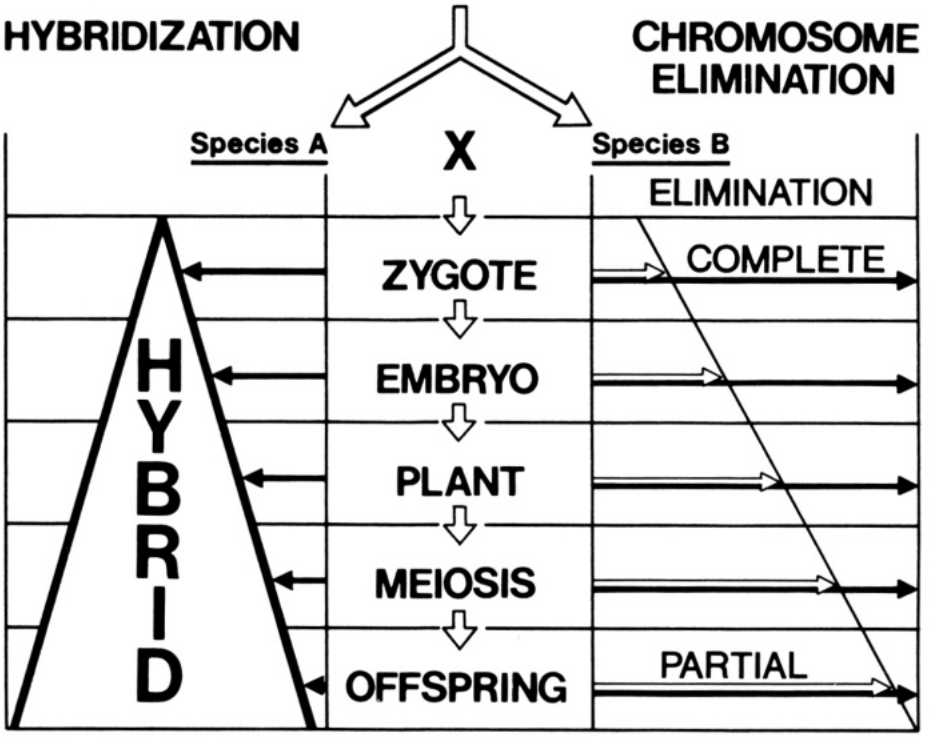

Fig. 6. Hybrid and haploid formation plasticity in wide species crosses followed by chromosome elimination. Normal—ly, one species and genotype determines partial or complete chromosome elimination. Now, cases exist where elimination of all the chromosomes of one parent can be completed after zygote formation, during embryo formation, or at later stages throughout plant development. This plasticity of hybrid haploid formation makes the system both versatile and interesting.

help improve some crop plants.

Expanded understanding is needed on rearing plants under artificial growing conditions (Jensen 1977, Langhans 1978), sporogenesis (Bennett 1976), sexual fertilization processes (Cass and Karas 1975, Jensen 1973, Nettancourt 1977, Norstog 1972), selection of pollen genotypes (Jensen 1977, Pickering 1980, Taira and Larter 1977), embryo induc—tion and formation (Brachet 1974, Dure 1975, Walbot 1978), genotypically and phenotypically controlled chromosome elimination (Bennett et a1 1976, Davies 1974, Falk and

Kasha 1981, Finch et al 1981, Fukuyama and Hosoya 1981, Fux 1974, Gupta and Gupta 1973, Ho and Kasha 1975, Humphreys 1978, Islam and Shepherd 1981, Kao 1977, Kasha 1974a, Orton and Tai 1977, Rhoades and Dempsey 1973, Sagar and Kitchen 1975), chemically induced chromosome elimination (Kasha 1974b, Nitzsche 1973, Pontecorvo 1974, Wheatley and Kasha 1980), in vitro organ and embryo culture (Clapham 1977, Foroughi—Wehr et al 1981, Jensen 1982, King et al 1978, Kruse 1974, Monnier 1978, Nitsch 1982, Nitzsche and Wenzel 1977, Sunderland 1980), plant production (Jensen 1977), and chromosome doubling procedures (Ignatova and Luk'yanyuk 1980, Jensen 1974, Theibaut et al 1979).

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FACTORS AFFECTING CALLUS FORMATION IN UNPOLLINATED OVARY CULTURE OF RICE

Zhou Chang, Yang Hong—yuan, Yan Hua, and Cai Sheng

Culturing the unpollinated ovary or ovule is an attractive area in current plant tissue culture. In practical application, it may offer an alternative to using anther culture for haploid production and it may form a new branch of theoretical research.

The technique has been tried on several species but until 1976 had resulted only in the formation of calli or plantlets from diploid tissue or from an unknown origin (Zhou and Yang 1981). San Noeum (1976, 1979) obtained haploid plants from unpollinated ovaries of barley. Subsequently, wheat and tobacco haploids were induced by Zhu and Wu (1979) and rice haploids were obtained independently by Asselin de Beauville (1980) and Zhou and Yang (1980) from cultured unpollinated ovaries. Embryological observations showed that proembryos and calli originated from the embryo sac. Now we know that the embryo sac, like the pollen, can be induced in vitro to pass through a sporophytic pathway, via proembryo development, callus formation, and organ differentiation, to plant regeneration (Zhou and Yang 1981).

Because improving culture techniques is an urgent problem, we conducted several experiments to study the factors affecting callus induction in rice ovary culture. The main results are reported here.

MATERIALS AND METHODS

Experiments were on 12 cultivars of Oryza sativa L., mostly japonica type. The basic method of ovary culture was:

- Young flowers at the vacuolated microspore stage were excised aseptically and their lemma and palea removed.
- Remaining parts, including the pistil and stamens, were left intact on the receptacle and inoculated as a unit on liquid medium N6 supplemented with 3% sucrose and 0.125 ppm MCPA (2-methyl—4—chlorophenoxyacetic acid). This medium has been used routinely in our laboratory instead of 2,4—D (Chou et a1 1978).
- The inoculums were floated on the surface of the medium and incubated in darkness at nearly 25°C.
- After 30—35days, ovaries were dissected and the

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percentage with visible gynogenic calli inside ovules was calculated.

RESULTS

First set of experiments

To study the effects of several factors on callus formation in rice ovary culture, we conducted a series of separate experiments.

Genotype. Using the basic method, 9 japonica and 2 indica cultivars were induced to form gynogenic calli. The induction frequency (percentage of ovaries producing gynogenic, calli) varied from 1.1 to 12.0% (Fig. 1). This showed that a wide range of rice cultivars could respond to our culture technique and that genotype is as critical a factor in ovary culture as it is in anther culture.

Embryo sac stage of inoculation. The pollen stage was used as an indirect criterion to test the effect of stage of embryo sac at inoculation. Preliminary observations were made on paraffin sections (Table 1).

Callus formation in three cultivars occurred only in materials inoculated at the middle or late stage. Materials inoculated at the early stage gave no response (Fig. 2). This means that the induction period is during the development of the female gametophyte, not during megasporogenesis.

Concentration of exogenous hormone. Four concentrations (0.125, 0.5, 2.0, and 8.0 ppm) of MCPA in the medium and a hormone-free control were compared with three cultivars (Fig. 3). Without MCPA, the majority of ovaries did not enlarge and produced no or very few calli. At 0.125 ppm MCPA, both the percentage of enlarged ovaries and the induction frequency increased significantly. The higher the concentration, the greater the number of enlarged ovaries. Induction frequency was highest at 0.125 or 0.5 ppm MCPA. It decreased as MCPA concentration increased. The 2 ppm concentration of 2,4-D or MCPA routinely used in anther culture media was not suitable for ovary culture.

Concentration of sucrose. Sucrose concentration can influence culture considerably. One percent sucrose was not sufficient to support ovary enlargement and callus formation. Three percent gave the best induction frequency. Nine percent somewhat inhibited callus formation (Fig. 4).

Mode of manipulation. Three modes of manipulation were tested:

- The lemma and palea were removed and the stamen and pistil were kept intact on the receptacle. This is

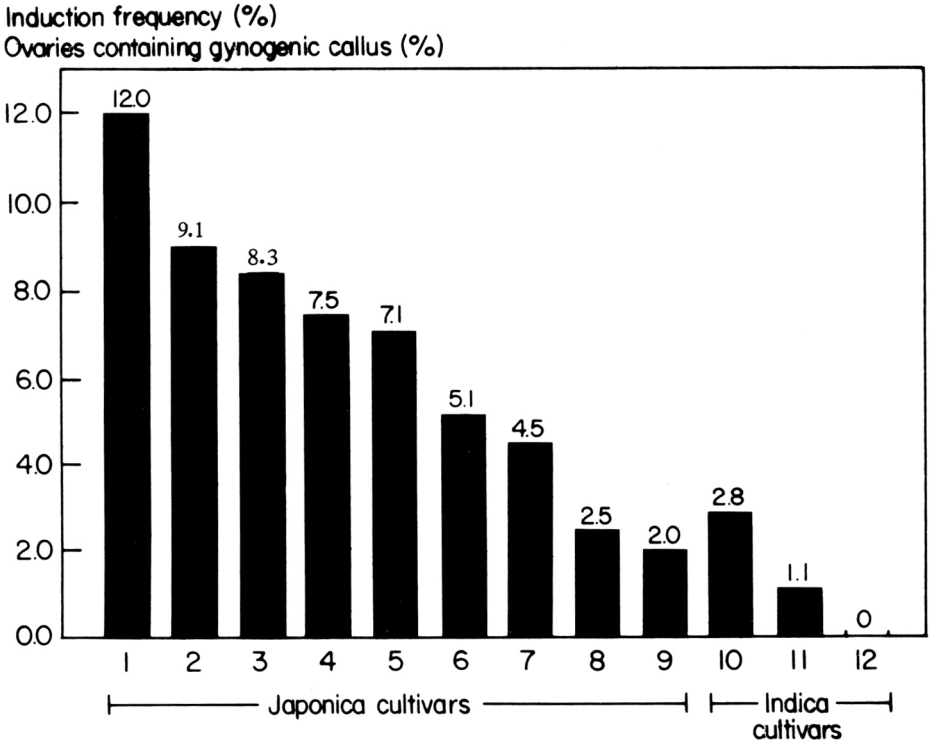


Fig.1. Gynogenic callus production in the culture of rice ovaries. Cultivars used: 1 = Nong Ken No. 4; 2 = Shi Yu; 3 = Xiao; 4 = Zao Geng No. 19; 5 = E Wan No. 3; 6 = Jing Hong No. 2; 7 = Hong Qi No. 16; 8 = Li Ming; 9 = Da Li No. 3; 10 = 5350; li = Jin Nan Te; 12 = Zhen San No. 97.

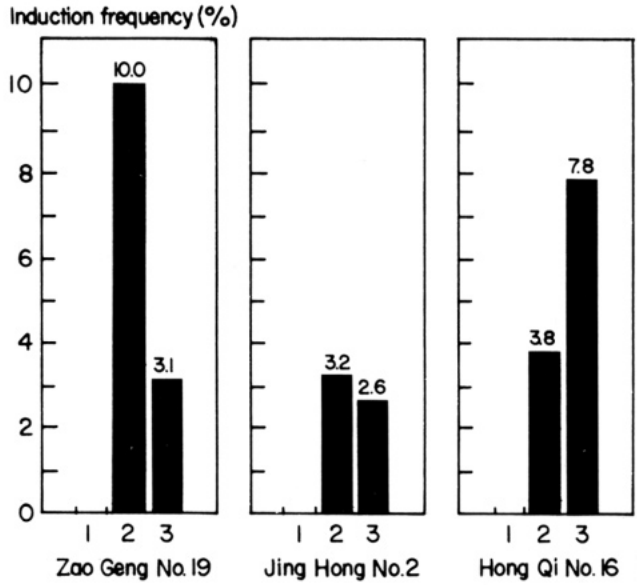


Fig. 2. Induction frequency of rice ovaries cultured at different stages. 1 = early; 2 = middle; 3 = late.

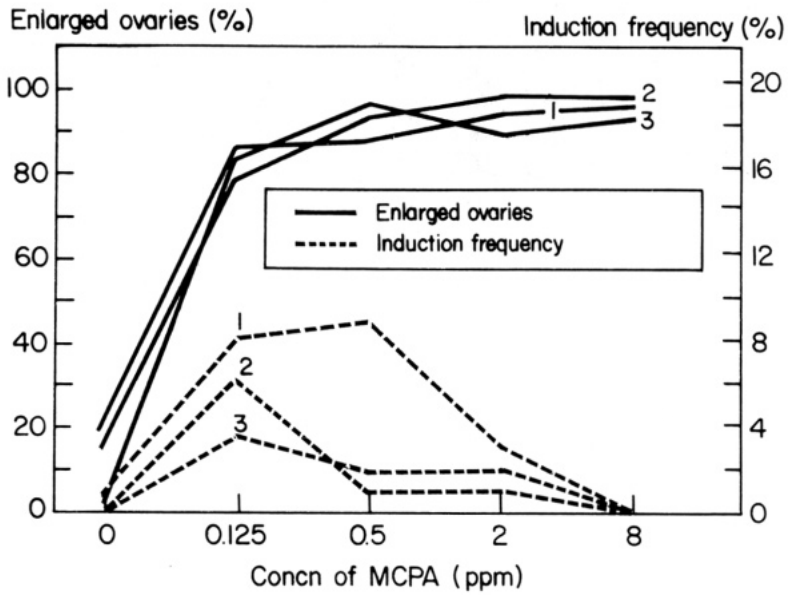


Fig. 3. Effects of hormone concentration on ovary culture of rices. 1 = Zao Geng No. 19; 2 = Jing Hong No. 2; 3 = Hong Qi No. 16.

Table 1. Relationship of embryo sac stage to pollen type.

Stage of inoculation	Flowers (No.)	Pollen type	Embryo sac (No.)				
			Megaspore tetrad	1—nucleate embryo sac	2—nucleate embryo sac	4—nucleate embryo sac	8—nucleate to mature embryo sac
Early	22	Nonvacuolated microspore	21	1			
Middle	35	Vacuolated microspore		23	7	5	
Late	21	2—cellpollen					21

our standard method.

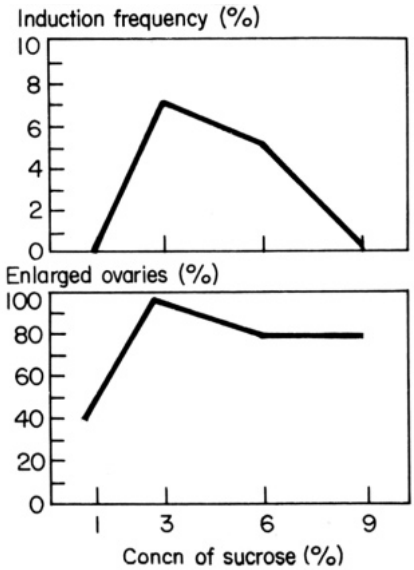


Fig. 4. Effects of sucrose concentration on ovary culture of rice.

- The lemma, palea, and anthers were removed, leaving only the pistil intact on the receptacle.
- The pistil was excised from the receptacle and all other parts were discarded.

Induction frequency was highest with stamen and pistil intact on the receptacle and next highest with only the pistil intact on the receptacle. The pis-til alone did not induce callus. In our experiment, it was difficult to induce gynogenesis from the ovary alone. Receptacle, sterile glumes, and anthers may all play an essential role in the induction process (Table 2).

Second set of experiments

In the first set of experiments, three features distinguished our technique from those used by previous investigators -- the mode of manipulation, the liquid medium, and the lower supply of exogenous hormone. To establish their role in ovary culture, a second set of experiments on 3 cultivars involved combinations of these three factors.

Table 2. Effect of mode of manipulation on ovary enlargement and induction frequency of gynogenic callus.

Mode of manipulation	Ovaries inoculated (no.)	Ovaries enlarged		Ovaries containing gynogenic callus	
		No.	%	No.	%
Stamenand pistil on receptacle	98	80	81.6	4	4.1
Pistil on receptacle	110	85	77.3	3	2.7
pistil alone	100	0	0	0	0

Ovary enlargement. Ovary enlargement was closely related to the mode of manipulation. Ovaries with stamen and pistil intact on the receptacle enlarged quickly and markedly. Those of excised pistil alone grew very slowly and slightly. The difference was clear—cut within 30 — 35days of culture (Table 3, Fig. 5 and 7). About 70 days after inoculation, part of the ovaries of excised pistils were still growing and en— larging very slowly. Obviously, the absence of receptacle and sterile glumes is a factor limiting growth of the ovary wall.

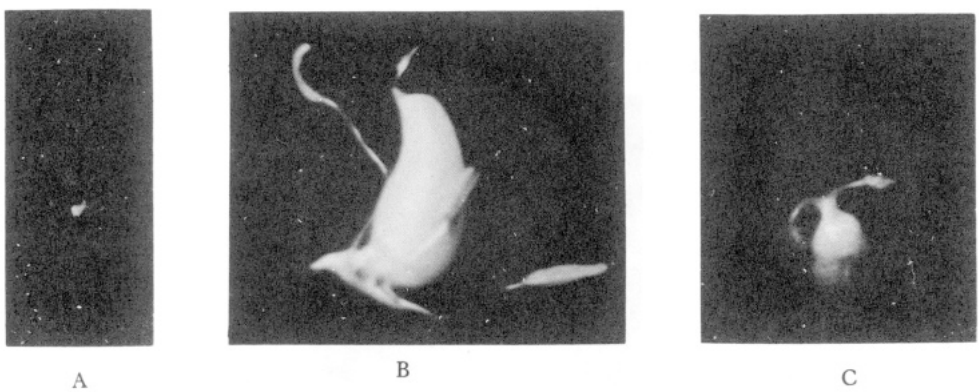
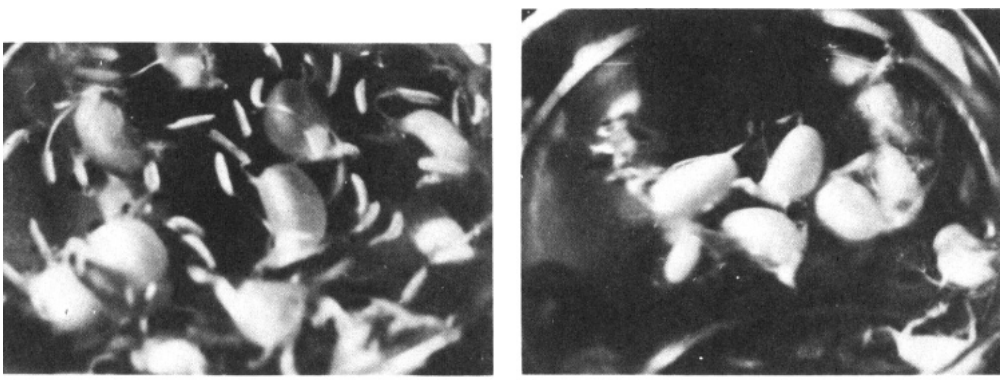


Fig. 5. Enlargement of ovaries was related to mode of manip— ulation. A = ovary at moment of inoculation; B = enlarged ovary with stamen and pistil intact on receptacle, 30 days of culture; C = slow—growing ovary with pistil alone, 30 days of culture.

Fig. 6. Two gynogenic calli dissected from surrounding ovular tissue.



A B

Fig. 7. Ovaries after 30 days culture on liquid medium with 0.125 ppm MCPA. A = stamen and pistil intact on receptacle; B = pistil intact on receptacle.

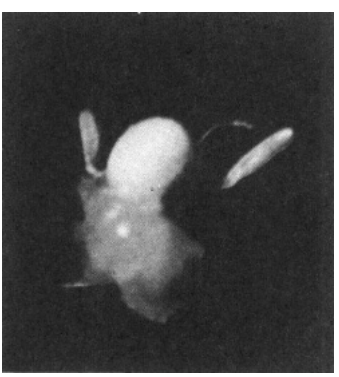


Fig. 8. Somatic callus formed around the base of an ovary cultured on solid medium with 2 ppm MCPA.

Table 3. Effect of treatments on ovary enlargement.

Treatment			Enlarged ovaries (%)		
Medium	MCPA (ppm)	Mode of manipulation	Nong Ken No. 4	Da Li No. 3	Xiao
Liquid	0.125	Stamen and pistil on receptacle	91.0	51.0	63.2
		Pistil on receptacle	85.6	46.0	65.0
		Pistil alone	0	0	0
	2.000	Stamen and pistil on receptacle	95.7	76.2	71.8
		Pistil on receptacle	87.1	53.0	60.8
		Pistil alone	0	0	0
Solid	0.125	Stamen and pistil on receptacle	63.7	55.0	15.3
		Pistil on receptacle	-	48.2	16.4
		Pistil alone	0	0	0
	2.000	Stamen and pistil on receptacle	73.6	65.0	40.0
		Pistil on receptacle	-	55.5	46.4
		Pistil alone	0	0	0

Table 4. Effect of treatments on induction frequency of gynogenic callus.

Treatment			Ovaries containing gynogenic callus (%)		
Medium	MCPA (ppm)	Mode of manipulation	Nong Ken No. 4	Da Li No. 3	Xiao
Liquid	0.125	Stamen and pistil on receptacle	7.8	5.5	8.5
		Pistil on receptacle	4.3	0	0.9
		Pistil alone	0	0	0
	2.000	Stamen and pistil on receptacle	2.2	0.8	1.3
		Pistil on receptacle	0	0	1.7
		Pistil alone	0	0	0
Solid	0.125	Stamen and pistil on receptacle	4.9	1.8	0
		Pistil on receptacle	—	1.1	0
		Pistil alone	0	0	0
	2.000	Stamen and pistil on receptacle	0	1.0	2.7
		Pistil on receptacle	—	0	0.9
		Pistil alone	0	0	0

Table 5. Effect of treatments on production of somatic callus.

Treatment			Ovaries with somatic callus (%)		
Medium	MCPA (ppm)	Mode of manipulation	Nong Keng No. 4	Da Li No. 3	Xiao
liquid	0.125	Stamen and pistil on receptacle	0	0	0
		Pistil on receptacle	0	0	0
		Pistil alone	0	0	0
	2.000	Stamen and pistil on receptacle	14.1	6.7	28.9
		Pistil on receptacle	9.1	13.0	1.7
		Pistil alone	0	6.7	0
Solid	0.125	Stamen and pistil on receptacle	0	0	0
		Pistil on receptacle	-	0	0
		Pistil alone	0	0	0
	2.000	Stamen and pistil on receptacle	46.2	22.5	5.5
		Pistil on receptacle	-	12.7	22.7
		Pistil alone	40.0	20.0	0

In most cases, the percentage of enlarged ovaries was higher with stamen and pistil intact on the receptacle than with pistil only on the receptacle. The existence of anthers may play a role in this aspect (Table 3). In addition, ovary enlargement had some relationship with two other factors. Ovaries were larger on liquid than on solid medium and at 2 ppm MCPA than at 0.125 ppm.

Induction of gynogenic callus. After 30—35 days of culture, ovaries were dissected. Some calli were found inside the ovule (Fig. 6). These calli originated from the embryo sac, as was shown in our previous work (Zhou and Yang 1980, 1981). They can be called gynogenic calli. Induction frequency varied considerably with treatment (Table 4). As a rule, liquid medium was better than solid and low concentration of MCPA (0.125ppm) was better than high concentration (2 ppm). Stamen and pistil on the receptacle was superior to pistil only on the receptacle, and the pistil alone gave no response (Fig. 7). That effect showed that the basic method used was best for the production of gynogenic callus.

Occurrence of somatic callus. In some cases, somatic calli that could easily be distinguished from gynogenic calli appeared around the base of the ovaries (Fig. 8). As a rule, somatic calli occurred at the high concentration of MCPA (2 ppm). There were more somatic calli on the solid than on the liquid medium. No correlation between the occurrence of callus and the mode of manipulation was found (Table 5). We can say that a high concentration of exogenous hormone is the decisive factor in inducing somatic callus.

DISCUSSION

Because ovary culture is a new branch of plant tissue culture, few examples of successful culture of unpollinated ovary have been reported so far and little work has been done on the technological aspects. During the last 2 years, ovaries from more than 10 rice cultivars have been induced to gynogenesis in our laboratory. Three distinguishing features are involved in our technique:

- inoculation of the whole flower, except the lemma and palea;
- float culture on a liquid medium, and
- use of a lower concentration of exogenous hormone.

It had been thought that success might be attributed to the effectiveness of technical points (Zhou and Yang 1981). The experiments reported here confirm this suggestion.

Comparison of three modes of manipulation revealed that the existence of the receptacle and sterile glumes plays an essential role in ovary enlargement and gynogenic development. The presence of anthers has a beneficial effect, too.

Perhaps the removal of the receptacle injures the ovary or some factors in the receptacle and anthers may favor ovary growth and callus formation. Such an interaction between different parts of an explant is often found to have significance in tissue culture, for example, the effect of calyx, lemma, palea, or pedicel in fertilized ovary culture (Maheshwari and Rangaswamy 1965), the effect of the placenta in fertilized ovule culture (Rangaswamy 1977), and the effect of embryo in endosperm culture (Johri and Bhojwani 1977).

Other work on unpollinated ovary culture in barley, wheat, tobacco, and rice were all done on solid media. Our work is the only experiment that used a liquid medium. Comparative studies show the advantage of such a modification. This result is in accordance with current thinking in tissue culture studies, that a liquid medium is often superior to a solid medium (Yeoman and Macleod 1977). A recent approach in anther culture technique, floating the culture on a liquid medium (Sunderland and Roberts 1977), also proved to be more productive in rice anther culture (Chen et al 1980).

The concentration of exogenous auxin used in other experiments on rice ovary culture was often high (2—4 ppm 2, 4-D) and no haploids were obtained (Nishi and Mitsuoka 1969, Yan and Zhao 1979). In our experiments, gynogenic calli were more easily obtained when the auxin level was low (0.125 ppm MCPA). At higher MCPA levels, the ovary wall swelled too quickly and somatic callus often occurred. On the other hand, ovary enlargement was difficult without exogenous hormone. In both cases, production of gynogenic calli was inhibited, although to different degrees. It seems that a certain delicate physiological balance between the growth of the ovary wall and the gynogenic callus is needed. For example, the growth of gynogenic callus requires a corresponding growth of ovary, but not an overgrowth. It is probable that the proper regulation of this balance is just one of the key points for the induction of haploids in rice ovary culture.

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GENETIC VARIABILITY IN RICE PLANTS REGENERATED FROM CELL CULTURE

Kiyoharu Oono

Callus culture of rice was first achieved by Furuhashi and Yatazawa (1964). They cultured the nodes of young seedlings on Heller's medium with vitamins and 2,4—D (2 ppm). Regeneration of plants from calli of various origins was done independently by five groups in 1968. Some phenotypic variations of regenerated plants, such as dwarf or twisted plants (Nishi et al 1968), albino (Niizeki and Oono 1968), and polyploidy (Nishi and Mitsuoka 1969, Niizeki and Oono 1971) have been reported.

Phenotypic variations may be induced by genomic, chromosomal, genic and cytoplasmic mutations and by physiological effects. The genic level of mutation in cultured tissues was suggested by rice plants regenerated by anther culture that had heritable characters of short culm or chlorophyll deficiencies. Some diploid regenerated plants ($A_1(2\underline{x})$)had either homozygous or heterozygous mutated characters, suggesting the occurrence of mutation before or after spontaneous chromosome doubling (Oono 1975).

The occurrence of somatic variations in plants regenerated by tissue culture was reviewed recently (Larkin and Scowcroft 1981). Most were found in vegetatively propagated plants and no further genetic analyses were reported. But the desirable mutations in plant breeding and in mutation studies are not chromosomal mutations but genic mutations, especially in cereal crops.

This paper summarizes studies of mutation in rice tissue culture. Regenerated plants are shown as A_1, A_2, A_3... for plants differentiated by anther culture and their successive generations, and D_1, D_2, D_3... for plants differentiated from somatic callus and their successive generations; ($\underline{x}$), ($2\underline{x}$) ... indicate ploidy level.

MUTATION IN HAPLOID TISSUE

Chromosome numbers in rice pollen callus obtained by anther culture seem highly variable. For instance, the frequencies of haploid cells in pollen calli obtained after 55 days of

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anther inoculation are 34.5% on callus induction media with 2,4-D and 31.1% with NAA. Phenotypic variations were observed in both regenerated haploids ($A_1(\underline{x})$) and diploids ($A_1(2\underline{x})$) from a single pollen origin. Some progeny of spontaneous diploids ($A_2(2\underline{x})$) carried mutated characters such as plant height and panicle length that correlated with those of haploids (Oono 1975).

Agronomic characters of plants regenerated from haploid somatic tissues were studied. Callus was induced from the nodes of a spontaneous haploid Nipponbare (supplied from

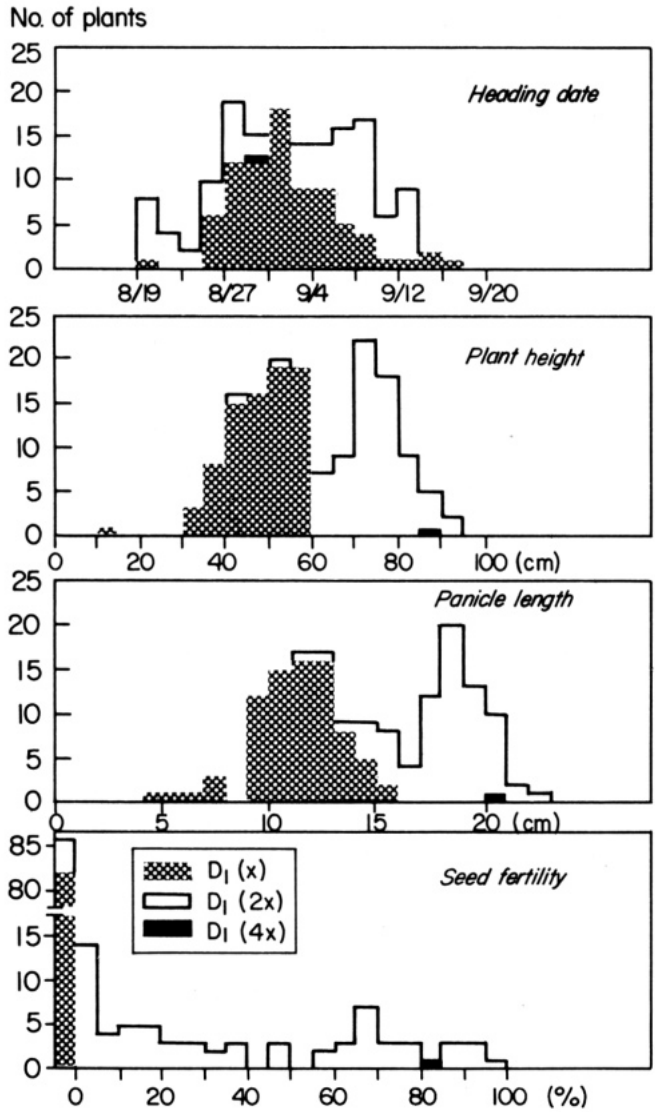

Fig. 1. Phenotypic variation in regenerated plants obtained from the somatic calli of a haploid rice.

N. Iwata, Univ. of Kyushu) using MS medium with 10^{-5}mol 2, 4—D, 70 g sucrose/liter, and either 10 mg BA/liter or 3 g YE/liter and 2 g CH/liter. More than 200 plants were regenerated. Agronomic characters were examined for 155 plants—74 haploids, 8 diploids, and 1 tetraploid (Fig. 1). Diploid frequency of more than 50% is about the same as that obtained by anther culture in rice. Spontaneous diploidization in the field is only 2/420 plants in vegetatively propagated haploids of Nipponbare. Heading date varied widely in both haploids and diploids. Plant height and panicle length also varied in haploids and diploids. Seed fertility of diploids varied from 0% to 97%.

MUTATION IN RICE SEED CALLUS

Dominant mutations will appear in plants regenerated from diploid tissues (D_1). Recessive mutations will segregate in D_2. True breeding lines in mutated characters may be obtained in D_3. Recessive lethal mutation also will segregate in D_2. Analysis of regenerated plants from diploid rice calli confirmed that spontaneous mutations frequently were induced in cultured cells.

Mutation analysis was applied to 1,121 plants (D_1) regenerated from 75 calli originating from rice seeds of a genetically pure line (progeny of a doubled haploid from a spontaneous haploid of Oryza sativa L. var. Norin 8). Seed fertilities of 489 D_1 plants showed wide variation—58.7% were less than 80% fertile and 18.2% were less than 40% fertile. Plant heights ranged from 77 to 124 cm (av, 101.4 ± 9.1 cm) and no dwarf type mutants were observed.

In D_2 plants, 19.5% had less than 80% seed fertility and 5% had less than 40% seed fertility. Plant heights ranged from 29 to 135 cm, including malformed or dwarf types (av. 107 ± 11.2 cm, control 116.9 ± 6.8 cm).

The correlation between D_1 and D_2 (means of D_1 progeny) was $g = 0.365$ (significant at 1% level) in seed fertility and $g = 0.168$ (not significant) in plant height.

Table 1 shows chlorophyll mutation in D_2. The mutation rate was 8.4%, comparable to the chlorophyll mutation of seed progeny following X—ray and X—ray irradiation.

An examination of 5 marker characters (seed fertility, plant height, heading date, morphology, and chlorophyll mutation) showed that only 28.1% of the D_2 lines were normal in observed characters and 28.0% carried 2 or more mutated characters (Table 2). Of 6,382 D_2 plants, 1,491 (23.4%) were considered mutants of recessive homozygotes or dominant heterozygotes, including chromosomal aberrations. Table 3 shows the estimated mutation frequencies in each seed callus.

In seed callus progeny with 23 D_2 lines, 12 mutated characters (tetraploid, low seed fertility, complete ste—

Table 1. Chlorophyll mutation in immediate progeny (D_2) of plants (D_1) regenerated from rice calli.

D_1 panicles examined (No.)	Mutated panicles (No.)	Number and relative frequency of mutation												Mutation frequency[a] (%)
		Albina		Xantha		Viridis		Striata		Others		Total		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
438	34	11	29.7	9	24.3	7	18.9	4	10.8	6	16.2	37	100	8.4

[a] Mutation frequency (%) = $\frac{\text{Number of mutations}}{\text{Number of panicles}} \times 100$.

Table 2. Character changes of mutants in D_2 lines derived from panicles of D_1 plants regenerated from rice calli.

Mutated characters	Lines	
	No.	%
Normal	241	28.1
Ploidy (4x)	12	1.6
Seed fertility (Fer.)	273	35.8
Plant height (Ht.)	19	2.5
Heading date (Hd.)	3	0.4
Morphological traits (Mor.)	1	0.1
Chlorophyll deficiency (Ch.)	27	3.5
Fer. + Ht.	74	9.7
Fer. + Hd.	14	1.8
Fer. + Mor.	7	0.9
Fer. + Ch.	59	7.7
Ht. + Ch.	3	0.4
Hd. + Ch.	1	0.1
Mor. + Ch.	1	0.1
Fer. + Ht. + Hd.	11	1.4
Fer. + Ht. + Mor	2	0.3
Fer. + Ht. + Ch.	27	3.5
Fer. + Hd. + Ch.	2	0.3
Fer. + Mor. + Ch.	1	0.1
Fer. + Ht. + Hd. + Mor.	1	0.1
Fer. + Ht. + Hd. + Ch.	9	1.2
Fer. + Ht. + Mor. + Ch.	1	0.1
Total	798	100

rility, low plant height, dwarf, early heading date, albina, xantha, varidis, striata, diseased leaf spot, and lopped leaf) were observed. The same types of mutations, but with different frequencies, were common among D_2 lines derived from a seed callus (similar to different sectors of muta— tions in a plant). This suggests that mutations are induced repeatedly in the process of cell growth and division in the callus, similar to clonic irradiation treatment.

Figure 2 shows the agronomic characters of 150 D_3 lines. Most are fixed with mutated characters. Some agronomically useful characters, such as early heading date, low plant height, long panicle length, and increased number of panicles or grains, were obtained.

Table 3. Estimated frequency of mutation in calli induced from 75 seeds of a genetically pure line, analyzed from the progeny of regenerated plants.

Seed Callus (No.)	Estimated frequency of mutation (No.)												
	0	1	2	3	4	5	6	7	8	9	10	11	12
75	1	10	14	9	18	11	6		4	1			1

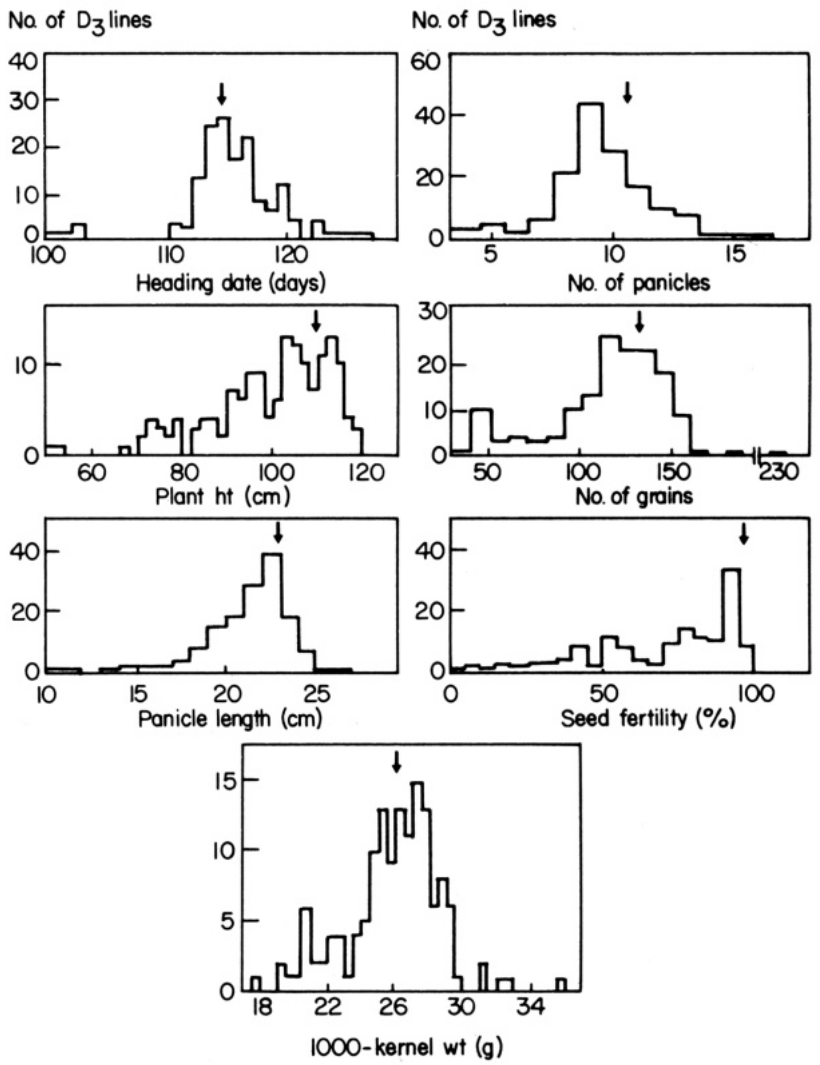

Fig. 2. Frequency distributions of seven agronomic characters of isolated mutants (D_3) of plants regenerated from rice seed calli.

MUTATION IN DIPLOID SOMATIC CALLUS IN RICE

Because frequent and repeated mutations are induced in rice calli, it is interesting to examine whether or not mutations in the chromosomes are random. Meristematic regions of the F_1 plant H32 were cultured and calli induced. H32 is a cross of LT—9/Murasaki—ine. LT—9 has characters of lopped leaf, chlorina, long glume, and waxy in single recessive genes harbored in different chromosomes. Murasaki—ine has characters of nonwaxy and purple plant color in dominant genes.

More than 40 D_1 plants were regenerated and selfed seeds were obtained. The D_2 lines with the most different segregation ratios of green and purple plants, H32—1 and H32—12, were compared with the F_2 generation of H32 in agronomic characters and frequencies of marker genes. The same numbers of green and purple plants in each progeny were planted in the field.

Heading date was later and plant height, panicle length, and seed fertility were significantly lower in H32—12 than in the F_2 of H32 (Table 4). However, variances of these four quantitative characters were larger than those of the F_2. Correlations between these quantitative characters also were larger than those for F_2. Lethal mutation (90/1000 plants) was segregated in H32—12 after the seedlings were trans—planted to the field. Analysis after classification of each character showed that lowering of plant height and seed fertility did not influence the gene frequencies of qualitative and quantitative characters.

It seems that these mutations were induced randomly in the chromosomes harboring marker genes. However, the possibility of the existence of hot spots of mutation in the chromosomes is not excluded. The genetic variability in somatic cell culture of F_1 is bigger than the range of F_2 segregation. This effect is comparable to the early experimental results of mutation effects by X—rays (Jalil and Yamaguchi 1965).

Mutation by somatic cell culture seems useful for broadening genetic variability. Regulation of shifts of such characters as lower plant height and low seed fertility is an interesting problem to be solved.

HOMOZYGOUS MUTATION

True breeding of mutated characters such as plant height and glaborous character in D_2 instead of in D_3, suggesting the occurrence of homozygous mutations in diploid tissues, was observed in plants regenerated from seed calli selected for by NaCl or S-(2-aminoethyl)-cystein resistance. For instance, the mean plant height of D_2 line NaCl 12—7 was 76.5 ± 2.87

Table 4. Comparison of nine characters in the second generation of regenerated plants (D_2) and the F_2 of the hybrid LT—9/Murasaki—ine.

Progeny	Plants (No.)	Days to heading	Plant height (cm)	Panicle length (cm)	Seed fertility (%)	Green/ purple (%)	Lopped leaf (%)	Chlo— rina (%)	Long glume (%)	Endosperm (%)
H32—1	1399	140.1	75.2	18.0	57	(49.8)	18.6	21.1	21.5	38.5(nonwaxy)
										22.2(waxy)
	(a^2)	8.5	259.6	10.8	817.0					29.9(mix)
										9.4(sterile)
H32—12	910	141.9	73.8	16.8	66	(40.2)	15.7	23.3	22.5	38.8(nonwaxy)
										19.7(waxy)
	(a^2)	6.8	308.9	13.4	1229.8					25.2(mix)
										16.4(sterile)
F_2:control	956	140.2	89.9	18.5	77	(46.1)	17.6	21.1	24.4	43.7(nonwaxy)
										26.8(waxy)
	(a^2)	5.8	165.0	6.5	405.9					29.3(mix)
										9.2(sterile)

cm (27 plants); Norin 8 was 121.2 ± 2.78 cm (29 plants). This character was fixed in D_3 and D_4. Reciprocal crosses with the control variety showed that this mutated character was not a maternal inheritance.

DISCUSSION

High frequencies of spontaneous mutation among plants regenerated from calli (only 28.1% of the plants regenerated from seed calli were normal in five observed characters) raise the obvious question of mechanism. Possible explanations are:

- The process of dedifferentiation or the dedifferentiated stage of cells is genetically unstable.
- Auxins or some other components act as mutagens.
- The culture media or conditions are not adequate for rice tissue culture.

Another experiment has been started to answer these questions.

The utilization of somatic mutations in vitro has great possibilities for practical rice breeding. First, we can manipulate large numbers of cells that may be regenerated into individual plants. Second, we can easily change the cultural environments by varying physical, chemical, or biological conditions. Third, mutations are induced repeatedly in the cells, similar to the effects of chronic irradiation. These may drift the cell population toward specific phenotypes and genotypes. The research on mutant selection by tissue culture shows the possibilities for application to practical plant breeding (Widholm 1977, Maliga 1978, Handro 1981).

At this moment, the drifting of phenotypes or genotypes caused by the cultural conditions of regenerated plants has not been studied for general tendencies. However, occurrences of lower plant height and seed fertility seem to be common phenomena in rice when MS or Miller's basic medium with 2,4-D is used for callus formation. At any rate, mutants with characters of practical importance were obtained in rice tissue culture. Induction and selection of other useful characters, such as salt tolerance and disease resistance, seem to be within our reach.

On the other hand, prevention of spontaneous mutation in cultured tissue is also an important problem if genetic resources are to be preserved in vitro. For studies of protoplast fusion and recombinant DNA technique, we should take into consideration that genetically stable cell culture is necessary. Otherwise, hybrids or transformants may acquire mutated characters spontaneously during the culture process.

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TOTIPOTENCY AND CYTOGENETIC STABILITY IN SUBCULTURES OF MAIZE POLLEN CALLUS

Gu Ming-guang, Zhang Xue-qin,
Cao Zi-yi, and Guo Cai-yue

The application of plant tissue and cell culture to plant improvement has been practiced in recent years. Because of the superiority of haploid cells in plant breeding, many researchers have been interested in culturing haploid tissues and cells to obtain mutants and pure lines. But two problems have hampered the utilization of plant tissue and cell culture: 1) the abilities of cells to proliferate and differentiate normally decrease or are lost in long-term in vitro cultures, and 2) callus with genetic stability is difficult to obtain in long-term cultures. Many reviews (Da'mato 1975, Heslop-Harrison 1967, Sunderland 1973, Torrey 1977) have discussed this situation. This paper deals with the potential of regeneration and the cytogenetic stability of callus clones derived from maize pollen in long-term culture.

MATERIALS AND METHODS

In 1978, Cao Zi-yi et al obtained a pollen callus of maize variety Ba-Tong-paiby anther culture. This callus had a high ability to differentiate and regenerate in subculture. It was subcultured at intervals of 3-4 weeks for 3 years. After a year in subculture, callus clones from the original callus mass were established by selection. Systematic obser vations were undertaken to better understand the ability of these clones to differentiate and regenerate and to examine their cytogenetic stability.

Cultures were incubated at 25-27°C in cool white fluorescent light (1500 lux) for 9-10 hours.

The medium for subculture contained inorganic salts Fe (Murashige and Skoog 1962), organic acids (Straus 1960) with 1.0 mg 2,4-D/liter, 3% sucrose, 0.8% agar, 500 mg. CH/liter, 100 mg inositol/liter, and 2,000 mg asparagine/liter. The differentiation medium was N_6 (Chu 1978) containing 0.1 mg NAA/liter, 1.0 mg K/liter, 500 mg CH/liter, 6% sucrose, 0.5% C, and 0.8% agar.

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Calli and root tips of regenerated plantlets were fixed directly in acetic acid and alcohol (1:3) for 12 to 24 hours. The preparations were stained with iron vitriol haematine crystal and aceto-carmine. The karyotype of cultured cells was studied by BSG Giemsa banding technique (Gu Ming-guang and Zhang Xue-gin 1981).

RESULTS

Totipotency of calli

Pollen calli with ability to differentiate and regenerate were maintained in a medium as subcultures. After 1 year, the differentiation/regeneration abilities of callus clones changed remarkably. Some still possessed high differentiation/regeneration capacity, others lost it. Four totipotency were observed:

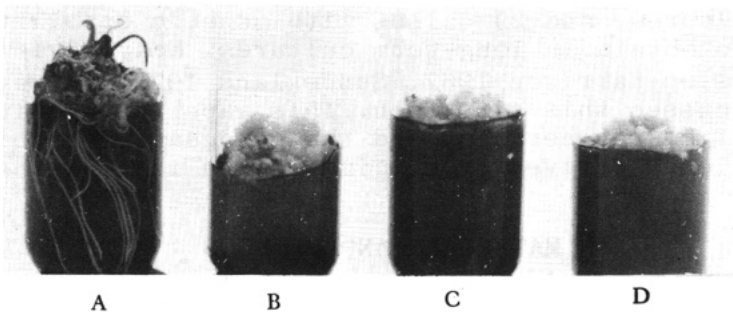


Fig. 1. Four types of totipotency observed in maize pollen callus after 1 year in subculture.

- Callus clones that completely lost the potential to differentiate and regenerate. (Fig. 1 D, Fig. 2A).
- Callus clones that differentiated and formed root-like structures with green spots. (Fig. 1C).
- Callus clones that grew into a few shoots, but did not develop into normal plants.(Fig. 1B).
- Callus clones possessed a strong ability to differentiate and regenerate. They developed embryoids as well as shoots without transfer to a regeneration medium. Soon after transfer, they readily regenerated into shoots and green plantlets with profuse roots (Fig. 1A, Fig. 2B-1, 2 B-2).

More than 200 plants have been regenerated from callus clones so far. Plant development is through somatic embryogenesis. Some plants have been transplanted into soil in the greenhouse. The majority of the regenerated plants have a normal appearance. Chromosomes counts show that they are haploids (Fig. 3).

Two conditions of plant regeneration from pollen callus

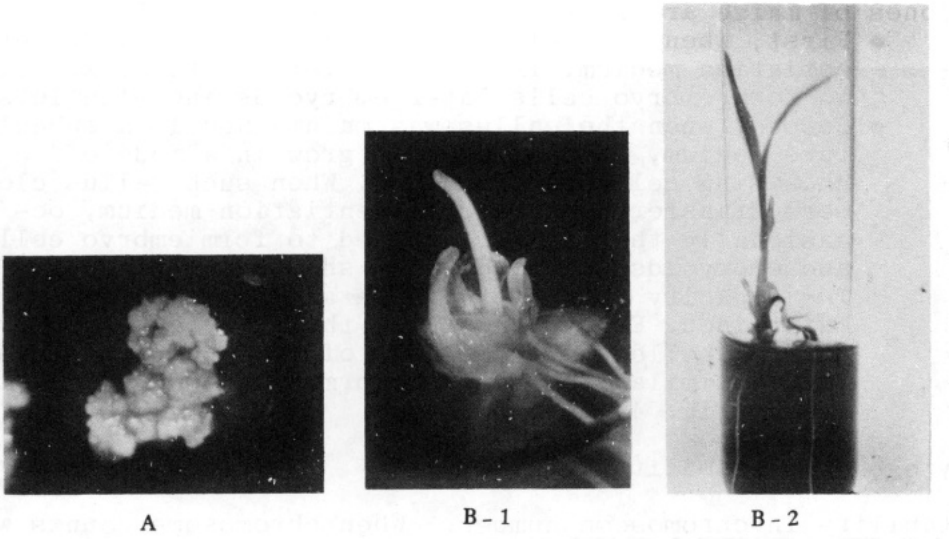


Fig. 2. Two extremes of totipotency observed in maize pol— len callus after 1 year in subculture. A = callus clones that completely loss their potential to differentiate and regenerate. B = callus clones that possessed a strong ability to differentiate and regenerate, developing em— bryoids (1) which regenerated into shoots (2) green plantlets.

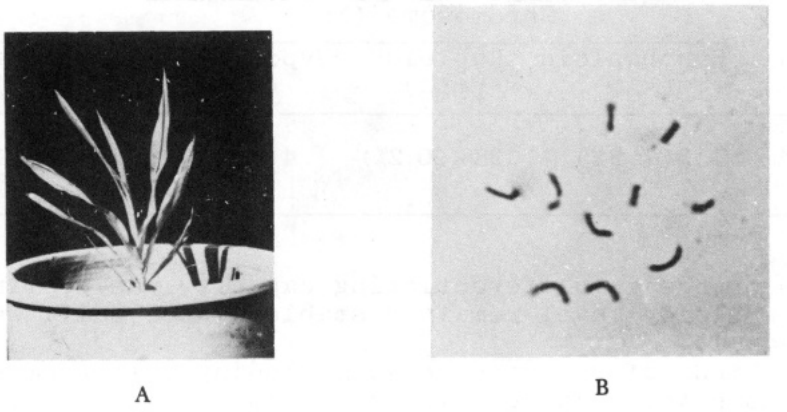


Fig. 3. Plant (A) regenerated from callus clone has normal appearances.Chromosome count (B) shows that it is haploid.

clones of maize are worth consideration.

- First, when the callus was transferred to a differentiation medium, it could differentiate at any time to form embryo cells later embryoids and plantlets.
- Second, when the callus was maintained in a subculture medium, it continued to grow in a mode of unceasing cell proliferation. When such callus clones were transferred to a differentiation medium, occasionally they differentiated to form embryo cells and embryoids and to develop shoots and plantlets. The capacity to differentiate and regenerate was maintained. Embryogenesis of the somatic cells derived from pollen callus clones of maize passed through stages similar to those of normal maize zygotic embryos (Fig. 4).

Cytogenetic stability in calli

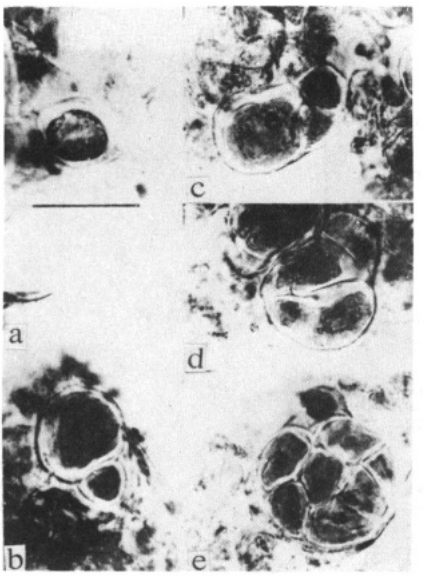
Stability in chromosome number. When chromosome counts were made of pollen calli maintained on a subculture medium for 1 year, less variation was found in chromosome number. Of 1,539 cells randomly chosen at the metaphase stage, 90% were haploid; only 10% were hypohaploids, diploids, and hypodiploids (Table 1).

Table 1. Chromosome numbers of maize pollen calli subcultured for one year.

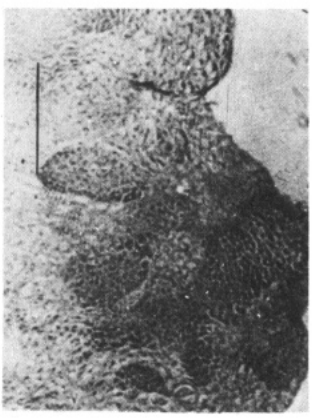
Cells examined (No.)	Chromosome (No.)			
	Hypohaploid (<10)	Haploid (=10)	Hypodiploid (<20)	Diploid (=20)
1539	115(7.5%)	1388(90.2%)	4(0.2%)	32(2.1%)

The change in differentiating capacity was prominent while the ploidy level remained stable. These calli were still haploid.

Two kinds of callus clones, including differentiating callus clone No. 1 and undifferentiating callus clone No. 250, were examined in subculture 3 times. The chromosome numbers in the calli of clone No. 1, which had a great ability to differentiate and regenerate, were not affected during subculture. Chromosome counts at the metaphase stage showed that more than 90% were haploid and less than 10% were cells with other chromosome ploidy. Among them, hypohaploid was not more than 7% and hypodiploid and diploid were not more than 3% (Table 2).



A



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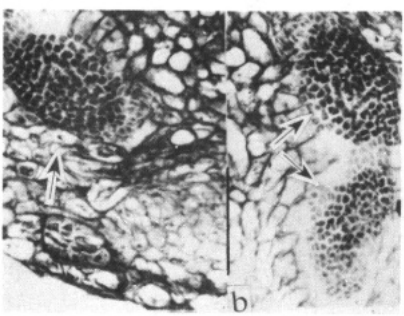
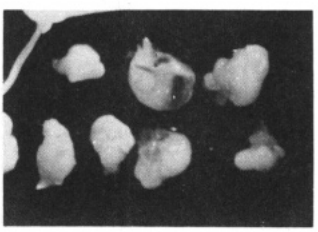


B



D

Fig. 4. Somatic cells derived from pollen callus clones of maize passed through embryogenesis stages (A, B, C, and D) similar to those of normal zygote embryos.

Table 2. Effect of long—term subcultures on chromosome numbers of callus clone cells.

Material	Subculture time (months)	Cells examined (No.)	Chromosomes							
			Hypohaploid (<10)		Haploid (=10)		Hypodiploid (<20)		Diploid (=20)	
			No.	%	No.	%	No.	%	No.	%
Clone No.1 a)	20	288	19	(6.6%)	260	(90.3%)	2	(0.7%)	7	(2.4%)
	25	172	4	(2.3%)	163	(94.8%)			5	(2.9%)
	27	1125	29	(2.6%)	1079	(95.9%)	2	(0.2%)	15	(1.3%)
Clone No. 250 b)	20	273	18	(6.6%)	246	(90.1%)	2	(0.7%)	7	(2.6%)
	25	145	6	(4.1%)	135	(93.1%)	2	(1.4%)	2	(1.4%)
	27	292	9	(3.1%)	282	(96.6%)			1	(0.3%)

a) Differentiating callus. b) Undifferentiating callus.

Despite a complete difference in ability to differentiate and regenerate, callus No. 250 showed similar results. Cytological investigation showed that haploidization and stability of chromosome numbers were not affected by a change in ability to differentiate and regenerate. This also may show the cytogenetical stability of the callus clone cells, however long the culture duration.

After 2 years in subculture, chromosome counts were made on clone No. 1 calli and on the root-tip cells of 35 plants regenerated from the clone. Table 3 shows that 89.7% of 427 calli cells and 87.4% of 645 root-tip cells were

Table 3. Chromosome numbers of clone No. 1 calli and root-tip cells of 35 regenerated plants.

Material	Cells examined (No.)	Chromosome (No.) Hypohaploid (<10)	Haploid (=10)	Hypodiploid (<20)	Diploid (=20)
Clone No.1	427	34(7.9%)	383(89.7%)	2(0.5%)	8(1.9%)
Root-tip cells	645	25(3.9%)	564(87.4%)	10(1.6%)	46(7.1%)

haploid. At the same time, about 1.9% of other calli cells and 7.1% of other root-tip cells diploidized by spontaneous chromosome doubling. It is reasonable to say that, in the early stage of development of the callus clones, more than 90% of the cells were haploid. But the diploidization of cells in regenerated plants prominently increased over that of the callus clones.

Another identification of the ploidy level of the 35 regenerated plants also revealed that 33 plants (94.3% of the total) were haploid. Only 2 plants (5.7%) were mixoploid. No diploid plants were found.

From callus to plantlet, most cells were haploid (Table 3). The changes in chromosome numbers of both callus and root tips of regenerated plants were the same. It may be concluded that the variation in chromosome numbers of root-tip cells was not affected by auxins and cytokinins in differentiation medium. In general, alterations in chromosome structure were not as frequent as those in chromosome number. However, at the metaphase and anaphase stages, nucleolus and fragmented, laggard, ring and dicentric chromosomes were occasionally observed (Fig. 5). These irregularities could have been produced by chromosome breakage and reunion. Chemical as well as physical components of the cultures may play some role in chromosome breakage.

Banding of karyotype. The karyotype of haploid cells and

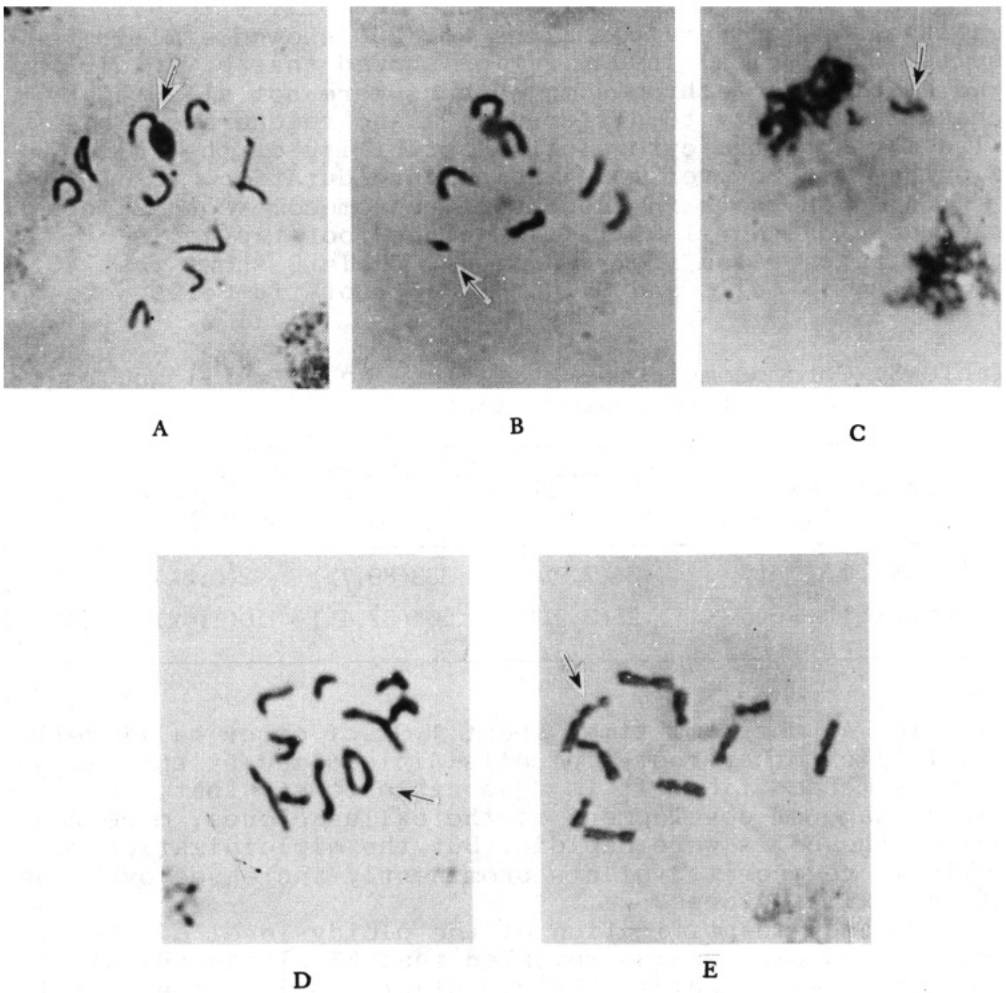


Fig. 5. At metaphase and anaphase stages of mitosis, (A,B, C,D and E) nuclealus and fragmented, laggard, ring and dicentric chromosomes were occasionally observed.

cells with spontaneously doubled numbers in callus clone No. 1 was studied by the BSG Giemsa banding technique. Prominent bands were present on 5 of the 10 chromosomes in 50 randomly selected haploid cells at the metaphase of mitotic (Fig. 6). There were large subterminal bands on the long arm of chromosomes 1,4, and 7 and a small terminal band on the short arm of chromosome 9. The nucleoli organ— izer and subterminal band on the long arm of chromosome 6 were clear, but the satellite stained slightly. The other five chromosomes did not show banding. The centromere of

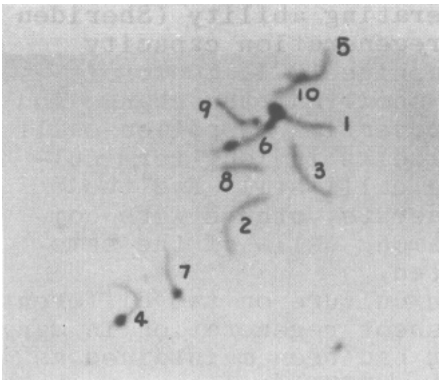


Fig. 6. Banding of karyotype of haploid cells on chromosomes 1,4,6,7, and 9.

all chromosomes did not have any banding. About 50 root—tip cells at the metaphase of plants regenerated from callus clone No. 1 had the same Giemsa banding pattern.

It has been reported that spontaneous chromosome doubling is often derived from endomitosis in the process of proliferation of cells in in vitro cultures (Jensen 1974). About 2% of the haploid cells from clone No. 1 in long—term subculture were diploid by spontaneous chromosome doubling. Banding patterns of these diploid cells were similar to those of the haploid cells (Fig. 7). Polymorphism and heterozygosity were not observed.

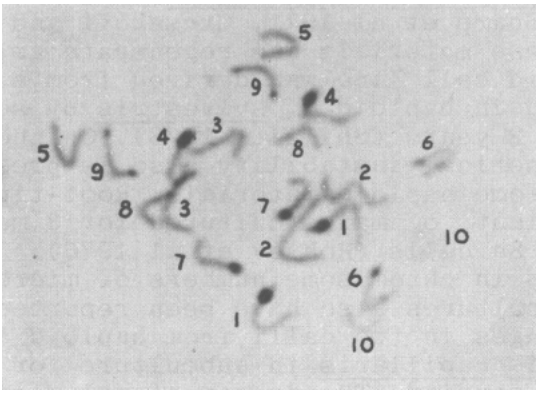


Fig. 7. Banding patterns of diploid cells from spontaneous chromosome doubling of haploid cells in long—term subculture.

DISCUSSION

It has been reported that the regeneration capacity in some species is not influenced by the age of callus (Murashige 1974, Scowcroft 1977). After 7 years in culture, Lilium

longiflorum still possessed regenerating ability (Sheriden 1975). However, in many species, regeneration capacity decline with time in culture (Murashige 1974, Scowcroft 1977, Sheriden 1975). Our results show that the change in differentiation and regeneration potential of pollen calli derived from the proliferation of cells in long—term culture was very great. Some of these calli still had the ability to differentiate and regenerate, others were completely undifferentiatable. Even among cells of the same callus mass, this phenomenon existed.

With this method of alternate culture on two different media, it was possible to induce shoot regeneration in many culture lines of haploid rape that had been maintained as calli for nearly 3 years. (Sacristan 1981).

We conclude that maintenance of the ability to differentiate and regenerate in culture is related not only to genetical differences in original materials and to the culture medium, but also to selection from generation to generation in the subculture.

A great cytogenetical stability has been found in culture calli derived from originally haploid materials. In some plants in longterm subculture, ploidy was stable. Examples of such materials are Antirrhinum majus, Nicotiana tabacum, and Lycopersicon esculentum (Melchers et al 1958, Carlson 1970, Sharp et al 1971, Gresshoff and Doy 1972). But whether these materials can regenerate is not explained. A stable haploid cell line was derived from a mixoploid that originated in haploid N. sylvestris by selection in subculture for 2 years (Shillito 1978). On the other hand, a great cytogenetical instability also is present in calli cultured from some haploid materials. Root—tip callus from haploid plants of maize cultured for 3 months included n, 2n, 4n, and 8n cells (Hohlov et a1 1976).

Variations in chromosome numbers of microspore callus of rice in subcultures also have been reported (Chen 1980). Karyotypic changes in two calli from haploid and diploid plants of Crepis capillaris in subculture for more than a year have been studied. The degree of polyploidization of the original haploid culture was considerably higher than that of the diploid culture (Sacristan 1971). Calli, cultures from haploid Brassica napus have been maintained for about 3 years as unorganized calli. Most regenerated plants of these calli were diploid; no plant was haploid (Sacristan 1981). The significant differences among material which has occurred may be related to the genetical background of the donor material of in vitro culture.

These results show that a great cytogenetical stability is present in the clones of pollen callus derived from anther culture of maize variety Ba—Tong—pai.Among them, the calli of clone No. 1 demonstrated unlimited totipotency by regeneration in subculture for 3 years. The calli clone with stability and totipotency may be a new approach to

cell culture methods for plant improvement.

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INITIAL TYPES OF WHEAT POLLEN CELLS AND THEIR DEVELOPMENT IN ANTHER CULTURE

Pan Jing—li, Gao Gong—hong, and Dan Hai

Since Fujii (1970) first obtained pollen callus from wheat anthers cultured in vitro, a number of experiments have been done on wheat anther culture (Zhu Zhi—qing et al 1978, Zeng Jun—zhi and Onyang Jun—wen 1980, Pan Jing—li and Gao Gong—hong 1978). To find more satisfactory conditions for diverting pollen into sporophytic developments, it seems necessary to study further the conditions for initiating dedifferentiation of wheat pollen and its morphogenetic process after the initial division.

MATERIALS AND METHODS

Anthers for culture were taken from the F_1 hybrid of Ai Feng No. 2—1/Cajeme F 71. Those containing microspores at the late uninucleate stage and suspending on N_6 liquid medium with 10% sucrose were subjected to cold treatment at 3—5°C for 72 hours before inoculation. Some pretreated anthers were suspended on N_6 liquid medium with 10% sucrose and others on N_6 liquid medium supplemented with 12 mg IAA/liter, 2 mg K/liter, 300 mg CH/liter, and 10% sucrose. Culture was under diffused light at 28 ± 1°C.

To examine cell dynamics, anthers were fixed with acetic acidalcohol (1:3) at regular intervals after inoculation and stained with parafuchsin (Feulgen Reaction).

EXPERIMENTAL RESULTS

Development after initial division

Abnormal divisions of pollen cells diverted into sporophytes were most prevalent on days 3—7 of culture. After day 7, the number of divisions gradually declined. In 20 anthers observed on days 3 and 7, the first mitosis of pollen diverted into sporophytes in vitro showed both equal and unequal divisions. Using staining intensity after the first division and participation in the formation of

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multicellular pollen, we classified abnormal pollen into eight types (Fig. 1):

Type A - vegetative nuclei resulting in cells after unequal division. First division of uninucleate pollen was unequal with a typical generative cell attached to the intine and a vegetative nucleus in the center of the pollen. The generative cell could not develop further and degen— erated. The vegetative nucleus divided continuously and formed multicellular pollen.

Type A_1 - vegetative nuclei in free state after unequal division. Pollen grains were similar to Type A during the first division but the vegetative nucleus did not form a cell wall after division. Daughter nuclei were in a free state. The generative cell degenerated.

Type B - vegetative nuclei resulting in cells after equal division. First division of uninucleate pollen resulted in two equal vegetative nuclei with diffuse chromatin. They continued to divide and formed multicellular pollen.

Type B_1 - vegetative nuclei in free state after equal division. Binucleate pollen was formed after the first equal division of uninucleate pollen but was not accom— panied by the formation of a new cell wall. Daughter nuclei continuously divided in a free state.

Type C - both vegetative and generative nuclei resulting in cells after unequal division. Behavior of first mitosis was similar to Type A, but both vegetative and generative nuclei divided and participated in the formation of mul— ticellular pollen during subsequent development Fig. 2).

Type C_1 - both vegetative and generative nuclei in a free state after unequal division. Nuclear behavior was similar to Type C, but daughter nuclei were free in the cytoplasm of the pollen. After one or two times in the initial division in forming a cell wall, nuclei were still in a free state (Fig. 3).

Type D - generative nuclei resulting in cells after equal division. Uninucleate pollen divided equally and gave rise to two identical daughter nuclei, both of which possessed the generative feature (condensed chromatin). They could go on dividing, resulting in the formation of multicellular pollen (Fig. 4).

Type D_1 - generative nuclei in free state after equal division. Behavior of nuclear division was similar to Type D, but daughter nuclei were free in the cytoplasm (Fig. 5 A—E) or were still in a free state after forming cell walls one or two times at the early stages of development.

In addition, pollen with micronuclei varying in size

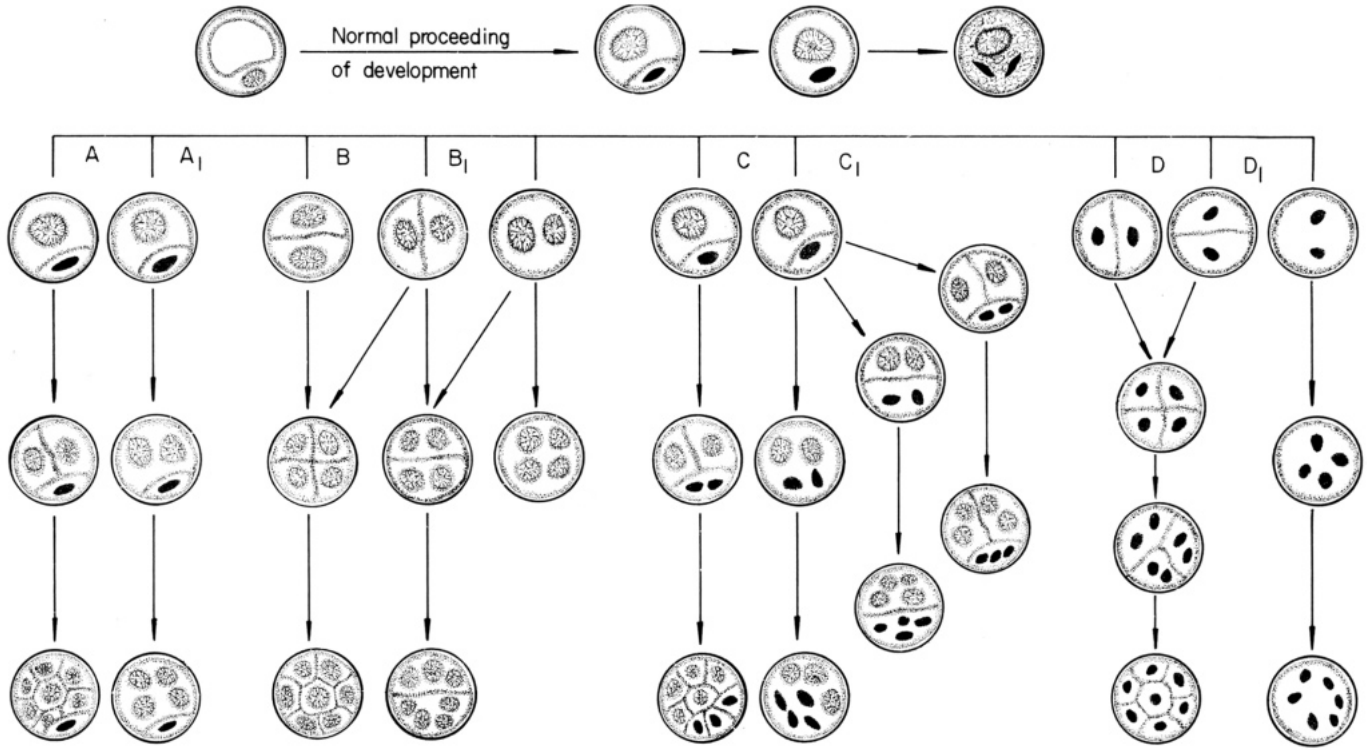

Fig. 1. Abnormal divisions of pollen cells diverted into sporophytes could be classified into 8 types. A = vegetative nuclei resulting in cells after unequal division. A_1 = vegetative nuclei in free state after unequal division. B = vegetative nuclei resulting in cells after equal division. B_1 = vegetative nuclei in free state after equal division. C = both vegetative and generative nuclei resulting in cells after unequal division. C_1 = both vegetative and generative nuclei in a free state after unequal division. D = generative nuclei resulting in cells after equal division. D_1 = generative nuclei in free state after equal division.

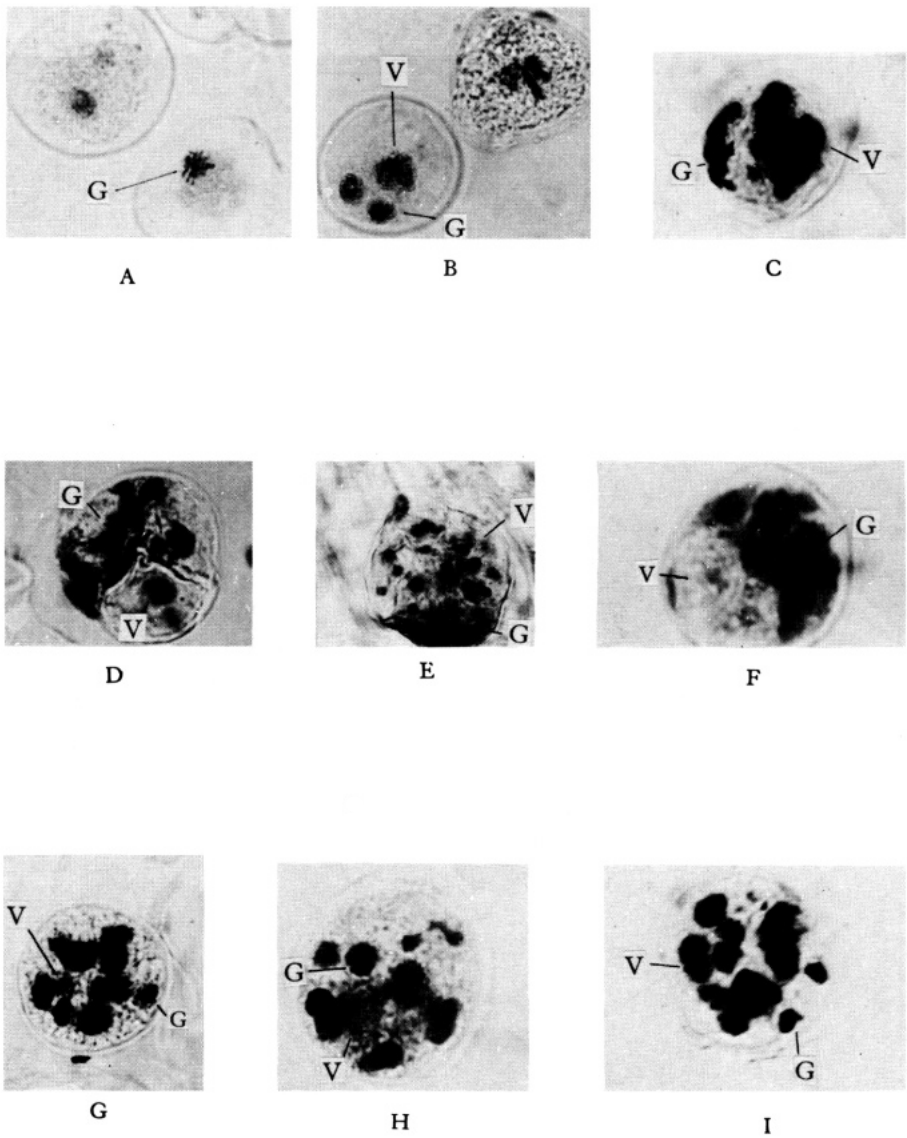

Fig. 2. Type C vegetative and generative nuclei divided and participated in the formation of multicellular pollen during subsequent development.

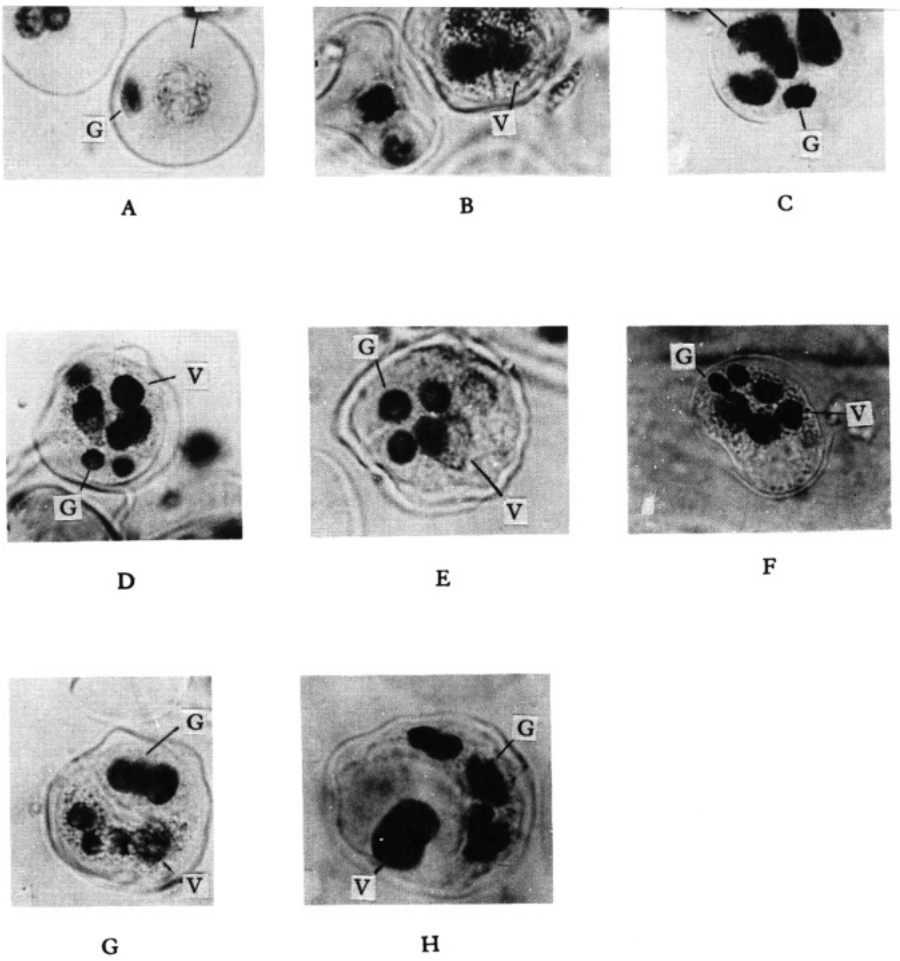

Fig. 3. Type C_1 daughter nuclei were free in the cytoplasm of the pollen, sometimes even after formation of a cell wall.

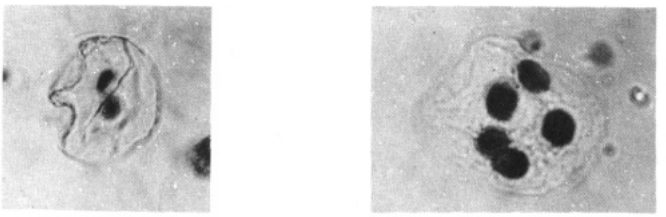

Fig. 4. Type D identical Generative daughter nuclei divided and participated in multicellular pollen.

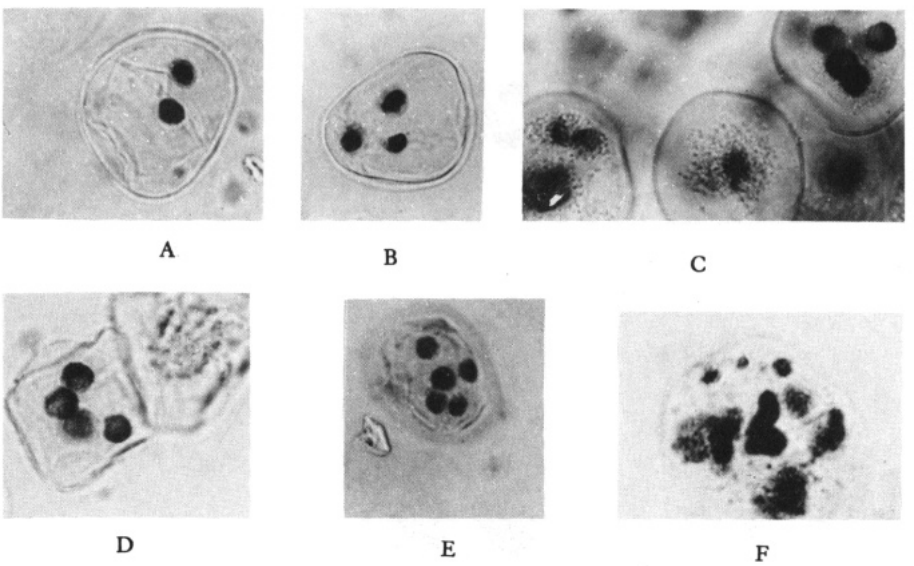

Fig. 5. Type D_1 generative daughter nuclei were free in the cytoplasm.

and number have been found (Fig. 5F).

Chromosome doubling often occurred in the vegetative components in Type A and Type B (Fig. 6). A doubled multicellular pollen might be formed if they continued to divide.

Multicellular pollen formation

Our previous experiments showed that, when anthers pret—reated at 3—5°C for 72 hours are cultured on solid N_6 medium supplemented with 12 mg IAA/liter, 2 mg K/liter, 300 mg CH/liter, and 10% sucrose, induction frequencies of abnormal pollen and multicellular pollen are higher (Pan Jing—li and Gao Gong—hong 1980). To find the optimal

Table 1. Development of multicellular pollen (day 15).

Medium	Anthers observed (No.)	Anthers with empty pollen (No.)	Multicellular pollen (cell No.)					Embryoids or cell masses with > 50 cells (No.)
			4—10	11—20	21—30	31—40	41—50	
N_6	70	27	322	297	50	19	2	9
N_6 +1AA^{12} +K^{2} +CH^{300} [a)]	62	35	575	566	101	33	5	48

[a)] K = kinetin, CH = casein hydrolysate.

condition for multicellular pollen formation, we made this test:

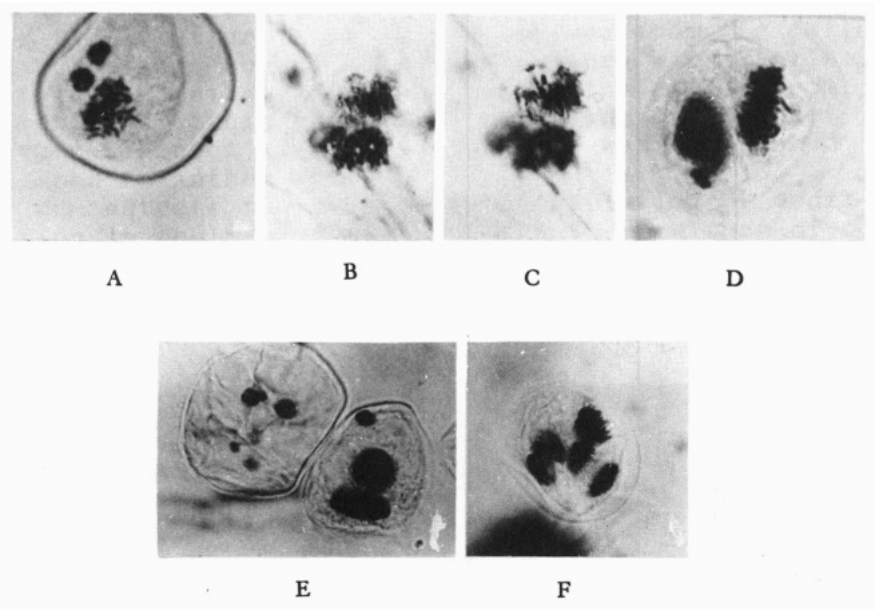

Fig. 6. Chromosome doubling occurred in the vegeta—tive components of types A and B pollen cells.

Anthers were floated on liquid N_6 medium with 10% sucrose at 3—5°C for 72 hours. Some of these pretreated anthers were suspended on medium N_6 with 10% sucrose and the others on N_6 medium with 12 mg IAA/liter, 2 mg K/liter, 300 CH/liter, and 10% sucrose.

Induction of any type of multicellular pollen growing on the medium with concentrated external hormones was much higher than induction on the medium without hormones (Table 1). The number of embryoids or multicellular masses on the hormone—supplemented medium was about five times more than that on the medium without hormones. Multicel—lular pollens per anther on the hormone supplemented medium averaged as high as 21.42 units, as compared to only 9.99 units on the medium without hormones. On the medium with more concentrated hormones, multicellular pollen scattered all over the surface of some anthers (Fig. 7). The yields of multicellular pollen per anther on both the medium with concentrated hormones and the medium without hormones were considerably higher than in any previous experiments. This show that the effects of pollen induction differ with the conditions in which anthers are pretreated. It is necessary to find more satisfactory conditions for anther pretreat—ment.

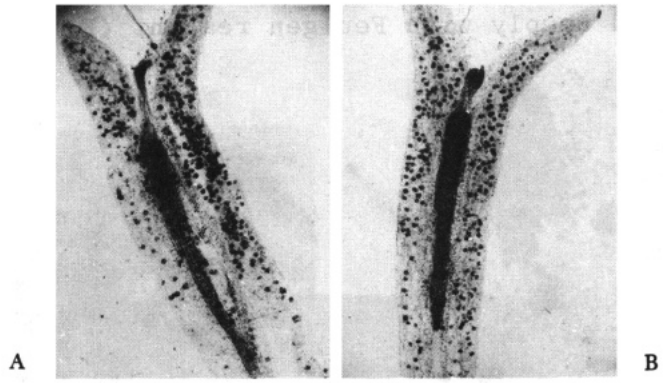

Fig. 7. Multicellular pollen scattered over the surface of some anthers plated on a medium with more concentrated hormones.

Influence of the state of anther wall cells

We also noticed that the development of the pollen cells is related to the state of the anther wall cells. And, the state of the anther wall cells is closely related to the external hormones provided. Although there were abnormal pollen and multicellular pollen on the medium without hormones, they were mostly in a degenerating state. The anther wall cells also were degenerating. Although there were formations of embryoid, the anther wall cells around

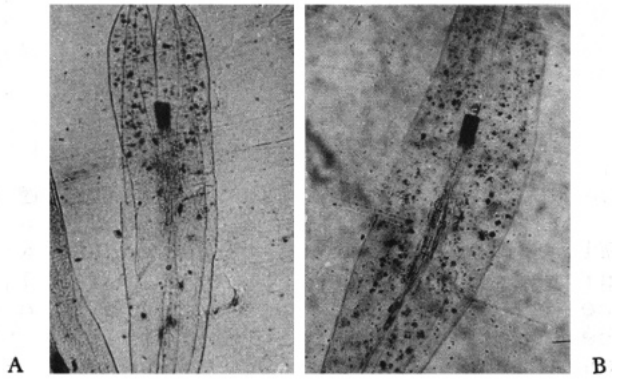

Fig. 8. Although embryoids formed on a medium without hormones, the anther wall cells around them were completely dead.

them were completely dead (Fig. 8). But in anthers with a large number of viable multicellular pollen, the anther wall cells - particularly those around the embryoids with stronger viability - were full of vitality. Sometimes we found that the anther wall cells surrounding the embryoids were very small. The protoplasm became dense and their

nuclei stained deeply with Feulgen reagent (Fig. 9).

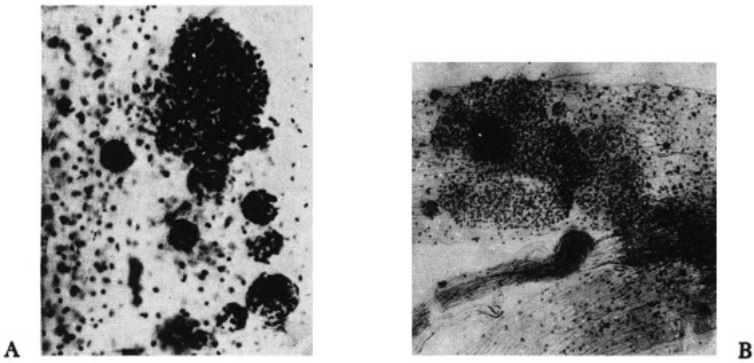

Fig. 9. In anthers with large numbers of viable multicellular pollen, sometimes anther wall cells surrounding embryoids were very small. The protoplasm became dense and their nuclei stained deeply with Feulgen reagent.

These phenomenon could be observed only on the medium with more concentrated hormones. We concluded that pollen developments are closely related to the viability of the anther wall tissues. External hormones could keep viability and prolong the life of the anther wall cells, improving further division and growth of the pollen diverted into sporophytic development.

DISCUSSION

Development pathways of pollen cells

Based on the behavior of the initial division of pollen and the origin of the embryoid in anther culture, Sunderland and Wick (1971) and Sunderland and Dunwell (1977) summarized that there are three pathways of pollen development, types A, B, and C. Types A and B also have been observed in wheat anther cultures (Zhu Zhi-qing et al 1978, Zeng Jun-zhi and Onyang Jun-wen 1980). The pollen plants developed from a vegetative cell formed after unequal division (type A) and two similar nuclei with vegetative feature formed after equal division (type B). In wheat, we observed not only the two basic types but also a type C similar to the E pathway in rice triticale and barley(Sun Ching-san 1978). Both the vegetative and generative components divide and participate in the formation of multicellular pollen. Zhu Zhi-qing (1978) also accidentally found this type in wheat.

We also observed that the cells or nuclei derived from

the generative nucleus participated in the formation of multicellular or multinucleate pollen. This we called type D.

There were certain numbers of types C and D pollen, suggesting that pollen plants also may be derived from multicellular pollens with generative component. It should be emphasized that, when the sections stained with Feulgen reagent were observed under a light microscope, the structures of the cell wall were not observed in the first divisions of most pollen diverted into sporophytic development. However, a lot of multicellular pollen with walls were found on day 15. This seems to suggest that initial divisions of pollen diverted into sporophyte followed the same pattern of segmentation as occurred in the formation of nucleate—type endosperm in the angiosperm, in which the nuclei in the early stages were in a free state. This remains to be ascertained.

Relationship between state of anther wall cell and pollen cell development

Sung Pei—lun et al (1978) pointed out that the tapetum has no direct relationship with the androgenesis of pollen in culture and that cells of both the anther connective and the middle layer of the anther wall were effective for the induction of androgenesis of pollen. The middle layer of the anther wall seems to be the principal factor. Liang Hai—man et al (1980) pointed out that timely degeneration of anther wall tissues, including tapetum, might be favorable for the initiation of dedifferentiation of pollen cells.

We found that, when wheat anthers produced more viable multicellular pollen on the medium with more concentrated hormones, the protoplasms of anther wall cells and the parenchyma cells of connective tissue became dense. But when the multicellular pollen in the anther were not induced or were poorly induced on the medium without hormones, the induced multicellular pollen themselves degenerated and the protoplasm of parenchyma cells of the anther wall became thin or degenerated and died. This suggests that the anther wall cells play an important role in further development of pollen and that plant hormones can keep anther wall tissues viable for a longer time (at least up to day 15 in culture). This improved the normal development of multicellular pollen.

Potentiality of diverted sporophytic developments

A current problem in the anther culture of all but a few species of cereal crops is inadequate yield. The induction frequency of pollen plants is low and haploid breeding is hindered. To find a way to overcome this inadequacy, we

attempted to study the development of pollen cells by dividing the process of pollen plant formation into different stages. First was a study of early pollen development, because whether or not division can be initiated to further develop into multicellular pollen is the determinant of anther yield.

The results showed that once conditions were greatly changed, the microspores characterized by gametophytic genotypes could divert into sporophytic development. The ratio changes as culture conditions change. For example, the results show multicellular pollen numbers of more than 100 in some anthers and that the potential for pollen developing into sporophytes is considerable.

The culture conditions may affect the embryogenic potential of the pollen. Because the yield of multicellular pollen increased continuously with further improvement of culture conditions, we believe that the induction frequency of pollen plants can be increased to satisfy the requirements for breeding during the process of finding the satisfactory conditions of nutrition and culture.

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TOWARD THE DEVELOPMENT OF A SINGLE CELL SYSTEM FOR GRASSES

Indra K. Vasil

Major advances in plant cell and tissue culture techniques during the last two decades have helped stimulate unprecedented research interest and activity in the plant sciences. Much of this interest is based on the demonstration that genetically modified plants can be produced without sexual reproduction by the fusion of somatic cells, by the selection of mutants/variants from anther-derived haploids or cell cultures, and by genetic transformation (Vasil 1980a, b; 1981). At the same time, remarkable developments of molecular biology and recombinant DNA technology, including the identification, characterization, and cloning of genetic material (Leaver 1980), have made it possible to attempt transformation of plant cells.

The potential of these powerful new tools for generating genetic variability has been demonstrated in model plant systems such as tobacco and petunia. However, the regeneration of plants from single cells is considered the most important basic requirement for applying the techniques of somatic hybridization, somatic cell genetics, and genetic modification to food crop species. The ability to grow plants from single cells of a model plant species was the key to the few but dramatic instances of success.

Unfortunately, progress in the development of similar single cell systems for important groups of crop plants such as grasses and legumes has been painstakingly difficult and slow.

Regeneration of plants from tissue cultures of most of the important species of cereals and grasses has been reported (Green 1978, Thomas et a1 1979, Vasil et a1 1979, Vasil and Vasil 1980a). But regenerative ability is sporadic, short-lived, and limited to a few genotypes of each species. Furthermore, plants often are formed by the depression of presumptive shoot primordia which proliferate adventitiously

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or, in rare instances, by the de novo organization of shoot primordia on the surface of callus tissues (Rangan 1974, Dunstan et al 1979, Nakano and Maeda 1979, Shimada and Yamada 1979, Springer et al 1979).

Shoot meristems in vivo and in vitro are believed to be multicellular in origin (Crooks 1933, Vasil and Hildebrandt 1966, Steffensen 1968, Coe and Neuffer 1978). Plants of multicellular origin cannot always be expected to be genetically uniform. They may be chimeras (Sacristan and Melchers 1969, Ogura 1976, Sree Ramulu et al 1976, Bennici and D'Amato 1978, Mix et al 1978) and unsuitable for mutation research, genetic analyses, maintenance of genetic stocks, and breeding.

Therefore, it would be advantageous to obtain plants from isolated single cells such as protoplasts or through the formation of somatic embryos which, like their zygotic counterparts, arise from single cells either directly or after the formation of a pro-embryonal complex (Halperin 1969, Backs-Husemann and Reinert 1970, Konar et a1 1972, Thomas et al 1972, McWilliam et al 1974, Street and Withers 1974, Haccius 1978).

Formation of embryos from somatic cells takes place both in nature and in vitro in a wide variety of angiosperms (Tisserat et al 1979). Until recently, the phenomenon of somatic embryogenesis in tissue cultures of cereals and grasses was considered rare. An early report described embryogenesis and the production of only albino plants from cell cultures of Bromus inermis (Gamborg et al 1970). There also are numerous reports of embryogenesis in anther cultures of many species of the Gramineae (Tisserat et al 1979, Vasil 1980c), but convincing histological evidence for the formation of embryos in most cases is not available.

This paper describes recent progress in our laboratory on the development of single cell systems for several species of grasses.

SOMATIC EMBRYOGENESIS IN CALLUS TISSUE CULTURES

Embryo-like structures have been formed from proliferating immature embryos of barley (Norstog 1970) and sorghum (Dunstan et al 1978, 1979). No plants were obtained from barley embryos. In sorghum, plant formation took place largely by the derepression and proliferation of presumptive shoot primordia in the process of microtillering.

The first detailed documentation for somatic embryogenesis in tissue cultures of grasses was provided in Pennisetum americanum (Vasil and Vasil 1981a). Since then, we have described the formation of somatic embryos and plants from a variety of tissue explants and species of grasses (Table 1). Immature embryos, young inflorescences, and young leaves proved to be equally suitable for inducing the formation of a compact and organized callus which formed numerous

Table 1. Somatic embryogenesis in tissue cultures of grasses.

Species	Source of explant	Type of cell culture	Reference
Panicum maximum	Immature embryo, young inflorescence, young leaf	Callus, suspension, and protoplast	Lu and Vasil (1981a,bc) Lu et al (1981)
Pennisetum americanum	Immature embryo, young inflorescence, young leaf	Callus, suspension, and protoplast	Vasil and Vasil (1980b, 1981a,b, 1982), Rangan and Vasil (unpubl.)
Pennisetum americanum × P. purpureum	Young inflorescence	Callus	Vasil and Vasil (1981a)
Pennisetum purpureum	Young leaf, young inflorescence, anther	Callus	Haydu and Vasil (1981), Wang and Vasil (1982)
Saccharum officinarum	Young leaf	Callus and suspension	Ho and Vasil (unpubl.)
Sorghum bicolor	Immature embryo	Callus	Boyes and Vasil (unpubl.)
Triticum aestivum	Immature embryo	Callus	Ozias-Akins and Vasil (1982)
Zea mays	Immature embryo	Callus and suspension	Lu and Vasil (unpubl.)

embryos. In the case of immature embryos (Vasil and Vasil 1981a, Lu and Vasil 1981a, Ozias—Akins and Vasil 1982), only the scutellar tissue gave rise to such an embryogenic callus. In leaves, the cells of the lower epidermis as well as meso—phyll cells near the vascular bundles proliferated to form embryogenic callus. Only the compact, generally white, opaque, organized callus showed the capacity to form somatic embryos. The soft, friable, transluscent, generally unorganized callus commonly formed from mature tissue explants did not form embryos. The embryogenic callus was composed of small, richly cytoplasmic, thin—walled cells which lacked conspicuous vacuolation but which contained many prominent starch grains. These cells are reminiscent of cells found in shoot and root meristems.

In almost all instances, the embryogenic callus was obtained on Murashige and Skoog's (1962) nutrient medium containing 1—10 mg 2,4—D/liter. Somatic embryos were obtained when the level of 2,4—D in the medium was either reduced or totally eliminated. Embryos formed in callus cultures ger—minated in vitro and the resulting plants were grown to maturity in soil.

Histological examination of cultured immature embryos showed that the somatic embryos arose from single embryogenic cells by internal segmenting division (Lu and Vasil, unpubl. observations of Panicum maximum; Vasil and Vasil, unpubl. observations on Pennisetum americanum), a process similar to that described for Ranunculus (Konar et al 1972), carrot (McWilliam et al 1974), and citrus (Tisserat and DeMason 1980).

In most instances, the somatic embryos formed in vitro were structurally similar to zygotic embryos and contained the characteristic organs of a grass embryo--a scutellum with storage reserves, a coleoptile, and a coleorhiza. However, significant variations were found in the degree of typical development (Table 2). For example, in wheat, where

Table 2. Degree and extent of somatic embryo—genesis observed in tissue culture of grasses. A high score indicates that wellorganized and typical embryoids are formed.

		SOMATIC EMBRYOGENESIS
Maximum	10	Panicum maximum Pennisetum americanum
	9	P. americanum x P. purpureum P. purpureum Zea mays
	7	Saccharum officinarum
	5	Sorghum bicolor
	3	Triticum aestivum
None	0	?

typical somatic embryos were seen only rarely, the embryos germinated precociously in vitro soon after their organization. The result was the formation of a green, leafy scutellum with characteristic trichomes and the development of multiple shoot meristems at the base of the scutellum (Ozias-Akins and Vasil 1982). Instances of precocious germination of embryos leading to the formation of a leafy scuttelum with many shoot meristems occasionally were found in Pennisetum americanum (Vasil and Vasil 1982).

There are many descriptions of the formation of leaves or leafy structures preceding the formation of shoot meristems in tissue cultures of grasses (Tamura 1968, Nakano and Maeda 1979). We believe that in these instances somatic embryos also are formed which germinate precociously and form a leafy scutellum subtended by many shoot meristems. This gives the erroneous impression that plant regeneration is through the direct organization of shoot buds. The embryos with multiple shoot meristems have been shown to have a scutellum and coleorhiza. Each germinates to produce several plants (Vasil and Vasil 1982).

SOMATIC EMBRYOGENESIS IN CELL SUSPENSION CULTURES

Somatic embryogenesis leading to the formation of normal green plants has been obtained in suspension cultures of grasses. We have used embryogenic callus cultures of various grasses to initiate and maintain embryogenic cell suspension cultures (Table 1) (Vasil and Vasil 1980b, 1981b, 1982; Lu and Vasil 1981b). The suspension cultures are composed of small, richly cytoplasmic cells and large, elongated, highly vacuolated cells. The cytoplasmic cells, identified as embryogenic, contain numerous plastids with starch and usually occur in tight groups of four or more cells, but occasionally as single cells. By manipulating the dilution ratios (cell suspension culture: fresh nutrient medium) and the duration of subculture, it is possible to obtain and maintain suspensions that consist almost entirely of embryogenic cells (Vasil and Vasil 1982, Lu et al 1981). The embryogenic nature of the suspensions can be maintained for at least 2 years. Embryogenic suspensions isolated from embryogenic callus initiated from immature embryos, young inflorescences, and young leaves are indistinguishable from each other and behave similarly in culture.

Somatic embryos to about the globular stage were formed in the suspension cultures. Mature embryos were obtained when the embryogenic suspension was plated in a 2,4-D-free or in a low 2,4-D agar medium. The addition of abscisic acid improved the efficiency of normal embryoid formation. The embryoids germinated in vitro to form plants which were successfully grown to maturity in soil. They were shown to be normal diploids.

SOMATIC EMBRYOGENESIS IN PROTOPLAST CULTURE

Protoplasts of graminaceous species have been found to be extremely recalcitrant and difficult to culture (Potrykus et al 1976, Vasil et al 1979, Potrykus 1980, Vasil and Vasil 1980b, Flores et al 1981). Nevertheless, sustained cell divisions leading to the formation of callus have been obtained in Hordeum vulgare (Koblitz 1976), Oryza sativa (Deka and Sen 1976, Cai et al 1978), Pennisetum americanum (Vasil and Vasil 1979), Sorghum bicolor (Brar et al 1980), Triticum monococcum (Nemet and Dudits 1977), and Zea mays (Potrykus et al 1979). In each instance, protoplasts were isolated from established cell cultures which had no morphogenetic capacity. Therefore, it was not surprising that the protoplast-derived callus also failed to form any embryoids, shoots, or plants. Vasil and Vasil (1980b) described the formation of somatic embryos and plantlets from protoplasts of Pennisetum americanum. More recently, embryoids and plantlets have been obtained from protoplasts of Panicum maximum (Lu et al 1981). In both cases, protoplasts were isolated from embryogenic cell suspension cultures which had been isolated from embryogenic callus initiated from immature embryos or young inflorescences.

The plating efficiency of Panicum protoplasts was improved considerably by using predominantly embryogenic suspension cultures for the isolation of protoplasts. Plantlets obtained from the protoplasts could not be grown to maturity in soil, but this is considered a technical problem not related to their protoplast origin. The plantlets are green and have several leaves, a good root system, and a well-organized shoot meristem with several incipient leaf primordia.

While we have concentrated on obtaining protoplasts from morphogenetically competent cell cultures, others have depended largely on the manipulation of nutrient media, conditions for growth of donor plants, and methods of protoplast isolation (Potrykus et al 1976, Galston 1978, Potrykus 1980). Several hundred thousand variations have been used. Unfortunately, none has led to the growth of protoplasts or to the regeneration of embryoids shoots, or plantlets. These experiences have led to suggestions that the lack of sustained cell division in grass protoplasts may be due to a mitotic block (Potrykus et al 1976, Kaur-Sawhney et al 1980). A gloomier forecast is made by Flores et al (1981): "Considerable evidence leads to the belief that cereal protoplasts are constitutionally incapable of sustained division.''

We believe these conclusions are misleading and unfounded. Our success in culturing Panicum and Pennisetum protoplasts and the experiences of many others with nonmorphogenic cell cultures show unequivocally that not only do grass protoplasts undergo sustained cell divisions in vitro, but that those isolated from morphogenetically competent cell cultures

will also form embryoids and plantlets.

Mesophyll protoplasts of grasses have never been successfully cultured. This is not because mesophyll cells of grasses are incapable of division or are morphogenetically incompetent, but because the correct conditions for their culture have yet to be determined. The totipotency of mesophyll cells of grasses is amply proved by recent success in the regeneration of embryoids and plants from young leaf tissues of several species (Haydu and Vasil 1981, Lu and Vasil 1981c) Wernicke and Brettell 1980).

The only other reports of embryoid, shoot, or plantlet formation from protoplasts of grasses is a recent poster paper presented at the XIII International Botanical Congress by Lorz et al (1981). They reported successful duplication of the results of Vasil and Vasil (1980b) on the culture of the protoplasts of Pennisetum americanum and said that "protoplasts isolated from cell cultures originally isolated from inflorescences and embryos can be cultured and regenerated. However, cell division and plant regeneration were, infrequent .... Special emphasis was made to observe real protoplast derived cell divisions, and to exclude non-digested, meristematic clumps." Plantlets derived from protoplasts were not grown to maturity.

Because of the serious difficulties still faced in the culture of mesophyll protoplasts of grasses and the success achieved with the culture of embryogenic protoplasts, more emphasis should be placed on the isolation and use of embryogenic cell suspension cultures as a source of totipotent protoplasts.

CONCLUSIONS

Extensive evidence of the regeneration of plants in tissue cultures of Gramineae by somatic embryogenesis has been discussed. We believe that somatic embryogenesis occurs or can be induced in tissue cultures of most other species of cereals and grasses. Reports on tissue cultures of this important group of plants indicated in many cases that embryonic tissue cultures were formed. Unfortunately, this was either not recognized or was misinterpreted as shoot morphogenesis. In other instances, initially slow-growing embryogenic tissues were either deliberately discarded in favor of faster growing but non-embryogenic tissues or were slowly diluted out during subculture by experimental conditions which favored the growth of non-embryogenic tissues.

The regeneration of plants by somatic embryogenesis offers a number of advantages. In comparison to the two other major methods of plant regeneration in vitro -- by formation of adventitious or axillary shoot buds -- many more plants can be obtained by somatic embryogenesis. Indeed, each embryogenic cell potentially is like a zygote and, under ap-

propriate conditions, can be induced to give rise to an em— bryo (embryogenic cell suspension cultures may contain as many as 15×10^6 cells/ml). Plantlets can be produced in relatively short periods of 3–6 weeks. Because such plantlets already have an integrated shootroot system, they are easy to establish in soil and grow rapidly into adult plants.

It is also possible to synchronize the development of somatic embryos in vitro, either by physical assortment of developmental stages or by manipulation of culture condi— tions. Such synchronized development is desirable for the establishment of automated systems and mass application. Embryogenic cell lines maintain their embryogenic competence for long periods and give rise to genetically uniform and normal plant populations. Embryogenic cell lines can be part— icularly valuable in studying the control of embryogenesis, in providing a suitable source of embryogenic protoplasts for culture and regeneration, and in developing single—cell systems. Nevertheless, a number of serious problems remain to be resolved.

Plant regeneration was obtained most easily from em— bryogenic callus cultures, with relative difficulty from cell suspension cultures, and with the most difficulty from pro— toplast—derived cell masses which yielded only plantlets. These differences in regeneration ability possibly are relat1ed to the initial degree of dissociation of the cells in culture and to cell—to—cell interactions.

In many cases, more than 70% of the callus cultures formed embryoids and plants, although the final number of plants formed was limited. The number of somatic embryos formed in callus and cell suspension cultures was large (almost unlimited in cell suspension cultures), but only a fraction reached maturity and germinated to form plants. This may be related to competition for space and nutrients.

For somatic embryogenesis to be of practical application in the rapid clonal multiplication of grasses, the number of embryoids reaching maturity and germination must be maxim— ized. To do this, a basic understanding of the process of somatic embryogenesis is essential. Although the phenomenon in tissue cultures was first described in 1958 (Steward et a1 1958, Reinert 1958), as yet little information is avail— able about cellular, physiological, and molecular controls of embryogenesis. An elucidation of these factors may allow better understanding and control of somatic embryogenesis in vitro.

No aneuploids or polyploids were encountered among the plants regenerated by somatic embryogenesis. Furthermore, no phenotypic variability was noticeable, even when regenerated plants were grown alongside the original donor plants in the field. It is not known whether this remarkable genetic uni— formity resulted from the selective development of embryoids and plants from only the cytologically normal cells present

within a heterogeneous population or whether the embryogenic cell cultures themselves are cytologically uniform and stable. We have not yet carried out any detailed and systematic cytological (especially meiotic) and genetic analyses of the regenerated plants. The possibility exists that some minor chromosomal changes, such as duplications, inversions, deletions, and translocations, did take place that could not be detected phenotypically.

Additional evidence substantiating early indications of the cytological and phenotypic stability/uniformity of plants regenerated by somatic embryogenesis will be of significant interest in agriculture as it will allow for the maintenance of genetic purity during rapid and mass clonal multiplication. The generation of genetic variability can be a serious disadvantage in cases where desirable improved genotype is being maintained and/or multiplied in vitro.

There is extensive evidence of cytological aberrations induced during plant cell culture accompanying genetic variability of regenerated plants. Therefore, the remarkable uniformity we observed is surprising. Although no clear reasons can be provided at this time, a number of possible explanations can be offered.

We believe that the method of in vitro plant regeneration is crucial to the question of the generation of genetically uniform or variable plant populations. It is suggested that callus cultures composed of enlarged and vacuolated parenchymatous cells are prone to cytological aberrations and soon give rise to chimeral tissues. Plants developing from such tissues are liable to express the variability inherent in the cultures.

On the other hand, embryogenic tissue cultures consist of small, richly cytoplasmic, nonvacuolated, starch-containing cells which resemble the cells present in the shoot meristems of plants that eventually participate in the formation of gametes. These cells, probably because of their unique cytological and cytoplasmic organization and tightly controlled cell cycles, are able to maintain their cytological and genetic integrity during countless cell divisions. Abnormalities induced in these cells often lead to the loss of their meristematic nature and even to abortion of gametogenesis.

Only such cytologically normal and stable cells as the embryogenic cells in vitro and the cells present in the meristem of an axillary or terminal bud can develop into normal embryos or plants. Thus, plants regenerated by the formation of somatic embryos or by the development of axillary buds in vitro generally do not display any genetic variability, in contrast to plants formed by the organization of adventitious buds. This implies that, even if cytologically abnormal cells are present in a population of embryogenic cells, they do not have the capacity to form embryos. Detailed cytological analyses and comparisons of

embryogenic and nonembryogenic plant cell cultures and plants regenerated from them may provide the answers.

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FACTORS THAT STIMULATE POLLEN EMBRYOGENESIS

Hiroshi Harada and Jun Imamura

Anther and pollen culture is important as a means to study physiological and biochemical processes in asexual em— bryogenesis. It also is a useful technique in the produc— tion of haploid plants for basic and applied research in breeding, mutation, and genetics.

After Guha and Maheshwari (1964, 1966) obtained hap— loid embryo formation from pollen of Datura innoxia using in vitro culture techniques, a number of similar experi— ments were done with many different species (Vasil 1980). However, the proportion of pollen that gives rise to plant formation has been small, regardless of the species used and even with considerable effort to increase it.

Pretreatment of tobacco anthers with low or high temperature or with water—saturated atmosphere has stimu— lated the proportion of anthers producing plantlets and the number of plantlets per anther (Nitsch and Norreel 1973, Keller and Armstrong 1979, Dunwell 1981).

Our recent results indicate that several important factors significantly enhance pollen embryogenesis and subsequent plantlet formation in tobacco anther or free pollen culture. These include the treatment of anthers or free pollen with low atmospheric pressure, anaerobic condition, water stress, abscisic acid, reducing agents, and amino acids.

Whether each treatment exerts its action through a different mechanism or whether certain common biochemical processes are involved among the different treatments needs to be confirmed.

MATERIALS AND METHODS

Plant materials

The anthers of healthy, uniform Nicotiana tabacum cv. Samsun plants grown in a greenhouse were used in all experiments except those using reducing agents. Flower

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buds at stage 3 (Imamure and Harada 1980a) were collected according to the corolla length (13—16 mm) which corresponded to pollen mitosis and early binucleate pollen stage. Anthers of Nicotiana tabacum cv. Mc were used in experiments with reductants. Flower buds collected at stage 3 had a corolla length of 14—17 mm.

Anther culture

Sterilized anthers were inoculated on 0.8% agar—solidified Nitsch's H medium (2% sucrose and 0.4% activated charcoal) in glass petri dishes and cultured under 16 hours light, 8 hours dark at 28°C.

Pollen culture

Pollen grains were isolated from anthers either after preculturing for 8 days or immediately after excision from plants. The anthers were homogenized with a 20—ml glass tissue homogenizer in Nitsch's H medium or in distilled water and filtered through 2 sieves with pore sizes of 74 µm and 37 µm. Pollen grains were rinsed twice in fresh medium or in distilled water by centrifuging at 150 x/g for 2 minutes. The supernatant was taken out and grains were suspended in Nitsch's H medium or in distilled water at a density of 3×10^4 to 1×10^5 pollen grains per ml. Three milliliters of each suspension was pipeted into 6—cm glass petri dishes and sealed with parafilm. Culture was under 16 hours light, 8 hours dark at 28°C.

Reduced atmospheric pressure

Anthers in open glass petri dishes were placed in a glass desiccator under reduced atmospheric pressure. Air was aspirated at 12 mm Hg for 0, 10, 20, 60, and 90 minutes and for 24 hours.

Anaerobic environment

Anthers in open glass petri dishes were placed in a glass desiccator. Either N_2 or a mixture of N_2 and O_2 (21% to 2.5% O_2) was flowed through the desiccator at 70 ml/min. N_2 treatments lasted 15, 30, and 60 minutes at 1 bar. O_2 concentrations of 2.5, 5.0, and 10.0% at 1 bar were controlled by an oxygen meter.

Chemical substances

Abscisic acid (ABA) and mannitol. Sterilized anthers were floated on liquid Nitsch's H media with ABA (10^{-5}M) and/or mannitol (0.5M) for 1 to 4 days. After rinsing 3 times in sterilized distilled water, each lot was placed on the

surface of an agar—solidified medium in glass petri dishes.
Reductants. Different concentrations of three reductants—ascorbic acid, mercaptoethanol (ME), and dithiothreitol (DTT) - were used.
Amino acids. The 8 amino acids tested for their effect on pollen embryogenesis were alanine (ALA), arginine (ARG), asparagine (ASN), aspartic acid (ASP), glutamic acid (GLU), glutamine (GLN), proline (PRO), and serine (SER).

Observation

After 40 days of anther culture, anthers showing no sign of plantlet formation were picked up. squashed on a glass slide, and checked under a binocular microscope for pollen development. After 14 days of free pollen culture, pollen showing no sign of embryo formation were checked. Embryos in which growth had stopped at the globule—, heart—, or torpedo—shaped stage were classified as aborted embryos. An average of 30 anthers was cultured in each petri dish. Each experiment was repeated 3 to 10 times.

RESULTS

Reduced atmospheric pressure

Treatment with reduced atmospheric pressure definitely increased the number of anthers producing plantlets as well as the average number of plantlets per anther (Table 1). Significant results were obtained with 10—, 20—,and

Table 1. Effects of reduced atmospheric pressure on pollen embryogenesis of Nicotiana tabacum cv. Samsun 40 days after beginning culture.

	Treatment duration					
	0 min	10 min	20 min	60 min	90 min	24 h
Anthers cultured (No.)	33	37	37	36	33	37
Anthers with plantlets (%)	6.1	64.9	54.1	50.0	48.5	0.0
Anthers with aborted embryos[a] (%)	12.0	3.0	2.7	2.8	3.0	16.2
Plantlets produced (No.)	57	447	640	603	343	0
Plantlets per anther (av.No.)	1.7	12.1	17.3	16.8	10.4	0

[a] Embryos stopped growth at globule–, heart–, or torpedo–shaped stage.

60—minute treatments. The 24—hour treatment completely inhibited plantlet formation.

Anaerobic environment

To investigate whether the stimulatory effect of reduced atmospheric pressure on pollen embryogenesis was due mainly to reduced air pressure itself or to decreased O_2 concentration, anthers were held under a 100% N_2 stream at 1 bar for 15, 30, and 60 minutes. The total percentage of anthers with either plantlets or aborted embryos was nearly the same in 15—, 30—, and 60—minute

Table 2. Effects of N_2 on pollen embryogenesis of Nicotiana tabacum cv. Samsun 40 days after beginning culture [a)]

	Treatment duration			
	0 min	15 min	30 min	60 min
Anthers cultured (No.)	32	32	32	31
Anthers with plantlets (%)	34(11)	44(14)	72(23)	71(22)
Anthers with aborted embryos [b/] (%)	16(5)	34(11)	9(3)	3(1)
Plantlets produced (%)	290	225	807	770
Plantlets per anther [c/] (av. No.)	9±4	7±2	25.2±5	24.8±4

[a)] Figures in parenthesis indicate the number of anthers.

[b)] Embryos stopped growth at globule—, heart—, or torpedo—shaped stage [±]Standard error.

treatments (Table 2). The 30— and 60—minute treatments increased the number of plantlets per anther but the 15— minute treatment gave fewer plantlets per anther than the control. These data indicate that, while embryo initiation was stimulated with the 15—minute treatment, further embryo development was not.

Treatment with N_2 containing 0, 2.5, and 5% O_2 increased the percentage of anthers producing plantlets and the average number of plantlets per anther (Fig. 1). Treatment with N_2 containing 10% O_2 gave a result similar to the control (natural air containing 21% O_2). These data indicate that quasi anaerobic treatment (2.5 or 5% O_2) is

more effective than pure anaerobic treatment (100% N_2).

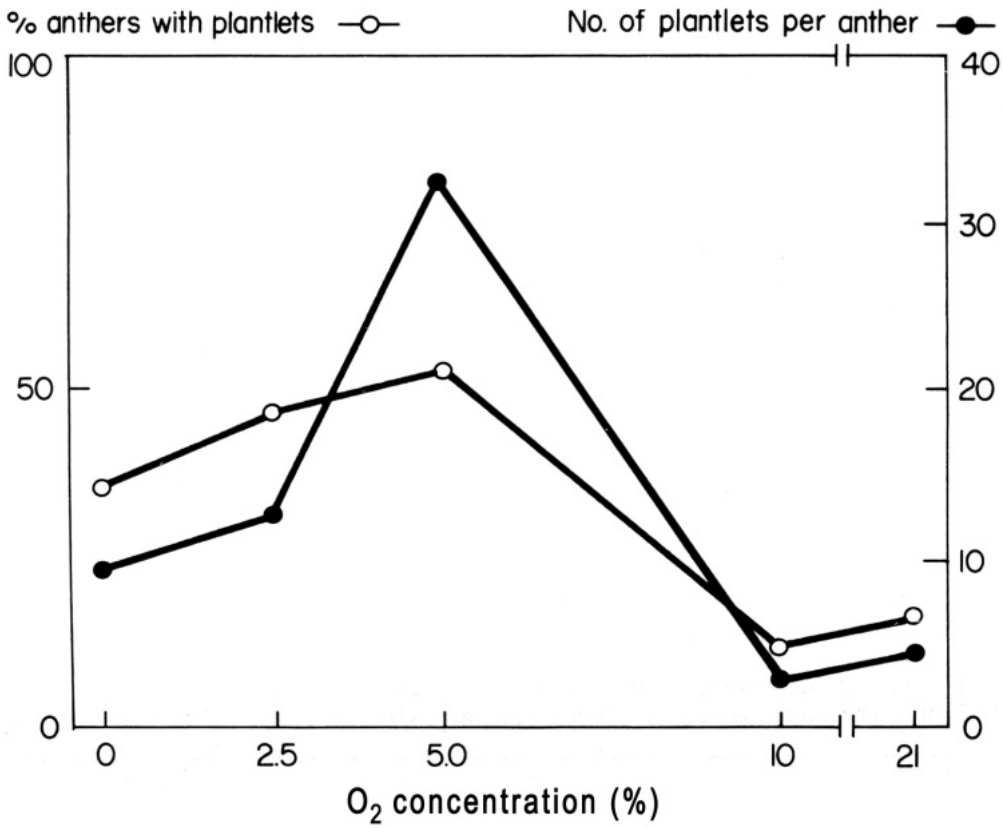

Fig. 1. The effects of varying concentrations of O_2 on pollen embryogenesis of Nicotiana tabacum cv. Samsun. One hour treatments, 34 anthers each. Zero on the abscissa indicates 100% N_2.

Water stress (treatment with mannitol)

Water stress given by adding mannitol (0.5M) to the medium showed a clear stimulatory effect on plantlet formation when the rate in the corresponding control was low (Table 3, Exp. 1). But, when the rate of plantlet formation in control was exceptionally high, the same water stress

inhibited plantlet formation (Table 3, Exp. 2).

Table 3. Effects of water stress on pollen embryogenesis of Nicotiana tabacum cv. Samsun.

	Treatment duration (days)			
	Exp. 1		Exp. 2	
	0	2	0	2
Anthers cultured (No.)	25	25	34	32
Anthers with plantlets (%)	28	80	94	72
Anthers with aborted embryos[a] (%)	36	4	6	13
Plantlets produced (No.)	103	165	473	215
Plantlets per anther (av.No.)	4.1	8.3	13.9	6.7

[a] Embryos stopped growth at the globule—, heart—, or torpedo—shaped stage.

ABA (short-term treatment)

The 3—day treatment with ABA (10^{-5}M) stimulated the rate of anthers producing plantlets and the number of plantlets

Table 4. Effects of short—term ABA (10^{-5}M) treatments on pollen embryogenesis of Nicotiana tabacum cv. Samsun.

	Treatment duration (days)			
	0	1	3	4
Anthers culture (No.)	32	30	29	22
Anthers with plantlets (%)	63	67	93	36
Anthers with aborted embryos[a] (%)	3.8	6.7	6.9	2.5
Plantlets produced (No.)	117	398	199	102
Plantlets per anther (av. No.)	3.6	13.3	6.9	4.6

[a] Embryos stopped growth at globule—, heart— or torpedo—shaped stage.

formed per anther (Table 4). Nearly 100% had either plantlets or aborted embryos. However, the 4—day ABA treatment inhibited plantlet formation while the 1—day treatment produced the highest average number of plantlets per anther.

Reductants

Continuous treatment with ascorbic acid at concentrations between 0.1 µg/ml and 100 µg/ml increased the percentage of anthers with plantlets and the average number of plantlets per anther (Table 5). ME at concentrations between

Table 5. Effects of ascorbic acid on pollen embryogeneis of *Nicotiana tabacum* cv. Imushi 40 days after beginning culture.

	Ascorbic acid concentrations (µg/ml)				
	0	0.1	1	10	100
Anthers culture (No.)	60	59	60	60	60
Anthers with plantlets (%)	12	29	42	23	25
Anthers with aborted embryos [a] (%)	8	19	27	18	5
Plantlets produced (No.)	157	592	584	323	303
Plantlets per anther (av.No.)	2.6	9.9	9.7	5.4	5.1

[a] Embryos stopped growth at globule—, heart—, or torpedo—shaped stage.

10^{-6}M and 10^{-3}M showed a clear stimulatory effect on the rate of anthers with plantlets and the average number of plantlets per anther (Table 6, Fig. 2). ME at 10^{-2}M was less stimulating. DTT also showed a marked stimulatory effect on pollen embryo formation (Table 7, Fig. 3).

Amino acids

The addition of glutamine to the culture medium increased the rate of embryo formation (Fig. 4). The optimum concentration was between 0.1 and 5 mM. Proline and aspartic acid stimulated embryo formation as the concentration increased. Asparagine and glutamic acid similarly stimulated embryo formation. A slight stimulation of embryogenesis was observed with the addition of alanine.

Table 6. Effects of mercaptoethanol on pollen embryogenesis of Nicotiana tabacum cv. Imushi 40 days after beginning culture.

	Mercaptoethanol (M) concentration					
	10^{-00}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Anthers culture (No.)	60	60	59	60	60	60
Anthers with plantlets (%)	12	62	50	40	57	32
Anthers with aborted embryos[a] (%)	8	12	22	30	23	32
Plantlets produced (No.)	157	601	570	354	586	210
Plantlets per anther (av. No.)	2.6	10.0	9.7	5.9	9.8	3.5

[a] Embryos stopped growth at globule—, heart—, or torpedo—shaped stage.

Table 7. Effects of dithiothreitol on pollen embryogenesis of Nicotiana tabacum cv. Imushi 40 days after beginning culture.

	Dithiothreitol concentration (µg/ml)				
	0	0.1	1	10	130
Anthers culture (No.)	60	60	59	59	60
Anthers with plantlets (%)	12	32	34	41	43
Anthers with abortive embryos[a)] (%)	8	28	27	27	28
Plantlets produced (No.)	157	575	337	642	688
Plantlets per anther (av. No.)	2.6	9.6	5.7	10.9	11.5

[a)] Embryos stopped growth at globule—, heart—, or torpedo—shaped stage.

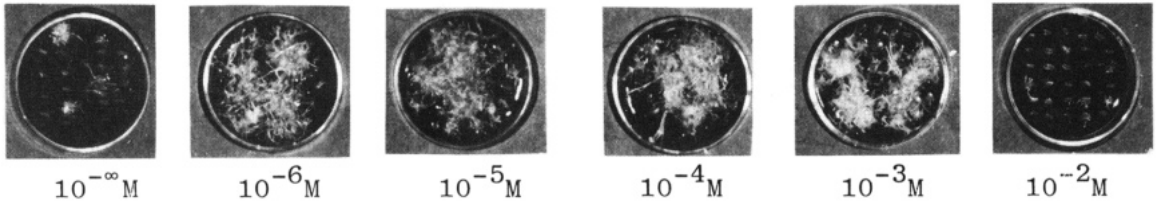

Fig. 2. Anthers of Nicotiana tabacum cv. Imushi cultured on agar—solidifiedmedia with varying concentrations of mercaptoethanol 40 days after beginning culture (×0.3). Basic medium was Nitsch's H with 2% sucrose, 0.4% activated charcoal.

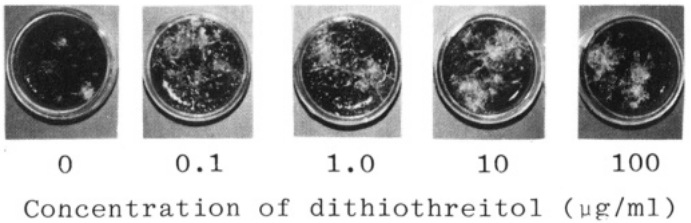

Fig. 3. Anthers of Nicotiana tabacum cv. Imushi cultured on agar—solidifiedmedia with varying concentrations of dithiothreitol 40 days after beginning culture (×0.25). Nitsch's H medium with 2% sucrose, 0.4% activated charcoal.

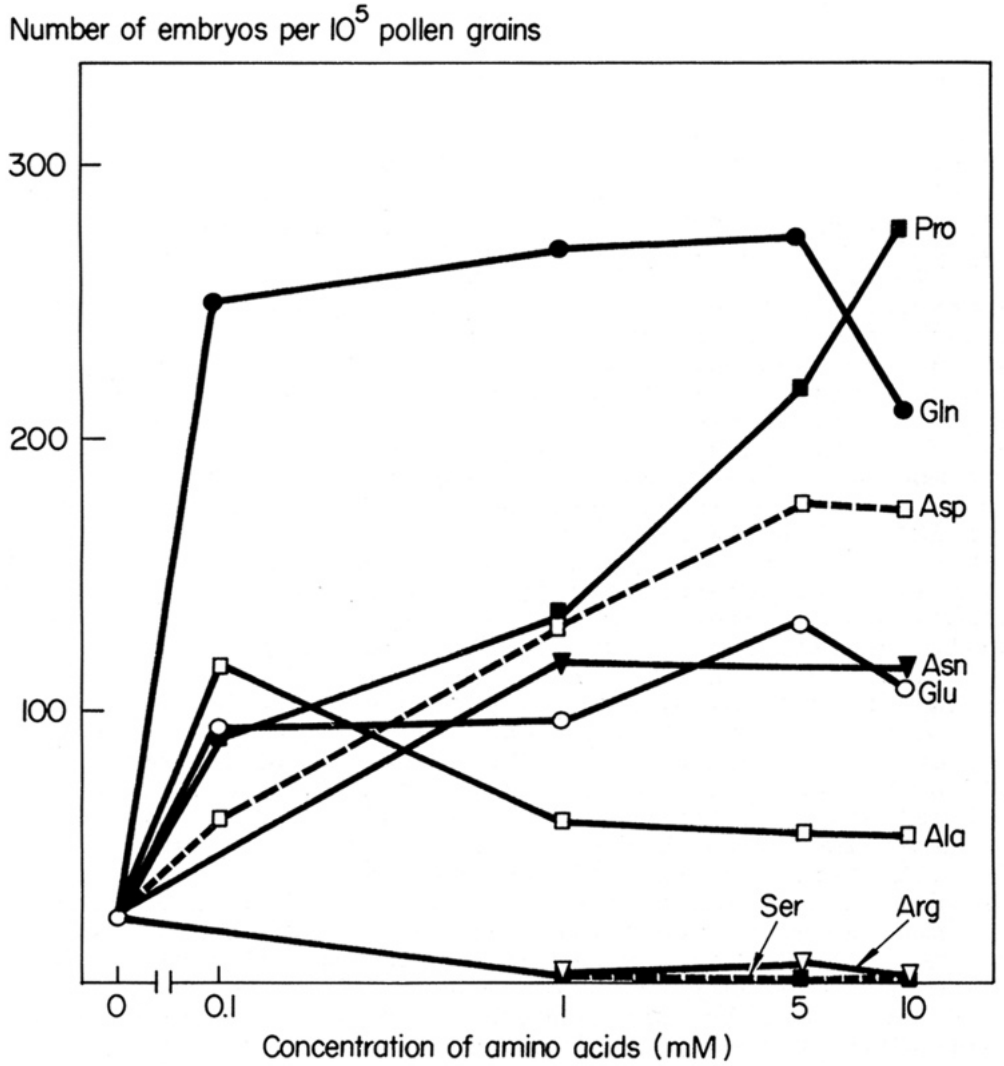

Fig. 4. Effects of varying concentrations of eight amino acids on pollen embryogenesis 14 days after beginning pollen culture.
ALA = Alanine, ARg = Arginine, Asn = Asparagine, Asp = Aspartic acid, Gln = Glutamine, GLu = Glutamic acid, Pro = Proline, Ser = Serine.

DISCUSSION

Tobacco pollen embryogenesis can be divided into two physiologically different phases: embryo induction and embryo formation. The duration of the embryo induction phase is about 8 days (Imamura and Harada 1980a). The type of pollen development can be determined in this period - for example, by switching its course away from gametophyte development, which is genetically determined, to sporophyte development,

The effects of different physiological stresses on embryogenesis were examined to elucidate the fundamental mechanism of pollen embryogenesis and to enhance the rate of haploid plant production from tobacco pollen. Pollen embryogenesis was stimulated in tobacco anthers exposed to different anaerobic conditions. Water stress produced by adding 0.5M mannitol to the medium also was effective. A temporary increase in the endogenous level of ABA was observed after water stress, suggesting that high endogenous ABA probably was responsible for enhanced pollen embryogenesis (Imamura and Harada 1980b). This interpretation is fairly plausible because tobacco pollen embryogenesis also was stimulated by short-term treatment with exogenously applied ABA. Imamura (1981) demonstrated that treating tobacco anthers with actinomycin D, an inhibitor of MRNA synthesis, stimulated pollen embryogenesis.

These results suggest that the stimulation of tobacco pollen embryogenesis by water stress and ABA treatments may be a consequence of mRNA synthesis inhibition at an initial stage of the pollen embryo induction. ABA is known to exercise an inhibitory action on mRNA synthesis.

In vitro formation of embryos from cultured tobacco pollen was stimulated when amino acids such as glutamine and proline were incorporated in the culture medium. It was not clear if this stimulation resulted directly from the external supply of basic amino acids to cells.

Tobacco pollen embryogenesis also was regulated by externally applied plant hormones. Its development was inhibited by continuous treatment with auxins, ABA, and BA and slightly stimulated by treatments with zeatin at 10^{-7} M and GA_3 at 10^{-6} M (Imamura 1981). Nitsch (1969) reported that ABA showed inhibitory effects on embryo formation at 10^{-6} M. No plantlets formed at 10^{-5} M. In our experiments, continuous application of ABA at 10^{-5} was strictly inhibitory but short-term application during the induction phase showed considerable stimulation of pollen embryogenesis. Continuous treatments with three reductants also were stimulatory, especially when the reductants were applied during the induction phase.

Except for certain plants belonging to a few genera, such as Nicotiana and Datura, the frequency of pollen embryogenesis generally has been low. Our work on tobacco anthers and free pollen indicates that similar treatments

also might increase the rate of embryo formation in other plants in which pollen embryogenesis previously has been considered difficult or nearly impossible. Even in tobacco, it has been difficult to obtain consistently high rates of embryo formation. These treatments make it possible to induce pollen embryogenesis at higher rates.

Our findings also may facilitate further physiological and biochemical studies on pollen embryogenesis. The fundamental mechanisms and physiological processes involved in this intriguing phenomenon are still mostly unkown. Further investigation is under way in an attempt to answer some basic questions:

- Why and how do these methods stimulate pollen embryogenesis?
- Does each treatment exert its action through dif— ferent mechanisms or are common biochemical processes involved?

To study the mechanisms of the stimulating factors and to analyze the biochemical processes taking place during the induction phase of pollen embryogenesis, our laboratory is searching for direct pollen culture techniques (without anther preculture) leading to high rates of haploid plant production. In preliminary experiments, the rate of surviving pollen grains was much higher when pollen grains were isolated from tobacco anthers immediately after excision and cultured in sterile distilled water or in different nutrient media without sucrose than when pollen was cultured in Nitsch's H medium containing 2% sucrose. Some pollen grains at stage 3 underwent further cell division and formed embryos when they were transferred from a medium without sucrose to a medium containing sucrose (2%) and glutamine (5 mM) and cultured for 2 weeks.

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SPOROPHYTIC DEVELOPMENT OF RICE POLLEN

Yang Hong—yuan and Zhou Chang

The successful anther culture of many angiosperm species indicates that microspores have the potential to develop either along a gametophytic pathway leading to normal male gametophytes (mature pollen grains) or along a sporophytic pathway resulting in pollen plants. But the mechanisms that control the switch from one pathway to another are not yet fully understodd.

MATERIALS AND METHODS

Rice cultivar Jinghong No. 2 (Oryza sativa L. subsp. japonica) was used as the experimental material. Young flowers at the vacuolated microspore stage were aseptically excised and manipulated, then inoculated on medium N_6 containing 5% sucrose and 0.8% agar with and without an exogenous auxin MCPA (2—methyl—4—chlorophenoxyacetic acid, 2 ppm) supplement. MCPA is a substitute for 2,4—D (Chou et al 1978). For callus formation, cultures were maintained at about 25°C in darkness. Anthers were sampled periodical—ly. Sampled anthers were fixed in acetomethanol (1:3), stained in toto in diluted Ehrlich's hematoxylin, and sectioned by the paraffin method for microscopic observa—tion.

RESULTS

Two successive experiments were done.

First experiment

Three modes of culture were used to find the condition in which pollen can maintain a normal gametophytic development and the condition in which development can be switched to a new sporophytic pathway: 1) The lemma, palea, and pistil were removed from young flowers and the latter were inocu—lated vertically with the pedicel inserted into the medium and the stamen standing free (vertical flower culture); 2)

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The lemma, palea, and pistil were removed from young flowers and the latter were inoculated horizontally with the anther in direct contact with the medium (horizontal flower culture); and 3) The anther was excised from young flowers and inoculated separately (anther culture).

These treatments had two common factors:

- Isolation. The explant was a flower in the first two cases, an anther in the third case.
- Nutritional passage. Nutrients passed into the anther through the normal vascular system in vertical flower culture and through the anther wall in horizontal flower and anther culture.

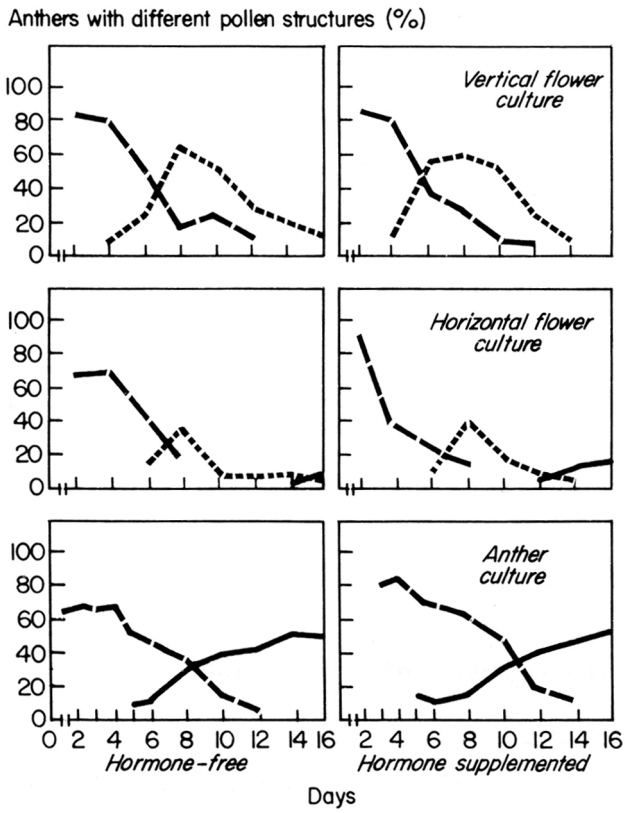

Fig. 1. Pollen development under different culture conditions. (Curves for aborted anthers omitted.)

Hormone supplement had no significant influence (Fig. 1). Most miscrospores passed their first mitosis, forming 2—cell pollen grains (with 1 vegetative and 1 generative cell). Differences occurred at later stages.

In vertical flower culture, most of the pollen passed a second mitosis, resulting in 3—cell pollen grains (with 1 vegetative cell and 2 sperm cells). Such pollen usually was filled with starch and could germinate into pollen tubes when pollinated artificially on fresh stigma.

However, some grains did not develop along the gametophytic pathway but turned to a sporophytic pathway. This was shown either by a first embryogenic division of the vegetative cell, by the division of the generative cell, or by the equal division of the microspore. As a result, multicellular grains were later observed in some anthers. This shows that there were two pollen populations—one gametophytic and one sporophytic - within the anthers cultured vertically.

In horizontal flower culture, the situation was similar, but sporophytic development went further, resulting in callus formation.

In anther culture, the gametophytic pathway to mature 3—cell pollen was blocked because of the absence of a second mitosis leading to sperm formation. The unique pathway was the sporophytic pathway which had been triggered to embryogenic division, which led finally to callus formation. We can see a tendency toward development deviation from the gametophytic pathway to the sporophytic pathway in the three modes of culture.

Several points can be made:

- The exogenous hormone is not indispensable for triggering the sporophytic development of rice pollen.
- The isolation of a flower is sufficient to trigger a sporophytic pathway, although it also continues to maintain a gametophytic pathway, as it does in vivo.
- To promote subsequent callus formation, anthers should be in contact with the medium.

To test these conclusions, a second experiment was done.

Second experiment

This experiment was in two—steps. First, vertical flower culture on MCPA—free medium, and second, excised anthers from vertically cultured flowers plated on MCPA—supplemented medium (subsequent anther culture).

Some anthers excised at 2—day intervals from cultured flowers were cultured for the second step and some were fixed for microscopic observation. During the second step, anthers were sampled and fixed for microscopic observation

at 4—day intervals (Fig. 2).

Four days after vertical flower culture, pollen that had completed a first embryogenic division were found in 8.3% of the anthers. This increased to 26.5% at day 10. Multicellular grains appeared in 3% of the anthers on day 8, reaching 54.3% by day 12. The rate of sporophytic development in vertical flower culture resembled that in ordinary anther culture (control). However, callus could not be produced from the flowers because of withering of the filament, which than could not supply further proliferation of multicellular grains.

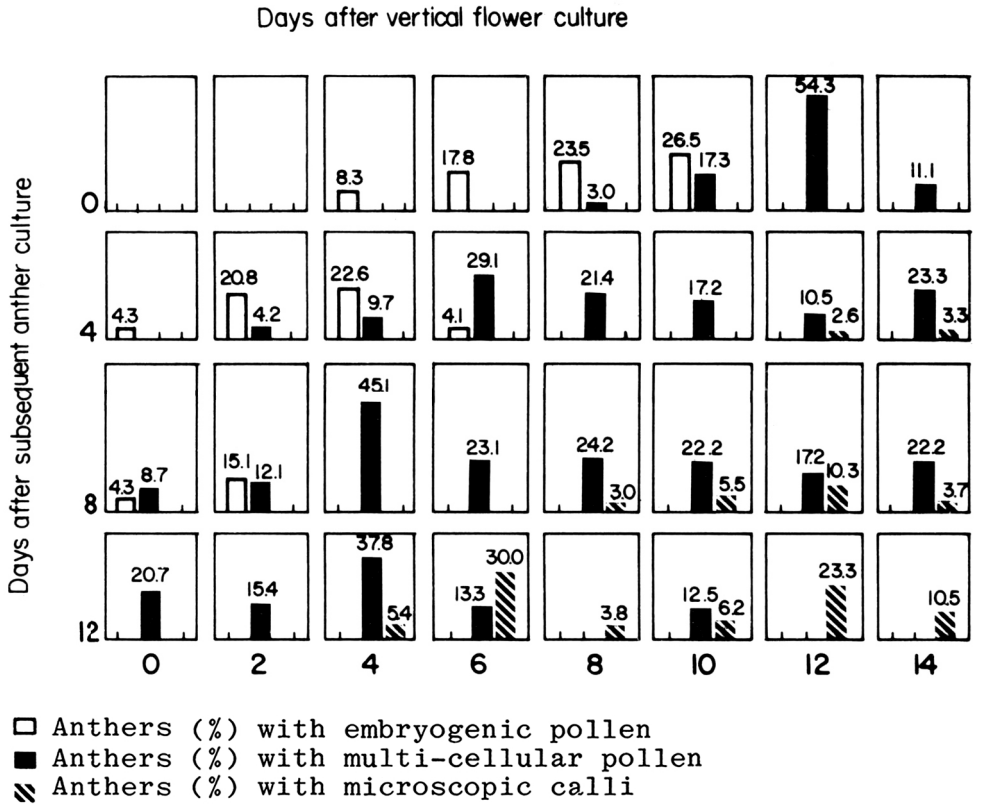

Fig. 2. Sporophytic development of rice pollen in vertical flower culture and subsequent anther culture.

Sporophytic development during vertical flower culture could continue when anthers were excised and cultured in step 2. Pollen grains that had reached the multicellular stage during the first step could develop into calli during the second step; the longer the first step, the shorter the second step. For example, the period of vertical flower culture and subsequent anther culture needed was 4 days plus 12 days in one case, 8 days plus 8 days in another,

and 12 days plus 4 days in a third. The total time in each case was about 16 days, indicating continuity of pollen development across these two culture steps.

DISCUSSION

According to Sunderland and Dunwell (1977), the switch from a gametophytic to a sporophyric pathway of pollen development is a result of the changed chemical and physical environment of the pollen caused by the release of restrictions imposed by other plant tissues. Excision alone would be sufficient to trigger a change. The culture procedure is only a means to promote the growth of predetermined units (Sunderland 1978). This view accords with the general idea of the essential role of isolation in cell dedifferentiation (Dyer 1976).

But as the first experiment predicted and the second experiment showed, sporophytic development of rice pollen can be divided into two distinct stages. The first is characterized by embryogenesis induction (a multicellular grain can reasonably be regarded as a kind of proembryo), resulted from isolation. A supply of exogenous hormone and contact of the anther with the medium are not necessary.

The second stage is characterized by callus formation from multicellular grains. Providing water, nutrients, and growth subtances continuously is essential. Evidently, direct contact of the anther with the medium and an exogenous hormone supply play an important role in this process.

The role of exogenous hormones in anther culture has been studied extensively. Species have been classified into two groups — hormone—dependent and hormone—independent All genera in which the emergent product is callus (for example, most of the cereals) belong to the first class (Sunderland and Dunwell 1977).

However, rice and wheat anthers cultured on hormone—free media have produced some pollen plants (Chu et al 1976, Zhou and Yang 1979). In wheat anther culture, an exogenous hormone was not necessary for triggering androgenesis but was important in preventing multicellular grains from aborting, allowing them to prow into calli (Zhu et al 1978). Our studies on rice confirm this.

So far, the routine method in anther culture is to place the anther in contact with the medium. If other parts of the flower are involved, the inoculum must be placed so that the anther comes into direct contact with the medium. The pollen will not grow if the filament lifts the anther clear of the medium. Hence, it is believed that the stimulus to growth cannot be transmitted via the filament (Sunderland and Dunwell 1977).

Nevertheless, supernumerary divisions of pollen grains havesometimes taken place in situ in many species. As an extreme example, a plantlet was produced from an anther

of Dianthus caryophyllus in vivo (Murty et al 1976, cited by Sunderland 1980). And, rice pollen could be induced to form multicellular grains during cold—pretreatment of excised panicles (Chen et al 1981).

Our work is evidence of massive multicellular grain formation during vertical culture of rice flowers. Under similar conditions, we also have induced barley pollen to form multicellular grains and even a few microscopic calli.

This means that the filament is able to transmit growth stimulus and that direct contact between anther and medium is not a critical factor for sporophytic development of pollen, especially during the early stage.

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USES AND APPLICATION OF ANTHER AND POLLEN CULTURE IN RICE

Hiroo Niizeki

The first successful haploid production using anther culture techniques was in Datura innoxia (Guha and Maheshwari 1964, 1966). Shortly after this success, the technique was applied to rice (Niizeki and Oono 1968, 1971) and tobacco (Bourgin and Nitsch 1967, Nakata and Tanaka 1968). Many laboratories throughout the world followed with work on many crop plants.

Recently detailed reviews have been published on the anther and pollen culture of various plants, including cereals (Chaleff and Carlson 1974; H. Hu 1978, 1981; Maheshwari et al 1980; Nitzsche and Wenzel 1977; Rush and Shao 1980; Vasil 1980; Wenzel 1979). Here I outline only the important points in the uses and application of rice anther culture in agriculture.

At present, the most promising approach for haploid production in rice is microspore culture within or in isolation from the anther. Since the first success of haploid rice production from anther culture in 1968, a great deal of progress has been made. Several cases of successful application of anther culture to the production of new rice varieties have been reported in China (H. Hu et al 1978, Rush and Shao 1980). However, convincing evidence of a general, simple, reproducible method applicable to a wide range of varieties or subspecies in rice is still lacking.

In 1980, the International Rice Research Institute indicated that there were still three problems in rice anther culture:

- Inadequate regenerating techniques.
- Inadequate techniques for creating diploid plants from haploids regenerated from pollen culture.
- High rate of mutation (abnormality) in pollen—derived diploid plants.

I want to add two more problems to the list, problems that need to be solved for the application of haploid breeding to rice varietal improvement:

- Too high an incidence of albino plants.
- Dedifferentiation of callus from cells of somatic tissue anthers.

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IMPROVING THE RATE OF REGENERATION

Plant genotype

The low rate at which haploid or dihaploid plants are regenerated possibly is caused by genetic differences in the varieties or subspecies used for anther culture. Reports on the influence of variety on haploid frequency (Chen and Lin 1976, Guha—Mukherjee 1973, Oono 1975) show that a high proportion of the anthers of some rice varieties produce callus or plantlets, whereas anthers of other rice varieties do so rarely or not at all. This does not make anther culture a practical breeding system. A recent attempt to accumulate the gene(s) favoring embryoid production used a clone of Solanum tuberosum (Jacobsen and Sopory 1978). It is also desirable to breed rice varieties with high efficiencies of haploid production.

Media

Most efforts to define the conditions that promote development of haploid plants from anthers have been done through modifications of the media. Efficient media for rice anther culture have been developed (Liang 1978, Rush and Shao 1980) but these do not induce enough haploids for rice breeding programs.

Recently, Inoue and Maeda (1981) developed a two—step culture technique that leads to a high frequency of shoot bud formation and plant regeneration in rice callus cultures The preculture medium was modified Linsmaier and Skoog's medium containing 10^{-5}M 2,4—D, 1 mg thiamine/liter, 200 mg inositol/liter, 3 g casein hydrolysate/liter, 30 g sucrose/liter, 9 g agar/liter, and 10^{-4} M abscisic acid (ABA). The initial callus was precultured for about a month. Then, the precultured callus was subcultured on a medium containing 5×10^{-5} M kinetin in the presence of a low level of thiamine (0.1 mg/liter). This method promoted shoot bud and plantlet formation and resulted in a severalfold increase in yield. This two—step culture method may be applicable to haploid induction.

Anther pretreatment

Anthers treated with low temperatures have produced calli at higher frequencies than did untreated anthers (Chaleff et a1 1975, Chen 1976, Genovesi and Magill 1979, Hu et a1 1978).

IMPROVING THE EFFICIENCY OF DIPLOID PLANT INDUCTION

Both haploids and diploids are included among the rice plants

regenerated by anther culture. Diploid plants are generally more numerous than haploid plants (Oono 1975). But, because many haploid plants are still regenerated, an efficient me—thod of chromosome doubling must be established.

Colchicine treatments have been used (Chin et al 1978, Watanabe 1975). Tanaka (1970) exposed haploid plants that had been propagated vegetatively from a single spontaneous haploid plant to chronic gamma rays. Diploid sectors frequ—ently were induced on irradiated haploid plants and more than 50% of the resulting diploid lines showed the same or higher purity than the mother variety. But only two of the five pure lines induced by radiation were mutants whose small but significant changes in characters bred true.

HOMOZYGOSITY AND GENETIC STABILITY

Many researchers have confirmed that pollen—derived lines are homozygous (Kwantung Institute of Botany, Laboratory of Genetics 1976; Oono 1975; Academia Sinica Institute of Genetics, 3rd Laboratory, Second Division 1974). About 90% of the plants produced were uniform and stable in all major traits.

But in the progeny of diploid plants obtained from the anther culture, segregation occurred in such simple char—acters as sterility, stripe on leaves, or short culm(Fujita and Uchiyamada 1981). Occurrences of segregation in some characters demand that pure breeding techniques be applied. This makes shortening the breeding period impossible.

Mutation frequently occurs in the process of callus culture. Because the rate of mutation in the process of developing a pollen embryoid to a plantlet seems to be low, this culture technique also might be applied to rice.

REDUCING THE APPEARANCE OF ALBINO PLANTS

Many rice pollen plants are chlorophyll deficient. More than 80% are albino (Oono 1975). The occurrence of albino plants diminishes the efficiency of haploid breeding.

Some studies on the mechanism of albino plant produc—tion have been done: disturbance of protein metabolism (Liang et al 1978), impairment of DNA (Sun et al 1978), metabolic deficiencies in the callus meristem (Liang et al 1978), effect of incubated temperature (Song et al 1978, Wang et al 1978), and loss of the capacity of synthesis for fraction I protein (Sun et al 1979). Albino formation might be caused by physiological factors that could be corrected using more suitable culture conditions.

PREVENTING DEDIFFERENTIATION OF CALLUS

Generally callus can be induced from diploid tissue of somatic origin, such as filaments or connective tissue, of cereal anthers, except in rice. Diploid callus originating from the somatic tissue of the anther usually shows better growth than haploid callus originating from pollen. Because of this, we cannot obtain a large amount of haploid callus from pollen, making haploid production still lower.

Gupta and Carlson (1972) suggested using para—fluoro—phenylalanine, a compound used to induce haploids of the fungus Aspergillus, as an agent to select for growth of haploid members of a heterogeneous population. We investi—gated the effectiveness of para-fluorophenylalanine treatment in tissue culture. A haploid plant of Oryza punctata (Niizeki 1977) and a low ploidy plant of Oyrza alta (Niizeki and Fukui 1978) were obtained from diploid callus originated from seed that had been cultured in the presence of para—fluorophenylalanine. These results suggest a possible al—ternative to anther culture as a method of obtaining haploid plants.

USES OF HAPLOID RICE CALLUS

Selection for resistance to amino acids is being carried out with haploid callus derived from anther culture. Chaleff and Carlson (1975) obtained a cell line resistant to lysine plus threonine and regenerated a plant from this line. They also selected lysine analogue S—2—aminoethylcysteine—resistant cell lines.

CULTURING ISOLATED POLLEN

Development of a technique for culturing isolated pollen (Wang et al 1978c, Hu et al 1978, Chen et al 1981) should increase the potential number of plantlets produced from F_1 plants. Maheshwari et al (1980) stated that "the solution of (these) problems lies in the use of isolated pollen culture." I agree with Maheshwari's view on rice improvement and an—ticipate a practical application of haploid production by pollen culture soon.

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PRODUCTION OF WHEAT POLLEN-DERIVED ANEUPLOID PLANTS THROUGH ANTHER CULTURE

Hu Han, Xi Zi—ying, Jing Jian—kang,
and Wang Xi—zhi

In addition to many haploid and dihaploid plants, heteroploid and aneuploid plants have been obtained through anther culture. The phenomenon has been observed in about 28 species belonging to 19 genera and 7 families of dicots and monocots of pollen—derived plants (Table 1). However, further investigations on pollen—derived heteroploid and aneuploid plants are sparse. The production of aneuploid plants of wheat through anther culture is reported here.

MATERIALS AND METHODS

In recent years,the induction frequency of green plantlets derived from wheat pollen has been steadily increased. Potato—II medium has been better than synthesized media N_6 and W_5 in wheat variety Orofen (Table 2). Incubation at a higher temperature (33°C) for 8 days after inoculation of anthers has increased induction (Table 3). The average frequency of induction of green plantlets has been increased to about 12% (Table 3) and the cytological method for identifying plant ploidy level has been improved. Aneuploids, such as mullisomic, trisomic, tetrasomic, and mixoploids, were obtained by means of our anther culture technique (Chung et al 1978, Ouyang et al 1973).

RESULTS

Production of aneuploids from intervarietal wheat hybrids

Pentaploids, octoploids, and mixoploids of wheat are obtainable through anther culture, in addition to about 90% haploids and homozygous diploids (Hu Han et al 1978, 1979, 1980). It was assumed that heteroploid and aneuploid plants also might be obtained by this technique. In 1978—80, aneuploid plants and seeds from semiwinter—, winter—, and spring—type intervarietal hybrids of common wheat were obtained.

Semiwinter intervarietal hybrids nullisomic (No. 498)

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Table 1. Heteroploid plants regenerated from anther culture.

Species	Chromosone characters of regenerated plants	Reference
Agropyron repens	3n, 4n or higher	Zenkteller et al (1975)
Hordeum vulgare	4n chimeras	Dale (1975), Foroughi—wehret al (1976), Wilson (1977)
Oryza sativa	3n, 4n, 5n chimeras	Nishi and Mitsuoka (1969), Niizeki and Oono (1971), Chen Ying et al (1974)
Secale cereale	Higher ploidy	Wenzel and Thomas (1974)
Triticum aestivum	5n, 8n, 12n, and aneuploids, mixoploids	Hu Han et al (1978, 1979, 1980) Buyser and Henry (1979, 1980)
Triticum vulgare/ Agropyron glaucum	Monosomic haploid	Wang, C.C. et al (1975)
Triticale	Mixoploids	Vnuchkova, V.A. (1979)
Triticale/Tr. aestivum (6x) (6x)	Various aneuploids and mixoploids	Wang, X.Z., and Hu Han (1981)

Table 2. Effect of media on induction of pollen—derived plants of wheat variety Orofen.

Medium	Anthers inoculated (No.)	Calli		Green plant lets	
		No.	%	No.	%
N_6	630	48	7.6	8	1.3
Potato—II (Sichuan potato)	630	149	23.6	27	4.3
Potato—II (Ke—1 potato)	630	162	25.7	32	5.1
W_5	630	46	7.3	10	1.6

Table 3. Effect of incubation condition on induction of pollen—derived plants of wheat variety Orofen.

Treatment	Anthers inoculated (No.)	Calli		Green plantlets	
		No.	%	No.	%
33°C for 8 days after inoculation	1186	715	60.3	194	16.4
24°C control	1186	516	43.5	92	7.8
Total	2372	1231	51.9	286	12.1

and nontypical nullisomic (No. 453) were discovered from pollen—derived plants (H_1) in 1978. Meanwhile, monosomic 6B, with a large satellite and lower arm ratio, was pre—liminary identified from the H_3 of plant No. 453 (Fig. 1 and 2).

Three plants were identified as monosomic in line A23 of pollen—derived plants from the F_1 of winter intervarietal hybrid Kotung 58/Norin 10 in 1979 (Fig. 3).

Nullisomic plant No. 5-87 was observed in the F_1 of the spring—type intervarietal hybrid Pitic 62/Hua Pei No. 1 in 1980 (Fig. 4). This plant was abnormally fertile and a few seeds set.

These intervarietal hybrids are summarized in Figure 5.

Production of aneuploids from common wheat varieties

To further verify the production of aneuploids through

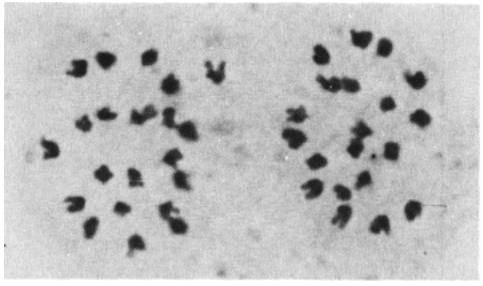

Fig. 1. Semi-winter, pollen-derived, intervarietal wheat hybrids plant No. 453 non-typical nullisomic-monosomic. Monosomic anaphase I, showing lagging chromosome.

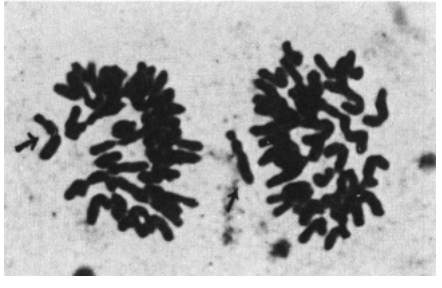

Fig. 2. Monosomic 6B with large satellite and lower arm ratio (see arrows) from H_3 of plant No. 453.

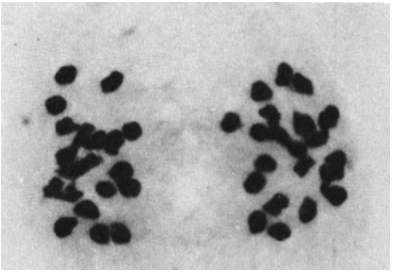

Fig. 3. Monosomic plant in Line A23 of pollen-derived F_1 plants of Kotung 58/Norin 10 wheat hybrid.

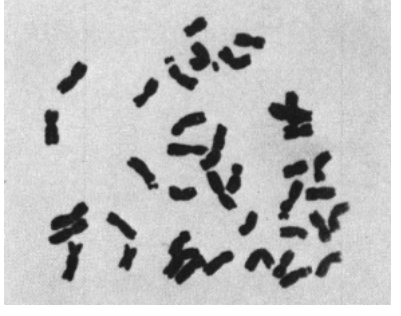

Fig. 4. Nullisomic plant No. 5-87 in the F_1 of Pitic 62/Hua Pei NO. 1 hybrid.

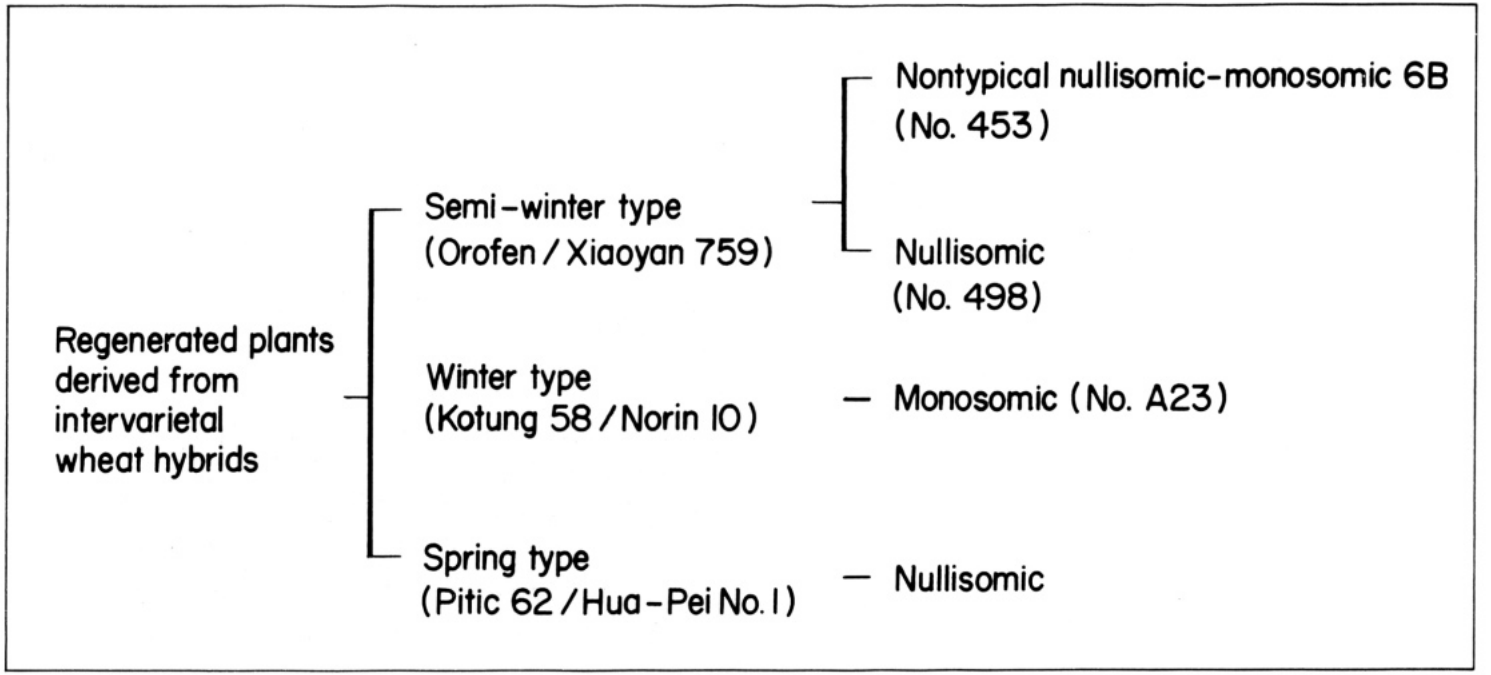

Fig. 5. Intervarietal wheat hybrids regenerated from common wheat.

anther culture, the common wheat variety Orofen was used. N_6, Potato—II and Maihe 5 (W_5) were used as basic media. In May 1980, 3,780 anthers were inoculated and 96 green plants were induced. Root tip and pollen mother cells of 96 green plants (H_1) were identified cytologically.

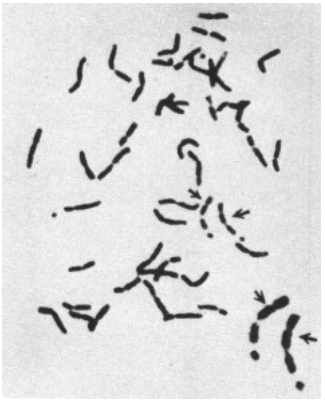

Fig. 6. Tetrasomic plant 5—92 with pair of di—centric 6B chromosomes (see arrows).

In addition to a great number of haploid and dihaploid plants, two tetrasomic (2n + 2 = 44) and one trisomic (2n + 1 = 43) were observed. One terasomic plant (Plant J—92) had a pair of di—centric 6B chromosome (Fig .6) One variant plant with a spelta—like spike was discovered (Fig. 7).

Four nullisomic plants originated from two pollen—derived lines of the common wheat variety Jinghong No. 5. These plants are characterized by long, thin leaf blades, crimped first leaves under spike, and poor fertility, although some seeds set.

Figure 8 summarizes plants regenerated from common wheat.

Fig. 7. Variant plant with spelta—like (right) compared with normal wheat spike (left).

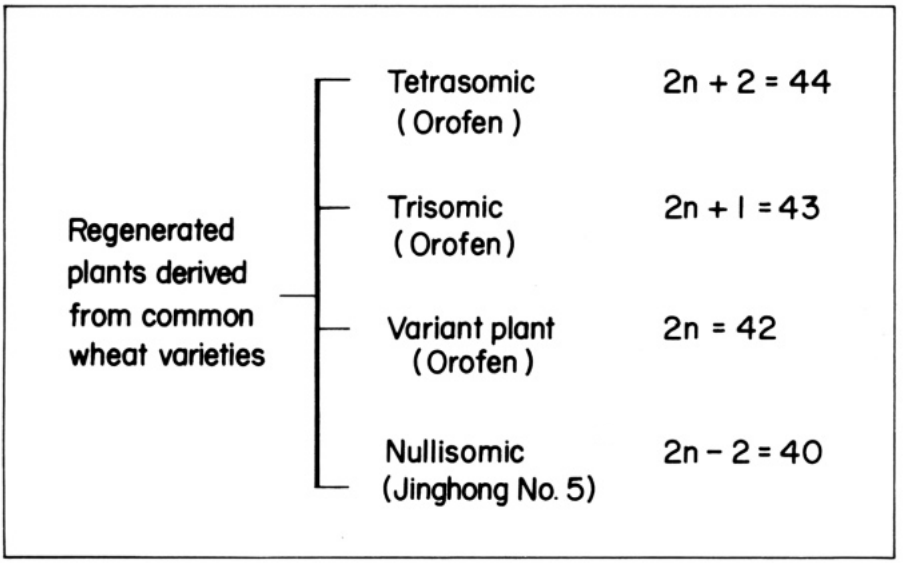

Fig. 8. Types of plants regenerated from common wheat.

Production of aneuploid from intergeneric hybrids

In 1979, the hexaploid triticale Rosner was used as the maternal plant in a cross with hexaploid common wheat Kotung 58. The F_1 hybrid was used as material in 1980 with B_5 as the basic medium. A total of 2,881 anthers were inoculated and 62 (2.2%) green plantlets were induced. Root tip cells of 27 healthy and strong pollen—derived plants were identified cytologically. Different aneuploid types - aneuhaploids, aneudihaploids, and mixoploid plants - were found (Table 4). The 21 + 3 (2n = 24) and mixoploid plants set seeds to different degrees.

Table 4. Pollen—derivedplants from hexaploid Triticale/ common wheat F_1.

27 pollen—derivedplants - { Aneuhaploid and haploid, 14 plants. Aneudiploid, 4 plants with 46 chromosomes (Mixoploid, 9 plants

Chromosome number of regenerated plants	21	22	23	24	25	26	27	46	Mix—oploid
Plants obtained(No.)	1	1	3	3	4	1	1	4	9

DISCUSSION

These results indicate that fertile aneuploids, such as nullisomic, monosomic, trisomic, and tetrasomic, can be obtained from intervarietal hybrids and common wheat varieties through anther culture. Plant J—92 derived from the variety Orofen was identified as tetrasomic with a dicentric chromosome 6B. This means that not only did chromosome number change, but also chromosome structure. Thus, the translocation line might be produced.

It is expected that different aneuploid plants, such as nullisomic and monosomic, without consanguinity to the Chinese spring could be obtained. Khush (1973) has said that, in general, nullisomic, monosomic, trisomic, and tetrasomic plants originate from haploids or from the progeny of hybrids between different ploidy levels of plants or by treatment with physical and chemical factors. Now, through anther culture, we have obtained different types of aneuploid plants directly from hexaploid wheat.

Research on different kinds of alien addition lines and alien substitution of wheat derived from hexaploid secondary triticale was reviewed recently (Muntzing 1979). Triticale was considered a classic material for investigation of chromosome engineering (Feldman and Sears 1981, O'Mara 1940). In our work, the maternal plant (cultivar Rosner) was identified as 14IIW + 7IIR by Shigenaga and Larter (1971). From a small population of 27 pollen—derived plants, many types of aneuploids and mixoploids could be obtained through anther culture. The F_1 hybrid between hexaploid triticale (Rosner) and hexaploid wheat (Kotung 58) was used as material.

Meanwhile, the pollen mother cells of aneuhaploid plants were observed. Results indicated that these aneuhaploid plants possess only univalents, with no bivalents

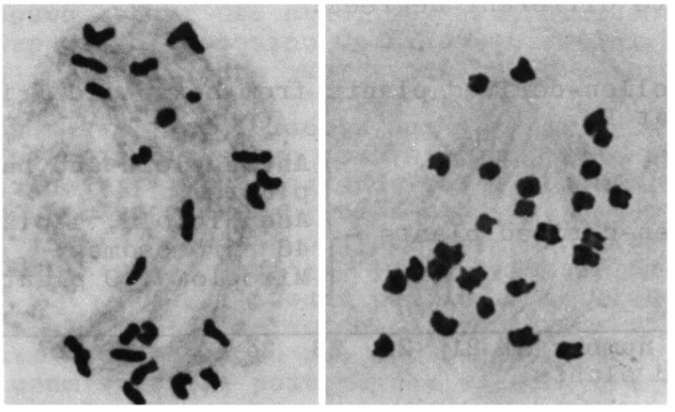

Fig. 9. Pollen mother cells of aneuploid plants with no bivalents, only univalents.

(Fig. 9). This means that their genomes might be ABDR, which are more valuable because homozygous alien addition lines or alien substitution lines and mixed amphiploid plants might be produced after chromosome doubling.

Although these investigations were carried out in different years (1978—80) and used different varieties and hybrids as materials, a number of fertile or partially fertile aneuploids were obtained repeatedly through anther culture. This anther culture method might be a useful tool to investigate chromosome engineering in higher plants.

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IMPROVING RICE BY ANTHER CULTURE

Shen Jin—hua, Li Mei—fang, Chen Yin—quan,
and Zhang Zhen—hua

The method widely used for breeding improved rice varieties in China has been intervarietal hybridization and systematic selection. In recent years, many promising varieties developed by conventional breeding methods have been released to farmers (Shen Jin—hua 1980). But to develop a variety by crossbreeding takes long, hard work.

In the 1960s, haploid plants of datura, tobacco, and rice were produced through anther culture (Guha and Maheshwari 1964, Bourgin and Nitsch 1967, Niizeki and Oono 1968). Natural and artificial chromosome doubling was necessary to make sterile pollen plants fertile and to ensure that they breed true. But a new technique had been developed to apply to rice breeding.

Anther culture techniques have been studied for their use in breeding rice since 1970. Progress has been made in the improvement of culture media and in the development of new rice varieties.

EFFICIENCY OF ANTHER CULTURE METHODS

Shortening the breeding cycle

The Chinese Academy of Agricultural Sciences Institute of Crop Breeding and Cultivation (CAAS—ICBC) has worked on rice anther culture since 1976. Pollen plants from F_2 anthers of the combination cross Jin—fu No. 9/IR24//Zao—feng were sent to the breeding base on Hainan Island for evaluation. Some plants with good agronomic characters and blast resistance were selected in March 1977. Their progeny were tested in a rod—row nursery in Beijing.

A superior line that matures early and is suitable for a wheat—rice double—cropping system was selected. Heading in this line was 2—4days earlier than heading in the standard variety Zao—feng and its yield was 6.7% higher. In 1980, line Zhong—hua No. 2 was released to farmers in Beijing and Tianjin.

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Developing this line from anther culture to its release as a new variety to farmers took only 3—4years, nearly half as long as conventional cross—breeding methods would have taken.

To improve a late Keng (japonica) rice variety in Yangzhou, the Institute of Agricultural Sciences used both anther culture and conventional breeding to inoculate the F_1 plant of Wan 87/America Rice No. 1 (Fig. 1).

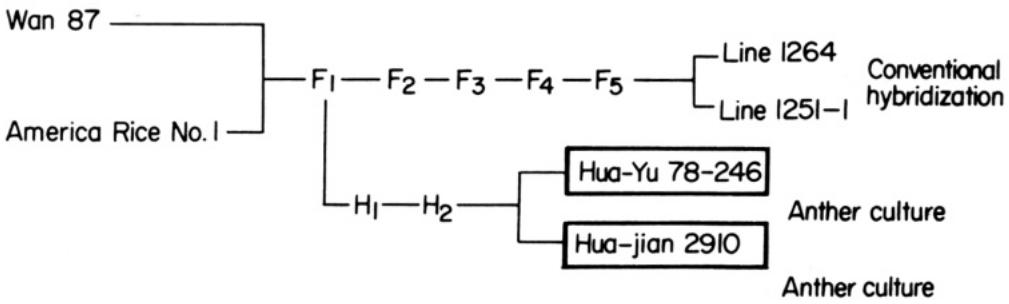

Fig. 1. Number of breeding generations needed to develop a variety by conventional hybridization and by anther culture.

In that work, two stable anther culture lines were selected at the H_2. By conventional breeding, two lines were not developed until the F_5. Anther culture can skip two generations. Uniformity of plant height, panicle length, grains per panicle, and length of flag leaf within the four lines were almost equal, but the coefficients of variation in characters were smaller in lines developed by anther culture than in lines developed by conventional breeding methods (Table 1).

The use of anther culture to shorten the breeding period for rice also has been shown by work at the Shanghai Academy of Agricultural Sciences, Institute of Crop Breeding and Cultivation, (1980), Yuxi Prefecture Institute of Agricul—tural Sciences, Yunnan Province (1980), Wujin Seed Company, Jiangsu Province (1980), and Hejiang Prefecture of Rice Sciences, Heilongjiang Province (2980).

Elevating breeding efficiency

Selection of hybrid progeny is decided by the distance between the characters of the parents as well as by the scale of breeding. Great differences between parents may result in varied genotypes and strong hybrid vigor, but a

Table 1. Uniformity among rice lines developed by conventional breeding methos and by anther culture.

Material	Culm length		Ear length		Grains per ear (No.)	
	$\bar{x} \pm S$	c.v.	$\bar{x} \pm S$	c.v.	$\bar{x} \pm S$	c.v.
Conventional						
Line No. 1264	70.4±4.11	5.83	17.87±1.27	7.10	179.5±28.1	15.69
Line No. 1251-1	91.6±23.0	25.11	22.19±1.57	6.87	237.0±40.7	17.17
Anther culture						
Hua—yu 78—264	79.4±3.56	4.48	17.7±1.16	6.55	192.4±28.8	14.96
Hua—jian 2910	103.4±3.74	3.50	20.6±0.89	4.31	196.0±25.1	11.98

large population is necessary for efficient selection. Because of the complexity of inheritance in characters, it is difficult to determine the optimal population size to conform to the general law of segregation. Conventional breeding not only demands a large experimental field and a large amount of labor, but accomplishing the breeding goal also is relatively uncertain.

These difficulties can be overcome by anther culture breeding. Investigations of pollen plants (H_1) have shown abundant variation. The character segregation of an H_1 Population provides diversity in genotypes (Yin Gue—da et al 1980, Li Mei—fang et al 1981b, Song Xian—bing et al 1980, Zhang Shu—hua et al 1980, Yuxi Prefecture Institute of Agricultural Sciences 1980, Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences, Central laboratory 1980). Some tend toward a particular parent, some are intermediate, and some exceed their parents. They always produce mutations. The experiments also have shown that tissue culture plants have high heritability in the H_1 pollen plant. In the H_2 produced in one step at the gametophyte stage, most lines are homozygous and stable. These pure lines can be applied directly or indirectly, through evaluation of characters and yield trials, to rice improvement.

A pollen plant can form a duplicate diploid through artificial or spontaneous doubling of the chromosome from the haploid. It may be obtained as a result of homozygosity of dominant or of recessive genes. Heterozygote genes do not exist within anther culture progeny.

Yi Gue—da et al (1980), Li Kei—fang et al (1981b), Zhang Shuhua et al (1980), and Xu Shi—huan (1980) investigated the segregation of such characters as sheath color (purple to nonpurple), awn (awned to awnless), disease resistance (resistant to susceptible), and heading date (early to late), that coincide in 3 to 1 and 1 to 1 ratios with both F_2 populations and H_2 lines. Also, because of the homozygosity of dominant or recessive genes that appear at H_1, it may be more effective to se1ect useful material s in the H_2 generations.

Hybridization between Hsien (indica) and Keng (japonica) rices is important to spread variation and increase genotypes for rice improvement in China (Liu Du—yin et al 1980, Chin Yi—min et al 1980). But serious obstacles often are produced within Hsien—Keng hybridizations — long segregation generation, high sterility, and differentiation of characters into two extreme opposites (Chin yi—min et al 1980, Liu Du—yin et al 1980). Recently, breeding for Hsien—Keng hybridization has been improved using anther culture techniques. Li Mei—fang et al (1980), found that by crossing varieties of Keng rice, the rate of ripened grain in the F_1 generally could be increased more than 85%. But it only increased 26—36% in

a Hsien—Keng F_1 hybrid. After backcrossing, the rate was raised to 45—50%. They subsequently suggested that the H_1 pollen plant's rate of ripened grains of more than 80% originated from the combination (Table 2).

Table 2. Fertility of Hsien-Keng (indica-japonica) hybrid progeny bred by anther culture.

Hybrid	Rate of ripened grain (%)		
	a) F_2	b) H_2	(M±SD)
Toride No. 2 /Nan 65	87.3		98±9.4
Tetep/Nan 65	36.0		80±10.7
Tadukan/Yorokobi-Mine	26.6		81±14.7
Aki-re/IR28//IR28	50.6		86±8.9
Jin-FengNo. 5/Tetep//Yorokobi-Mine	46.9		85±8.9
Toyo-hikari/Tetep//Nan65	32.7		85.6±11.7

a) Inoculative material. b) Plant line of second generation from H_1 anther culture.

Doubling the chromosomes of haploid pollen plants has been used for early stabilization of segregated characters in hybrid progeny. Zhao Chang—zhang and Lang Li—yuan (1981) investigated 108 pollen plants of Hsien—Keng crosses in which Hsien—type rice, Keng—type rice, and their interme—diates have been segregated. Characters such as plant height, heading date, and 1,000—grain weight from an in—termediate type in the H_2 or H_3 generations showed stabi—lization. It is impossible to obtain this rapid stabiliza—tion in conventional breeding.

The CAAS—ICBC inoculated the anthers of the F_2 plant Toyo—Nishiki/Jing—fengNo. 5//C4—63 (Hsien rice) in 1976. The next year, a stable line was selected from its progeny. Through yield trials in 1978—80, Zhong Hua No. 5, a Hsien—Keng intermediate type, was developed. Another stable line was developed from the F_1 of Er—jiu—qing (early Hsien)/ Xian Rong (Keng) and later Zhe—hua No. 1, a new variety with the superior characters of both Hsien and Keng rices was developed.

This illustrates that anther culture is the only feasible approach to resolving the problems of breeding a

Hsien—Keng hybrid.

Remote hybridization in rice always has appeared to be frenzied segregation, an obstacle to selection. Song Xian—bing et al (1980) began anther culture with Kaoliang rice in 1974 and obtained stable progeny rapidly, providing an effective way to resolve the instability in the progeny of remote hybrids.

TECHNIQUES IN ANTHER CULTURE RICE BREEDING

Selection of material

In anther culture breeding, the characters of two parents can be made complementary at an early generation and a plant can immediately be made homozygous. Because the characters in anther—cultured rice are controlled by dominant and recessive genes and interallelic complementation, selecting moderate parents according to a breeding target is important (Din Hui—jing et al 1980, Li Mei—fang et al 1981b, Zhang Zhen—hua et al 1980). The experience of the Rice Haploid Breeding Cooperation Group of Hubei Province (1980) suggests that the range of variation within pollen plants correlates closely with the genetic bases and heterogeneity of inoculative materials. To spread the genetic bases, it is necessary to use distant parents. Compound cross F_1, F_2, or F_3 plants usually are used as inoculative materials (Yang Xue—rong 1980).

Because F_1 or F_2 individuals can be used to evaluate such qualitative characters as disease resistance in both the laboratory and the field, they are the most suitable inoculative materials (Li Mei—fang et al 1980b).

Anther culturability (the rate of callus formation and green plantlet differentiation) is also an important character. Culturability is linked with inoculative materials (Ding Xue—ying et al 1980; Qinzhou Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region, 1980). Zhang Shu—hua et al (1980), Xu Shi—huan (1980), and Li Mei—fang et al (1980b) suggest that the progent of Keng/Keng have greater culturability than the progeny of Hsien/Keng. Experimental data on rice anther culture 1975-1977 at the Hunan Academy of Agricultural Sciences, Institute of Rice Crop Sciences, showed that different combinations of various rice types have a distinct effect on culturability. They suggested that the order of culturability is: Keng/Nou (a glutinous rice) — Keng/Keng — Hsien/Keng — Hsien type A/Hsien type C hybrid (a sterile line crossed with a restorer line) — Hsien/Hsien.

Because of obvious differences between characters of Hsien and Keng rices, different combinations of Hsien—Keng

crosses produced greater variation on culturability. However, different combinations of hybrid rice also produced culturability divergence. Shan-you No. 2 (the F_1 of Zhen-Shan 97A/IR24) has a greater rate of green plantlet differentiation than other Hsien-type hybrid rices (Zhou Xiotao et al 1980; Yulin Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region 1980). Din Hui-jing et al (1980) and Liu Su-lan et al (1980) showed that culturability is higher in the progeny of Keng type A/Keng type C than in the progeny of Keng/Keng, but differs among combinations of Keng-type hybrid rices. Zhou Xiong-tao et al (1980) reported higher culturability of parents in the exotic varieties IR24, IR26, IR30 IR1529-680-3-2, and Labelle. In the native varieties Ai-jiao-tang-zhu,Ji-dui-lun, Er-jiu-qing and Zhen-Shan 97, the parents have higher culturability. This shows the importance of careful selection of the parents of inoculative materials. Li Chao-zan 1980 and Li Mei-fang et al (1980b) proposed that culturability diversity was affected by reciprocal crosses and reported a higher rate of green plantlet differentiation in Hsien/Keng than in Keng/Hsien crosses. To raise the efficiency of anther culture breeding, inoculative materials should be evaluated to find genes of high culturability. These genes then should be introduced into released varieties.

Data in Table 3 show that culturability also is affected by the media selected (Hunan Academy of Agricultural Sciences, Institute of Rice Crop Sciences, 1980; Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences Central Laboratory 1980, Zhou Xiong-tao et al 1980, Cai Yang-hua et al 1980).

Selection and cultivation of the H_1 gereration

The segregation of morphological and physiological characters as well as of various chromosome ploidy may appear in the H_1 generation. Therefore, H_1 becomes the selection base in anther culture breeding. Zheng Zu-ling (1980) discovered various ploidy in H_1 populations. Plants were divided into 35.7% haploid, 58.7% diploid, and 1.5% polyploid as well as 3.1% chimera and aneuploid. Ding Hui-jing et al (1980) obtained similar results. Yuxi Prefecture Institute of Agricultural Sciences, Yunnan Province (1980), found fewer diploid than haploid plants in the H_1 generation, but Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences Central Laboratory (1980) and Yunnan University, Research Group of Breeding Lines by Anther Culture, (1980) reported that the occurrence was about 34—35%of both haploid and diploid. The Yuxi Institute also suggested that anther culture progeny from hybrid rice produced more sterile haploid plants and mutants than had been produced from conventional material, but their

results in inducing plants of diploid, tetraploid, and aneuploid showed the opposite. When a large number of haploid plants were discovered in the H_1 generation, a desirable doubling of chromosomes may be obtained by dipping roots of haploid seedlings into a solution of 0.2% colchicine and 20% dimethyl vanadite. (Zhou Pu-hua et al 1980).

Table 3. Adaptation of materials for inoculation in different media (Zhou Xiong—taoet al 1980).

Combination[a]	Culturability			
	He 5	Synthesis	N6	He 2
Dissi 1—2/37/Non—shuNo. 4	0.5	0	0	0
Dissi 52/37/Zhen—ding28	0	1	0	0
Zhen—shan97/Dissi 52/37//Zhai—ye—qing	6	0	0	0.5
Zao—zhon—shan/Gui—fuNo. 3	0	0	0.5	0

[a] Hsien/Hsien.

Because of the high correlations between haploid and diploid on important economic characters, investigating the appearance of haploids before treatment is useful (Zheng Zu—ling 1980). In vitro culture of young tissue from haploid pollen plants gave rise to a high frequency of double haploid plants. Using young haploid tissue for the regeneration of plantlets increased the frequency of chromosome doubling twice as much as did using chemical treatment (Sun Li—hua and Yu Jian—ming 1980, Zhu De—yao 1980). Extending the population of diploids has practical value in anther culture breeding.

A large number of albino plants always appears in the H_1 genetation. The rate of albino differentiation generally reaches 50% of the total number of seedlings (Yang Xue—rong 1980). Reducing the rate of albino differentiation would be significant in increasing breeding efficiency.

Diploid pollen plants in the H_1 generation usually are of many types as a result of segregation and recombination of different characters of the parents. Yin Gue—da et al (1981) studied the segregation phenomenon in H_1 plants induced from hybrid rice (Shan—yu No. 2, Wei—yu No. 2, and Ai—yu No. 2). A wide range of variation occurred: from 165 to 195 days in growth period, from 50 to 115 cm in plant height, from 25 to 190 grains in average grain number per panicle, and from 18 to 31 g in 1,000—grain weight.

Experiments showed that induction of callus and dif—

ferentiation of green plantlets cannot yet be completely synchronized. Meanwhile, plantlets can be grown only during the abnormal growth season for rice. Many test tube plantlets cultured in the second crop are planted one after another in the greenhouse. Plantlets of the first crop must be transplanted in the season of the second crop or sent to Hainan Island for accelerated propagation. For this reason, the plants of H_1 generations cannot normally be compared with each other. But some physiological characters, such as disease resistance, pest resistance, drought tolerance, cold tolerance, heat tolerance, and salinity tolerance, can still be evaluated for effective selection (Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences Central Laboratory 1980).

At present, the relatively low rate of differentiation of green plantlets is a key problem in anther culture breeding. According to Mendel's law of inheritance, the scale of the F_2 population needed for conventional breeding is about 4,000——5,000plants in combination. By extrapolation, 140——150plants are enough in an H_1 generation.

So far, the rate of green plantlet differentiation (on the basis of anthers inoculated) has been raised to 1.0% in Hsien—type rice (Zhong Ming—hui et al 1980) and to 10.2% in Keng—type rice (Yuxi Prefecture Institute of Agricultural Sciences, Yunnan Province, 1980). This makes it possible to reach the breeding scale needed.

Selection and cultivation of the H_2 generation

H_2 plants are duplicate diploid and become isozygoty lines. But a few segregation lines appear. Among H_2 populations, plant lines of uniform appearance occur 60-90% of the time (Ge Mei—fen et a1 1980, Zhang Shuhua et a1 1980, Li Da—mo et a1 1980, Zhao Chang—zhang et a1 1981, Yin Gue—da et al 1980, Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences Central Laboratory 1980; Yunnan University, Research Group for Breeding Three Lines by Anther Culture 1980; Yulin Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region, 1980). The main factors for producing segregation are: 1) mutation of genes or chromosomes, 2) diploid plant induced from somatic cell of anther wall, and 3) allogamy by revealed stigma of sterile line (Yulin Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region 1980; Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences 1980).

Yin Gue—da et al (1980) used the H_4, H_6, H_7, and H_8 of anther culture line 75——3obtained from the hybrid Ai—you No. 2 under similar conditions. The results show that the important economic characters plant height, grains per panic—

le, and 1,000—grain weight were stable in the H_3—H_8 generations (Table 4). Anther experiments also affirmed that no notable variation nor degeneration occurred with increasing generations as soon as the line was stabilized (Liu Su—lan et al 1980; Ge Mei—fen et al 1980; Tongline Institute of Agricultural Sciences of Anhui Province 1981a; Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences Central Laboratory 1980; Yulin Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region 1980; Shanghai Academy of Agricultural Sciences, Institute of Crop Breeding and Cultivation, 1980). Character tests and yield trials were conducted to evaluate progeny in H_2 generation to increase not only the effect of anther culture breeding, but also reduce the scale of breeding.

Procedures for anther culture rice breeding

The successes achieved in anther culture represent a new breeding approach in China. The working procedure and flow of materials developed by CAAS—ICBC are shown in Figure 2. Genetic resources provide germplasm for improving local rice varieties. Hybridization includes varietal crosses and Hsier Keng crosses (single cross, backcross, and compound cross) as well as production of hybrid rice seed. Inoculative materials are F_1, F_2, F_3, M_1, and sterile line (A)/restorer line (C).

Test tube plantlets are cultivated in clusters in pots and divided into single seedlings for transplanting in the field. The aneuploid is eliminated through selection.

In the H_2 generation, one row is cultivated per plant. Investigations on morphological characters and physiological characteristics are made to select good lines to enter in yield trials the next year. Evaluation of characteristics is made continuously through two to three yield trials. As well as evaluation of characteristics, the most superior lines are entered in regional tests to determine their response to regional conditions and the value of further popularization.

SUCCESSES IN BREEDING RICE BY ANTHER CULTURE

Anther culture breeding methods have the combined advantages of speed and efficiency. A number of new varieties (lines) have been developed by anther culture in the last 10 years. Early stage improved varieties include Mo—hua No. 1, Dan—feng No. 1, Tonghua No. 1 and No. 2, Xin—Xiu, and Zhen—nan (Shanghai Academy of Agricultural Sciences, Institute of Crop Breeding and Cultivation 1980).

The Chinese Academy of Agricultural Sciences, at a national conference on rice anther culture in Yangzhou,

Jiangsu Province, October 1980, collected information on new varieties. About 81 varieties or lines -- 49.5% Keng/Keng crosses, 10.5% Keng/Hsien, 2.0% Hsien/Hsien, 34.0% Hsien type hybrids and 2.0% Keng type hybrids -- have been developed through anther culture. Some, such as late Keng 959 (Rice Haploid Breeding Cooperation Group of Hubei Province 1980), Xin—Xiu (Shanghai Academy of Agricultural Sciences, 1980), Late Keng 76 (Song Zong—miao et al 1981), and Tong—hua No. 2 (Tongling Institute of Agricultural Sciences of Anhui Province 1980b) are planted to more than 10 thousand ha. Many other anther culture—bred rices have been released or demonstrated.

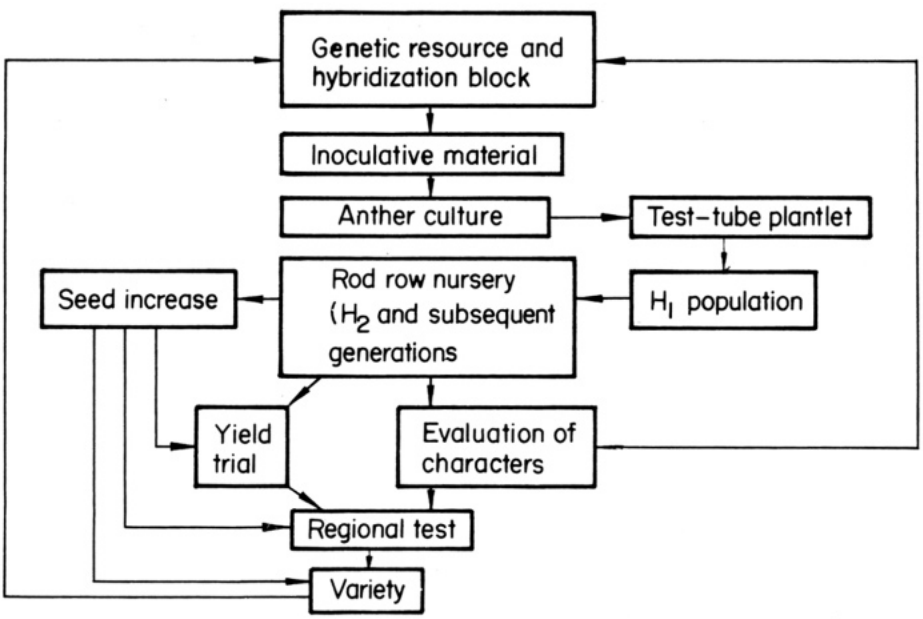

Fig. 2. Working procedure and flow of materials in breed—ing rice by anther culture.

The primary accomplishment in anther culture breeding has been to develop varieties adapted to specific environ—mental conditions.

Applications in varietal crosses

Selection for early maturity. Heilongjiang Province is the most northerly rice belt in China, 48°-50°N. The rice grow—ing period is only 100-120 days. The climate during the rice growing period is temperate with a long day length, but rice

Table 4. Comparison of major agronomic characters in different generations of anther culture lines of 75—3.

Generations	Plant height (cm)	Ears (No.) per plant	Length of ear (cm)	Grains (No.) per ear	1,000-grain weight (g)	Growth duration (days)	P—value 0.05	P—value 0.01
H_4	69.8	9.4	19.1	96.7	20.5	136		
H_6	69.7	8.0	19.2	101.8	21.1	136		
H_7	69.9	8.0	18.6	95.0	21.0	137		
H_8	69.6	8.3	18.9	98.2	21.1	136		
F—value	0.031	0.333	2.300	0.996	2.946	1.301	3.86	6.99

always encounters temperatures below 20°C before or after heading. To select an early—maturing variety tolerant of low temperatures is important to stabilize yields in that district.

Hejiang Institute of Rice Sciences, Heilongjiang province, started anther culture breeding in 1977. He—dan 76—085, an early—maturing blastresistant, high—yielding variety has been developed (Xu Shi—huan, 1980) (Fig. 3).

Breeding for blast resistance. Rice blast is a common disease in China, especially in the Keng rice district. The CAAS—ICBC and the Yangzhou Prefecture Institute of Agricultural Sciences, Jiangsu Province, have introduced the blast-resistant genes of Toride No. 1 and No. 2 (Shen Jin—hua et al 1981) into local, high—yielding varieties. ICBC has developed two varieties, Zhong—hua No. 8 and No. 9 (Fig. 4). Rice variety Hua—jian 7902 was bred later (Ge Mei—fen et al, 1980) (Fig. 5). All these varieties not only have high yields but also are resistant to local virulent races of blast.

Selection for low—temperature tolerance. Low temperature at the flowering stage influences rice yields in the southern rice district of China. Selecting a variety tolerant of low temperatures is important to stabilize yields, especially in the Changjiang (Yangtze River) basin. During 1973—80, the Shanghai Academy of Agricultural Sciences, Institute of Crop Sciences, developed Hua—han—zao, an early second-season Keng rice (Fig. 6). Multiple recombination between lines of pollen plants tolerant of low temperature at meiosis or the flowering stage was used.

Breeding for salinity tolerance. Large areas of rice fields are in the sea border region, where salinity affects 0.1—0.5% of the crop. To breed a tolerant variety adapted to these soils, Tianjin Institute of Agricultural Sciences and Institute of Genetics, Academia Sinica (1980) cooperatively selected a new anther culture variety Hua—yu No. 1 (Fig. 7). It was selected from the combination of Nihon—bare, with disease resistance, and Qian—jun—bang, with favorable growth in the local saline soils. Hua—yu No. 1 was evaluated in local trials for normal growth in fields with 0.2—0.3% salinity (Tianjin Institute of Agricultural Sciences and Institute of Genetics, Academia Sinica 1976). At present, it is popular in a large area of Tian jin district.

Breeding for high—yielding Hsien variety. Anther culture ability (culturability) of progeny from varietal crosses of Hsien rice is low and success in breeding by anther culture is always slower than it is in Keng rice. Recently, improved techniques have made possible the development of a number of high—yielding Hsien varieties. Min—hua No. 1, No. 2, and

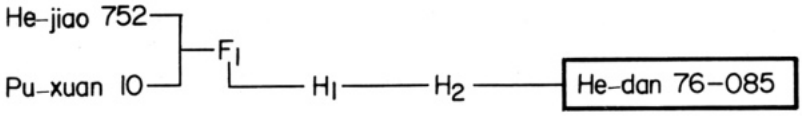

Fig. 3 Pedigree of He—dan 76—085.

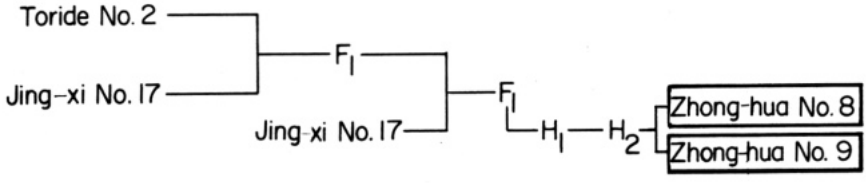

Fig. 4. Pedigree of Zong—hua No. 8 and No. 9.

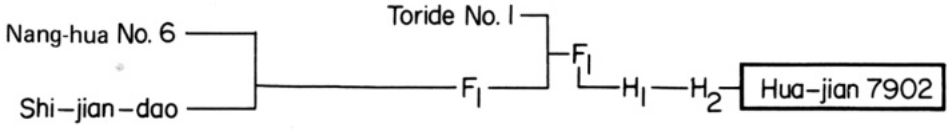

Fig. 5. Pedigree of Hua—jian 7902.

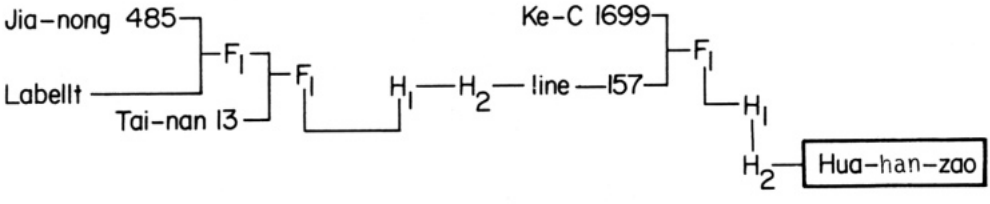

Fig. 6. Pedigree of Hua—han—zao.

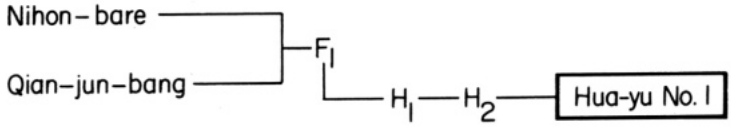

Fig. 7. Pedigree of Hua—yu No. 1.

Table 5. Agronomic characters of Nan—hua No. 5, No. 11, and No. 2.

Variety	Growth duration (days)	Panicles per m^2 (No.)	Grains per ear (No.)	Sterility (%)	1,000—grain weight (g)	Yield (t/ha)	Yield increase (%)
Nan—hua No. 5 [a)]	122	249	132	30.9	26.0	7.25	22.5
Nan—hua No. 11 [a)]	137	267	111	18.1	28.2	6.92	16.9
Nan—yu No. 2 [b)]	124	183	159	47.8	26.5	5.92	0

[a)] Anther culture rice. [b)] (Er—jiu—nanA/IR24) F_1.

No. 3 were developed by the Fujian Academy of Agricultural Sciences, Institute of Rice and Wheat Sciences (Ding Xue—ying et al 1980).

Improving hybrid rice

Breeding new varieties from hybrid rice by anther culture is an important achievement. Anther culture—bred rice strongly displays its hybrid vigor in grain yield. But one problem is that a large amount of hybrid seed is needed every year. The complexity of the procedure and the strictness demanded by the techniques of seed production delay the popularization of hybrid rice.

Scientists expect to induce a homozygous diploid with high yield and the same gene type as its originator. For this purpose, they have adopted anther culture methods to breed a number of varieties that have the same high yield ability as hybrid rice.

Breeding for yield increase. The Tongling Institute of Agricultural Sciences, Anhui Province, has released Nan—hua No. 5, No. 11, and No. 22 from the anther culture—bred progeny of Nan—you No. 2. Nan—hua No. 5, and No. 11 lines had 16.9—22.5% higher yields than Nan—you No. 2. (Table 5). The Lichuan Institute of Agricultural Sciences, Jiangxi Province, developed Shan—you No. 2 through anther culture. Six lines of anther culture rice (Shan—hua 7701, 7706, 78-1, 791, 792, and 793) were developed in 1977—1979. Morphological and economic characters of the new lines are nearly the same as Shan—you No. 2. They yielded 15% more than Shan—you No. 2 when cultivated as late—maturing varieties for a single crop. Yields decreased 5-13% when they were planted as late—maturing varieties for a second crop (Li Zu—xin1980).

The Luodian Institute of Agricultural Sciences and the Guizhou Academy of Agricultural Sciences Central Laboratory (1980) through anther culture developed Qian—hua No. 1 from progeny of Nan—you No. 2. Qian—hua No. 1 grows in areas 370-1085 m above sea level at 14.9-19.9°C average temperature. Grain production is 10.3 t/ha in moderately fertile soil, almost as high as the yield of Nan—you No. 2. Qian—hua No. 1 has more panicles as well as stronger resistance to drought and disease (blast and blight) than Shan—you No. 2 and is the recommended variety in Guizhou Province.

The Guilin Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region (1980), inoculated anthers of hybrid rice Yin—you No. 2 (Lu—yin a/ IR24). Through a 3—year selection, it developed variety Hua—you No. 2. Its yield is 13.4% more than that of Guangxuan No. 3.

Breeding for grain quality. Grain of Hsien—type hybrid rice has been characterized as too sticky, with lower cooking

quality. A homozygote variety developed through anther culture has the improved grain quality of hybrid rice. Experimental results from Yulin Prefecture Institute of Agricultural Sciences, Guangxi Autonomous Region (1980) suggest that anther-cultured rice varieties Yin-hua No. 3 and Shan-hua No. 8 are free of the adverse characters of poor grain quality. They originated from hybrid rice Yin-hua No. 1 and Shan-you No. 5. Qian-hua No. 1 shows similar characters in Guizhou Province (Luodian Institute of Agricultural Sciences and Guizhou Academy of Agricultural Sciences Central Laboratory (1980).

For hybrid rice of the Keng type, the Institute of Improvement and Utilization of Saline and Alkaline Soil, Liaoning Province, selected line 77-7-5, bred by anther culture from the progeny of Zao-feng A/C64. This line had yields 7-8% more than the yields of cultivar Toyo-nishiki (Liu Su-lan 1980).

Regardless of the type of hybrid rice - Hsien or Keng- excellent varieties with homozygous superior characters have been selected by anther culture and the grain quality of hybrid rice has been improved. One success was to resolve the problem of annual seed production, so that anther-cultured rice might be of practical value in production.

Breeding for three lines. Anther culture has been used to breed three pure lines: male-sterile, maintainer, and restorer. The restorer line of Keng-type hybrids is from the progeny of a Hsien/Keng hybrid. Annual testing of restoration and purifying seed is a large task.

The Kunming Prefecture Institute of Agricultural sciences, Yunnan Province, has developed a Dian-hui (Yunnan's restorer) line by anther culture (Cun Zhen-yang 1980). The Yulin Prefecture Institute of Agricultural Sciences,Guangxi Autonomous Region, has selected by anther culture some male-sterile and maintainer lines from hybrid rice.

Breeding a parent for chemical emasculation. Another achievement is applying anther culture to the breeding of parents of hybrid rice by chemical emasculation. The Zhaoqing prefecture Institute of Agricultural Sciences, Guangdong Province, has discovered a line with compact plant type, short flowering time, outer emergence of stigma, and good economic characters. With chemical emasculation, this line can be used as a mother parent for hybrid rice (Zhou Li-nong and Zhong Min-hui 1980).

Stabilizing remote hybrids. Anther culture also has been used in developing remote hybrids. In 1976, the CAAS-ICBC used anther culture of F_1 plants of 25 crosses of Kaoliang rice with local superior varieties to improve disease resistance and grain yields of Kaoliang rice. They developed a few

Table 6. Some improved rice varieties developed in China.

Local variety

Ai—jiao—tang—zhu(矮脚塘竹)
Er—jiu—qing(二九青)
Guang—xuan No. 3 (广选 3 号)
He—jiao 752 (合交 752)
Jin—fu No. 9 (津辐 9 号)
Jing—feng No. 5 (京丰 5 号)
Jing—xi No. 17 (京系 17)
Jia—nong 485 (加农 485)
Ji—dui—lun (鸡对伦)
Late 76 (晚 76)
Nan 65 (南 65)
Nong—hu No. 6 (农虎 6 号)
Pu—xuan No. 10 (普选 10 号)
Qian—jung—bang (千钧棒)
Shi—jian—dao (实践稻)
Wan 87 (晚 87)

Hybrid rice

Ai—you No. 2 (矮优 2 号)
Guang—lu—yin A (广陆银 A)
Nan—you No. 2 (南优 2 号)
Shan—you No. 2 (汕优 2 号)
Wei—you No. 2 (威优 2 号)
Yin—you No. 2 (银优 2 号)

Anther culture

Dan—fe (单丰 1 号)
Hua-yu No. 78-246 (花育 78—246)
He—dan76—085 (合单 76—085)
Hua—jian 2901 (花鉴 2901)
Hua— jian 7902 (花鉴 7902)
Hua—han—zao (花寒早)
Hua—yin—you (花银优)
Hua—yu No. 1 (花育 1 号)
Hua 110 (花 110)
Mo—hua No. 1 (牡花 1 号)
Min—hua No. 1 (闽花 1 号)
Min—hua No-. 2 (闽花 2 号)
Min—hua No. 3 (闽花 3 号)
Nan—hua No. 5 (南花 5 号)
Nan—hua No. 11 (南花 11 号)
Nan—hua No. 22 (南花 22 号)
Shan—hua No. 8 (汕花 8 号)
Tong—hua No. 1 (铜花 1 号)
Tong—hua No. 2 (铜花 2 号)
Wan—keng 959 (晚粳 959)
Xin—xiu (新秀)
Yin—hua No. 3 (银花 3 号)
Zhu—hua No. 1 (浙花 1 号)
Zhen—nan
Zhong—hua No. 2 (中花 2 号)
Zhong—hua No. 5 (中花 5 号)
Zhong—hua No. 8 (中花 8 号)
Zhong—hua No. 9 (中花 9 号)

lines, such as Hua line 110 (Kaoliang rice/A 7505//Zao—feng), with growth periods of about 140 days, plant heights of 110 cm, an average 100 grains per panicle, 1,000—grain weight of 27 g, and blast resistance. These were demonstrated in the suburban district of Beijing (Son Xianbing et a1 1980).

Combining with mutation breeding. In recent years, an early—maturing dwarf mutant line has been obtained by using chemical mutagens or radiation on an excised rice anther

and its pollen plants (Hu Zhong et al 1980, Zhuang Cheng—Ji et al 1980). Stable mutation has been achieved with an artificial mutant of hybrid rice.

It appears that comprehensive application of hybridization, intervarietal crosses, hybrid vigor, anther culture, and artificially induced mutation will be a direction to expand the field of rice breeding (Li Da—mo et al 1980, Liu Du—yin et al 1980).

CONCLUSION

The use of anther culture for breeding improved rice varieties has steadily increased in China. A large number of rice varieties with superior characters have been developed and are becoming popular in production (Table 6). And, the techniques and working procedures for anther culture breeding have been systematically accumulating. The next achievement to be gained from anther culture breeding is the stabilization of segregation of characters in early generations of the progeny of intervarietal crosses. Remote hybridization and artificially induced mutation are being used.

Through practical experience, we have found that anther culture can be used not only to breed new varieties, but also to synthesize new, specific genotypes never before found in local or exotic germplasm. This means that anther culture has an important function to play in the establishment of a new germplasm bank.

Problems still exist in anther culture. The appearance of albino and aneudiploid plants inhibits the expansion of H_2 populations. Many mutations also have been discovered in progeny produced by anther culture. These are interesting problems in genetics.

To raise the efficiency of rice breeding, it is necessary to strengthen research work on the techniques of anther culture and to increase the number of theoretical studies. To do this, international cooperation among agricultural scientists (in China, IRRI, Japan, USA, and countries of southeast Asia) involved in anther culture breeding of rice must be promoted by such means as exchange of materials and experiences, symposia and coordinated studies. These will benefit the development of the science and technology of rice breeding by anther culture in China as well as in rice-growing countries of the world.

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When available, names of scientists in China are given in the traditional Chinese order: surname followed by full given name with no comma. Given names are hyphenated, with only the first syllable capitalized. Initials of given names normally are not used.

Names of all authors of articles, publishers, and places of publication were not always available. Details may be requested from the author of the paper.

CALLUS INDUCTION AND REDIFFERENTIATION OF DIFFERENT HYBRID RICE PLANT PARTS

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Since callus tissue induction was first reported from the stem nodes of rice (Furuhashi and Yatazama 1964), other organs such as the root, leaf sheath, seedlings, embryo, and seed have been used to induce callus formation. Nishi et a1 (1968) successfully redifferentiated rice callus tissue into intact plantlets. Tsai (1978) reported similar success of green plantlet formation from the shoot apex, and Shu Li—hui et a1 (1980) from young panicles.

Our experiment studied the abilities for dedifferentiation and redifferentiation of a variety of explants, such as seed, shoot apex leaf, and young spikelets at different developmental stages. It sought to find the organ for plant tissue culture that could be used for the large—scale cell propagation that would be required in the industrial manufacture of seedlings.

MATERIALS AND METHODS

The materials used were spikelets of rice subspecies Keng Ting, Oryza sativa L., from the Liaoning Academy of Agriculture Science. Young spikelets were selected from plants growing vigorously in the field. The main stems were cut about 5—10 cm from the base, scrubbed with 75% alcohol, dipped in alcohol for 10 minutes, and stripped of their internal leaves under sterile conditions. Young spikelets at different developmental stages and the shoot apex were excised.

The young spikelets (YE) were divided into 6 developmental stages according to length: YE1 (0.2 cm), YF2 (0.5 cm), YF3 (1 cm), YE4 (1.5 cm) YE5 (3 cm), and YE6 (5 cm). Seeds were cleaned with soap solution, rinsed in running water for 24 hours, dipped in 75% alcohol for 5 minutes to remove glumes, sterilized with $HgCl_2$ solution for 10 minutes, and rinsed 3 times with sterile water. Excised rice leaves were sterilized with 10% Ca—hypochlorite solution for 8 minutes and rinsed in sterile water 3 times.

The sterilized materials were inoculated on MS medium

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supplemented with 2 ppm, 2,4—D, 0.2 ppm IAA, 0.2 ppm KT, and 500 mg casein hydrolysate. After 20—30 days, calli were transferred to a redifferentiation medium with 2 ppm KI, 0.2 ppm IAA, and 800 mg casein hydrolysate. Temperature in the culture room was about 27°C. Cultures were kept in complete darkness for dedifferentiation and callus induction and in an 8 hours light, 16 hours dark cycle for redifferentiation and regeneration of plantlets. Light intensity was 1600 lux.

RESULTS

Callus induction

Callus formation was induced in seeds, shoot apices, and young spikelets at all developmental stages, including just sprouting out. The peak period of callus formation and growth and callus induction frequency varied significantly among the different materials. Young spikelets about 1—3 cm long were the earliest to reach peak callus formation. They grew most vigorously and gave the highest induction

Table 1. Callus formation on different plant organs.

Materials[a)]	Number inoculated	Peak callus—forming period (days)	Ca11us (No.)	Induction frequency (%)
Seed	40	10	28	70
Leaf	40	0	0	0
Shoot apex	40	16	21	52.5
YE1	40	16	30	75
YE2	40	7—10	32	80
YE3	40	5—7	40	100
YE4	40	5—7	40	100
YE5	40	5—7	34	85
YE6	40	15	21	52.5
YE ready to emerge	40	25	2	5

[a)] YEl = young spikelets 0.2 cm long, YE2 = young spikelets 0.5 cm long, YE3 = young spikelets 1 cm long, YE4 = young spikelets 1.5 cm long, YE5 = young spikelets 3 cm long, YE6 = young spikelets 5 cm long.

frequency (Table 1, Fig. 1).

Induction frequency was lowest (52%) with the shoot apex and increased gradually with spikelet development. It peaked with young spikelets about 1—1.5 cm long, then decreased to only 5% when young spikelets were about to

Fig. 1. Callusing young spikelets of hybrid rice. (Left to right: YE5, YE4, YE3, and YE2).

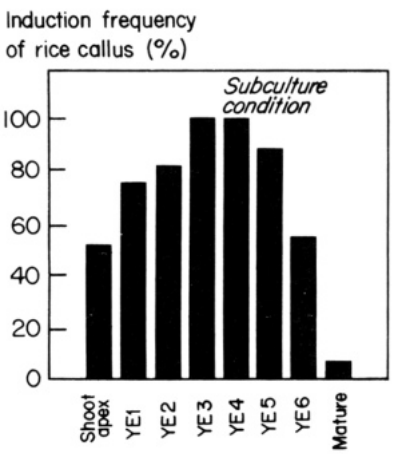

Fig. 2. Callus induction in young rice spikelets cultured at different stages of development.

sprout from their leaf sheath (Fig. 2).

Regeneration of green plantlets

All the calli induced from explants were transferred to the same redifferentiation medium. Most finally redifferentiated into green plantlets. The redifferentiation frequency was notably different with different explants. Redifferentiation frequency was highest in young spikelets 1—1.5 cm long (YE3 and YE4) and lowest in those derived from YE6 (5 cm long) callus. Callus induced from spikelets that were about to sprout from the leaf sheath could not redifferentiate at all (Table 2).

Table 2. Redifferentiation of green plantlets from callus formed on different plant organs.

Materials[a)]	Green plantlet formation period (days)	Callus (No.)	Green plantlets (No.)	Redifferentiation frequency (%)
Seed	14	28	21	75
Shoot apex	18	21	18	85.7
YE1[a)]	18	30	26	86.7
YE2	14	32	32	100
YE3	12	40	40	100
YE4	12	40	40	100
YE5	12	34	30	88.2
YE6	12	21	13	61.9

[a)] YE1 = young spikelets 0.2 cm long, YE2 = young spikelets 0.5 cm long, YE3 = young spikelets 1 cm long, YE4 = young spikelets 1.5 cm long, YE5 = young spikelets 3 cm long, YE6 = young spikelets 5 cm long.

Redifferentiation of green plantlets in subculture

Callus induced from different explant materials was subcultured for 85 days before transferring into MS redifferentiation medium. In general, the redifferentiation frequency (Fig. 3), the period needed for green plantlet formation, and the vitality of the plantlets all declined. Seedlings grew very slowly, reaching 1.5 cm long with very poor root redifferentiation 35 days after being transferred to the

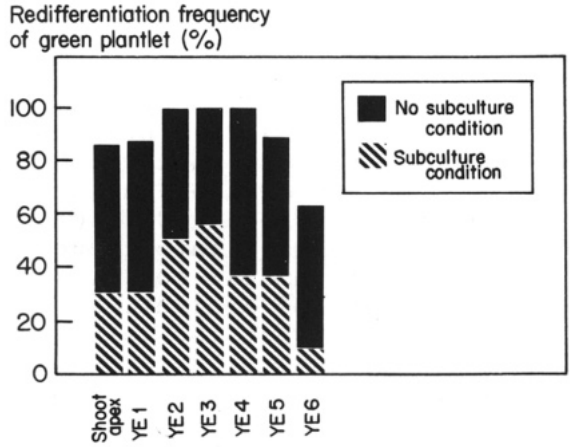

Fig. 3. Effect of subculture on rediffer— entiation frequency of green plantlets from young rice spikelets cultured at different stages of development.

redifferentiation medium. In contrast, those not subcul— tured grew well and rooted normally (Fig. 4).

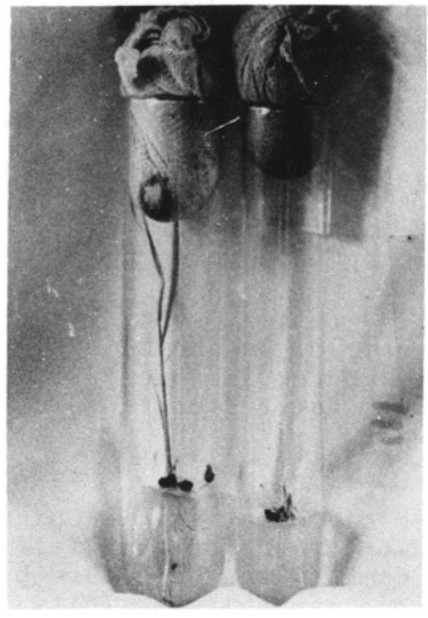

Fig. 4. Growth of green plantlets from callus that had been subcultured (right) and from callus that had not been subcultured (left).

DISCUSSION

The important question is whether plant cells can grow as quickly and easily as microbes. If they can, it should be possible to maintain heterosis and to produce seedlings in the laboratory by somatic cell culture. More than 100 labs throughout the world are engaged in such investiga— tions. In theory, plant cells, being totipotent in nature, should be able to redifferentiate into intact plants. But in practice, the effects of somatic cell culture vary greatly with species, organs of a plant, and developmental stages of an organ. It seems that the materials themselves, rather than the medium components, are more instrumental in callus induction and green plantlet formation (Shu Li— Hui et al 1980).

We found that young rice spikelets can be congenial material for tissue culture, not only because they can be easily sterilized to avoid contamination and can be manipulated conveniently, but also because they produce highly efficient results. A great deal has been reported in rice tissue culture about using different organs, such as seed, stem node, root, ovary, embryo, leaf sheath, and rachis branches. Callus induction frequency has varied from 20 to 80% and differentiation frequency of green plantlets has varied from 40 to 70%. The results of our experiments show that both induction and redifferentiation frequency can reach 100% (with young spikelets 1—1.5 cm long). This fact alone suggests the favorable nature of this material.

However, in prolonged subcultures, the frequency of redifferentiation of green plants from callus showed a significant decrease and growth of plantlets derived from these subcultures was markedly poor.

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RECENT PROGRESS IN RICE EMBRYO CULTURE AT IRRI

Emerita V. de Guzman

One of the most useful applications of plant embryo culture is in overcoming the inviability of hybrid embryos. Intergeneric and interspecific crosses sometimes are unsuccessful because of the degeneration of hybrid embryos. Abortion of the embryo is attributable to the failure to form normal endosperm.

F_1 hybrids from otherwise unsuccessful interspecific crosses have been obtained in rice through embryo culture (Bourhamont 1961, Li et al 1961, Iyer and Govilla 1964). Embryo culture also has been used to overcome nongermination or poor germination of mature hybrid rice seeds (Nakajima and Morishima 1958). The culture conditions and first germinative stage of immature rice embryos were studied by Amemiya et al (1956).

Transferring cultured seedlings to potted soil can pose a survival problem. Iyer and Govila (1964) improved survival rates by growing cultured seedlings in a nutrient solution before transferring them to soil. A primary consideration for transplant survival is seedling vigor, which can be determined by the growth medium and other culture conditions.

But embryo-cultured hybrid plants of rice and other species are sterile (Li et al 1961, Davies 1960, Konzak et al 1951, Morisson et al 1959, Schooler 1960). Schooler was able to induce fertility in F_1 plants with cochicine treatment. Fertile F_1 interspecific hybrids of cotton were obtained through treatment of combined chemical spray, embryo culture, and colchicine application in vitro (Liang et al 1979).

The objectives of embryo culture studies at IRRI are:

- To determine the first germinative stages and morphogenetic potentials of immature rice embryos with different media.
- To obtain F_1 hybrids from otherwise unsuccessful interspecific and even intraspecific crosses.
- To develop an efficient method of colchicine treatment to overcome sterility when immature embryos are used.
- To regenerate fertile plants from tissue cultures of

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Table 1. Size of spikelets and embryos harvested at different ages after anthesis.[a)]

Variety	Age (days)	Embryos (no.)	Intact spikelet		Dehulled spikelet		Embryo	
			Length (mm)	Width (mm)	Length (mm)	Width (mm)	Length (mm)	Width (mm)
H_4	4	20	8.5	2.9	5.0	1.4	0.24	0.18
	5	20	8.6	3.2	6.2	2.3	0.46	0.29
	6	20	8.7	3.3	6.4	2.4	0.90	0.51
F_5	4	20	6.8	3.6	4.6	1.5	0.17	0.13
	5	20	7.1	3.6	5.4	1.7	0.49	0.33
	6	20	6.9	3.6	5.8	2.4	0.89	0.59

[a)] Plants were grown in the phytotron at natural day length, 29/21°C day—night temperatures.

embryos from sterile hybrids.

In this study, embryo culture refers to the culture of mostly immature and excised embryos to induce germination and some morphogenetic response.

MATERIALS AND METHODS

Rice varieties Fujisaka 5, IR8, and H_4 were grown to maturity in the phytotron under natural daylight and 29/21°C day—night temperature. Spikelets were dated at anthesis and panicles were harvested at appropriate stages (Table 1).

Whole spikelets were immersed in 70% ethyl alcohol for 5 minutes, then sterilized with 5% calcium hypochlorite solution and Tween 20 (ca. 3 drops/50 ml solution) for 30 minutes.

Embryos 6 to 11 days old were excised by slicing the base of the spikelets and pressing the hulls with forceps. Embryos 4 and 5 days old were excised under a dissecting microscope. Each embryo was inoculated directly onto the culture medium.

Modified White's (W) (1963) and Murashige and Skoog's (MS) (1962) media were used in most experiments, MS at 114 strength except for callus formation and regeneration. The MS medium contained myo—inositol. Other growth substances were added as needed. In early experiments on the culture of 6—day—oldand older embryos, Nitsch and Nitsch vitamins were used. MS vitamin formulation was used in all other cultures. Coconut water from mature nuts (150 ml/liter) was added be—fore or after autoclaving, if needed. If added after auto—claving, coconut water was aseptically extracted. Monnier's (1978) mineral salt formulation was used instead of MS min—eral salts in a few trials. Embryos 4 and 5 days old were cultured in petri dishes, older embryos in individual 18 x 150 mm test tubes.

RESULTS

Culture of 6—day—oldand older embryos

In general, the more mature embryos germinated by the second day after inoculation. Maximum germination was attained in 2 days by the more mature embryos and a few days later by 6—and 7—day—oldembryos (Tables 2, 3, 4). Initial germina—tion was lowest in the W medium, especially with embryos excised at younger stages. Accelerated germination of very immature embryos was obtained by adding coconut water to the W medium or by using MS medium.

Cultures were terminated 2 weeks after inoculation. At this stage, seedlings had overreached the cotton plugs, with well—developed root and shoot systems (Tables 5, 6, 7). The

Table 2. Germination and viability of excised immature Fujisaka 5 rice embryos.

Embryo age (days)	Medium [a]	After 2 days		After a minimum of 14 days		Viability [b] (%)
		No.	%	No.	%	
6	W	63	35.2	63	96.8	96.7
	WC	55	77.3	56	98.2	92.6
	1/4 MS	57	84.2	55	96.4	94.5
	1/4 MS+CW	56	95.0	55	96.4	96.3
7	W	54	81.5	55	90.9	94.2
	WC	52	92.3	51	94.1	93.8
	1/4 MS	52	96.2	44	97.7	93.2
	1/4 MS+CW	47	97.9	44	97.7	93.0
8	W	44	93.2	32	100	
	WC	55	100	28	100	
	1/4 MS	54	100	33	100	
	1/4 MS+CW	39	100	27	100	
9	W	42	90.5	32	100	
	WC	44	95.5	34	100	
	1/4 MS	43	93.0	36	100	
	1/4 MS+CW	23	95.7	34	100	

[a] W=Modified White's medium, WC + W + coconut water, MS = Modified Murashige and Skoog's medium, MSCW = MS + coconut water. [b] Based on number of germinated embryos.

Table 3. Germination and viability of excised immature IR8 rice embryos.

Embryo age (days)	Medium[a]	After 2 days		After a minimum of 14 days		Viability[b] (%)
		No.	%	No.	%	
6	W	54	4	43	100	62.2
	WC	32	40	28	100	106
	MS	54	29	46	96	76.7
	MSCW	31	62.5	21	100	100
7	W	46	37.9	39	98.2	96.7
	WC	37	58.8	29	100	89.5
	MS	47	58.7	42	100	84.9
	MSCW	30	80	27	100	82.4
8	W	31	70	28	100	
	WC	30	90	20	100	
	MS	33	90.3	27	100	
	MSCW	30	86.7	22	100	
9	W	21	60	20	100	
	WC	19	67.8	18	100	
	MS	20	79.8	16	100	
	MSCW	22	86.7	17	100	
11	W	7	85.7	7	100	
	WC	7	100	6	100	
	MS	7	100	6	100	
	MSCW	7	100	3	100	

[a] W = Modified White's medium, WC = W + coconut water, MS = Modified Murashige and Skoog's medium, MSCW = MS + coconut water. [b] Based on number of germinated embryos.

Table 4. Germination and viability of excised immature H_4 rice embryos.

Embryo age (days)	Medium[a]	After 2 days		After a minimum of 14 days		Viability[b] (%)
		No.	%	No.	%	
6	W	22	14	12	75	100
	WC	16	50	12	75	100
	MS	17	35	8	100	100
	MSCW	17	35	9	100	100
7	W	12	25	12	100	91.7
	WC	11	46	6	100	100
	MS	11	100	10	100	100
	MSCW	11	91	9	100	100
10	W	10	90	10	100	
	WC	10	100	5	50	
	MS	10	100	5	100	
	MSCW	10	100	3	100	

[a] W = Modified White's medium, WC = W + coconut water, MS= Modified Murashige and Skoog's medium, MSCW=MS+coconut water

[b] Based on number of germinated embryos.

Table 5. Growth of immature Fujisaka rice embryos 14 days after culture.

Embryo age (days)	Medium[a]	Leaves (no.)	Shoot length (mm)	Roots (no.)	Root length (mm)	Dry wt (mg)
6	W	4.5	94.3	4.0	72.3	4.0
	WC	4.3	78.8	4.2	72.2	4.4
	MS	5.0	144.9	4.5	71.6	6.9
	MSCW	4.8	112.8	4.2	86.1	6.8
7	W	4.6	117.1	4.5	99.0	5.6
	WC	4.2	95.1	4.4	72.9	5.8
	MS	4.7	165.7	4.8	66.8	9.2
	MSCW	4.8	134.3	4.7	87.6	9.4
8	W	4.6	140.9	5.2	127.3	8.1
	WC	4.4	96.8	5.1	82.0	7.4
	MS	4.8	171.5	5.7	73.3	12.5
	MSCW	4.9	139.5	5.4	95.5	14.2
9	W	4.7	136.3	5.1	86.9	8.3
	WC	4.4	84.2	4.4	67.4	7.3
	MS	5.1	161.4	6.5	74.5	13.7
	MSCW	5.0	147.2	5.0	101.1	13.3

[a] W = Modified White's medium, WC = W + coconut water, MS= Modified Murashige and Skoog's medium, MSCW = MS + coconut water.

Table 6. Growth of immature IR8 rice embryos 14 days after culture.

Embryo age (days)	Medium[a]	Leaves (no.)	Shoot length (mm)	Roots (no.)	Root length (mm)	Dry wt (mg)
6	W	2.8	28.7	1.1	34.8	2.1
	WC	4.1	36.2	3.6	16.1	2.6
	MS	4.5	113.0	5.4	63.8	6.8
	MSCW	3.8	44	4.0	26.5	2.7
7	W	3.9	50.5	2.0	42.6	3.3
	WC	3.7	39.8	8.1	23.2	3.8
	MS	4.0	113.6	4.7	56.8	7.9
	MSCW	3.6	41.2	2.6	16.8	4.6
8	W	4.1	75.0	3.4	60.6	5.7
	WC	4.5	66.3	4.0	48.3	6.7
	MS	3.7	107.7	6.3	51.0	7.6
	MSCW	4.3	64.1	4.0	29.3	7.0
9	W	4.6	86.7	3.3	65.8	6.6
	WC	3.6	40.3	3.3	17.6	5.2
	MS	3.8	86.7	4.3	42.2	10.1
	MSCW	4.1	54	4.6	22.7	5.9
11	W	4.3	97	4.9	65.1	8.2
	WC	4.0	63.8	3.0	19.0	7.1
	MS	4.2	91.8	5.5	44	7.5
	MSCW					

[a] W = Wodified White's medium, WC = W + coconut water, MS = Modified Murashige and Skoog's medium, MSCW = MS + coconut water.

younger the embryo at excision, the less the final growth attained. Growth was best in MS medium. However, adding coconut water to MS was inhibitory at all ages in varieties IR8 and H_4. Whether the superiority of MS to W was due to the presence of NH_4^+ ions and myo—inositol is not yet determined.

Culture of 5—day—oldembryos

High germination was obtained with 5—day—oldembryos of all varieties used (Table 8). Viability was high, especially with MS medium. Some plants with green shoots and good root systems could be transfered to potted soil. But some seedlings developed only shoots or roots.

Six days after anthesis, embryos acquired an opaque, ivory white appearance and a firm texture. Five—day—old embryos were translucent and soft, somewhat like a hard jell. Histological differentiation varied from cotyledonary to

first leaf.

Table 7. Growth of immature H_4 rice embryos 14 days after culture.

Embryo age (days)	Medium[a]	Leaves (no.)	Shoot length (mm)	Roots (no.)	Root length (mm)	Dry wt (mg)
6	W	2.3	14.8	2.4	12.4	2.2
	WC	3.8	30.8	3.2	10.9	2.2
	MS	3.9	113.8	4.1	52.6	4.8
	MSCW	3.8	46.4	3.3	18.2	2.6
7	W	3.6	66.5	2.5	41.5	2.7
	WC	3.9	39.0	4.0	9.4	2.7
	MS	3.9	141.7	5.6	42.9	6.6
	MSCW	4.0	84.9	5.1	29.7	4.3
8	W	3.9	91.1	3.9	43.8	3.6
	WC	3.6	34.1	3.3	45.5	2.2
	MS	4.6	178.4	6.7	70.3	10.6
	MSCW	4.0	118.7	7.0	34.1	7.2
9	W	3.7	100.1	3.8	58.3	5.4
	WC	3.8	34.2	3.5	5.6	2.9
	MS	4.3	181.5	7.4	64.6	11.6
	MSCW	4.0	79.9	4.1	29.0	5.2
10	W	3.7	77.5	3.9	53.8	4.3
	WC	4.0	34.0	3.3	6.9	1.9
	MS	4.4	193.8	8.8	73.2	12.7
	MSCW					

[a] W = Modified White's medium, WC = W + coconut water, MS= Modified Murashige and Skoog's medium, MSCW = MS + coconut water.

Culture of 4-day-old embryos

Germination of 4-day-old embryos ranged from 6% to 86.5% (Table 9). In some experiments, 100% of those germinated were viable after 2 weeks of culture. Unfortunately, microbial contamination prevented prolonged observations.

Some cultures developed into complete seedlings whereas others formed only shoots or roots. In all varieties, many cultures had roots but no viable shoots. Two IR8 and one Fujisaka 5 4-day-old embryo cultures produced 6—4green shoots per plant. In the older embryo cultures in nonregeneration media, multiple shoot formation of the degree observed with the 4-day-old embryos did not occur. The multiple shoots occurred only in MS media supplemented with coconut water. W medium, whether supplemented or not supplemented with coconut water, did not produce complete seedlings. No shoot

Table 8. Growth and development of 5—day—oldrice embryos.

Variety	Medium[a]	Embryos (no.)	Germination[b] (%)[d]	Shoot[c] or root (%)[e]	Viable[c] plants (%)[e]	Transferable[c] plants (%)[e]
Fujisaka 5	W	52	83	13	58	2
	WC	45	85	7	56	14
	MS	32	100	6	80	18
	MSCW	25	79	0	67	0
H_4	W	30	70	7	9	7
	WC	31	80	0	20	11
	MS	29	48	0	58	33
	MSCW	41	65	4	44.2	29
	2%	26	81	0	60	37
	4%	23	76	6	18	0
	8%	24	17	0	0	0
	10%	19	4	0	0	0
IR8	W	10	60	17	0	0
	WC	18	33	31	39	31
	MS	10	100	0	100	50
	MSCW	15	93	0	93	50

[a] W = Modified White's medium, WC = W + coconut water, MS = modified Murashige and Skoog's medium, MSCW = MS + coconut water. [b] After a minumum of 1 week. [c] After a minimum of 2 weeks. [d] Based on total number of embryos. [e] Based on number of germinated embryos.

Table 9. Growth and development of 4-day-old rice embryos.

Medium [a]	Embryos [b] (no.)	Germination (%)	Root only [c] (%)	Shoot only [c] (%)	Root and shoot [c] (%)	Viability [c] (%)	Multiple shoots [c] (%)	Max. per plant (no.)
				Fujisaka 5				
MS	32	22	0	5	60	50		
MSCW	10	50	20	40	40	100		
MS15AT	15	86	46	0	38	84		
MS30AT	10	30	100	0	0	0		
MS60AT	10	30	90	0	10	100		
MS15AS	8	62	20	40	20	100		
MS60AS	10	30	33	0	67	100		
Mon	20	0	0	0	0	0		
Mon30AT	10	50	20	0	60	80	20	6
				H_4				
W	30	20	0	0	0	0		
WC	23	14	0	0	0	0		
MS	40	23	25	0	12	38		
MSCW	28	15	0	0	0	0		
MS15AT	17	6	50	0	0	0		
MS30AT	9	0	0	0	0	0		
MS15AS	15	13	25	0	75	100		
MS30AS	15	27	38	0	38	75		
Mon	14	0	0	0	0	0		
				IR8				
W	10	10	100	0	0	100		
WC	10	40	25	25	0	50		
MS	30	32	12	5	18	22		
MSCW	10	50	0	12	0	40		
MS15AT	19	32	0	0	50	50	16.7	4
MS30AT	14	42	0	0	50	75		
MS60AT	8	25	0	0	0	0		
MS30AS	15	40	0	0	75	75	33.3	5

[a] MS = Modified Murashige and Skoog's medium, MSCW = MS + coconut water, MS15AT = MS + 1% autoclaved coconut water, MS15AS = MS + aseptically extracted coconut water, Mon = modified Monnier's.
[b] After a minimum culture of 1 week. [c] After a minimum culture of 2 weeks, based on number of germinated embryos.

formation occurred in unsupplemented media.

Four-day-old embryos were mostly undifferentiated, with root meristems initiated only in advanced cases,

Morphogenetic responses

Immature embryos five days old can be developed into seedlings whose morphological features are similar to those germinated in the intact seed under natural conditions. The potential to develop into a complete plant is determined by the histological state of differentiation of the embryo attained at the stage of first leaf formation of cultures in simple media. Inducing normal germination in 4-day-old embryos is difficult because they lack the requisite state of differentiation.

The formation of multiple shoots by 4-day-old embryos is morphogenetically significant. These cultures eventually appeared as clumps of green shoots with well-developed roots. This type of response was rare, but recurred in two different experiments with two different varieties. Because it was never observed in cultures of more mature embryos, the indication is that the very immature embryo has a greater tendency to regenerate shoots.

To test this possibility, immature embryos were cultured in a shoot regeneration medium with kinetin and NAA. Slight callusing appeared around the base of the coleoptile about a week after inoculation. Then shoot regeneration occurred. Cultures of 6-day-old embryos showed a high degree of multiple shoot formation (Table 10). Because the embryos were subcultured, no data on number of shoots per culture were taken. The average number of shoots per plant was 9.21 from 8-day-old embryos and 4.0 from 10-day-old embryos. Shoots produced very good roots.

Table 10. Multiple shoot formation in immature Fujisaka 5 rice embryos.

Embryo age (days)	Embryos (no.)	Shoot formation[a] (%)				shoots per culture[b] (av no.)
		Profuse	Moderate	Slight	1-3	
6	16	56.2	43.7	0	0	-
8	17	11.7	35.2	11.7	41.2	9
10	13	0	0	0	100	4

[a] After 26 days. [b] After 7 weeks.

Multiple shoot formation also was obtained from 6- and 7-day-old embryos in MS medium supplemented with benzyl amino

purine, with or without coconut water (Table 11). In this case, formation of shoots was not preceded by callus formation. This might be considered induced tillering. Viability with 6—day—oldembryos was less than with 7—day—oldembryos, but the number of shoots per plant was higher in 6—day—old—embryos. Five—day—oldembryos formed multiple buds in regen—eration media.

Table 11. Multiple shoot formation in immature H_4 rice embryos.

Embryo age and medium [a]	Embryos (no.)	Germinated embryos (no.)	Viability [b] (%)	Shoots per culture [b] (av no.)
6 days				
5 ppm BA	13	9	69.2	6
5 ppm BA+CW	12	10	83.3	5
10 ppm BA+CW	12	5	41.6	5
20 ppm BA+CW	12	7	58.3	10
7 days				
5 ppm BA+CW	12	12	100	2
10 ppm BA+CW	6	6	100	4
20 ppm BA+CW	12	11	91.6	3

[a] BA = benzyl amino purine, CW = coconut water. [b] After 7 weeks.

The immature embryos had good callusing ability. In media with 1 ppm kinetin and 5 ppm NAA, both callus and' shoots are formed.

Culture of interspecific and intraspecific hybrid seeds

Seed set in a cross between IR36 and different accessions of O. minuta, but the developing seeds started to degenerate. Seeds which were practically empty except for a tiny embryo mass were collected and grown aseptically in 1/4 MS media. Many of the seeds germinated and gave complete seedlings transplantable to soil.

An F_1 Mgl_2/IR8 cross gave mature, normally filled grains but formed only the coleoptile when germinated using usual methods. The seeds germinated aseptically in MS media could produce a callus type of growth in MS medium without growth hormones (Table 12). A germinating seed first formed a coleoptile, then callus at the basal portion, and finally roots. This seedling was grown to maturity and flowered when subjected to a short photoperiod. Mature seeds harvested from the plant gave good germination (20 out of 25 seeds) on 1/4 MS medium and second—generation seedlings produced good shoot and root systems.

Table 12. Germination in vitro of mature intraspecific hybrid seeds.

Medium	Embryos (no.)	Germination (no.)	Germination (%)
	F_1 $Mg1_2$ / IR8 [a]		
1/4 MS	10	3	30
1/4 MS	10	10	100
	F_2 IR8/$Mg1_2$ [b]		
1/4 MS	20	3	15
5 NAA [c]	20	5	25
10 NAA	20	5	25
20 NAA	20	6	30
40 NAA	20	4	20
5 GA	20	1	5.0
10 GA	20	2	10.5
20 GA	20	3	15
40 GA	20	1	5.0

[a] Seed glumes cut at pollination. [b] Seeds dehulled before sterilization. [c] Growth substances added with 2% dextrose 1/4 MS.

Mature F_2 seeds from an IR8/$Mg1_2$ cross also filled normally but showed no germination under ordinary conditions. Germination in vitro varied from 5% to 30% in different media (Table 12).

Further studies are needed to determine whether the irregular seed germination is due to morphological differentiation of the embryo or to the composition of the endosperm.

An embryo culture method using colchicine treatment

Fertility can be induced in hybrid plants from intergeneric and interspecific crosses with colchicine treatment (Schooler 1960, Peto and Young 1942, Liang et al 1979). This makes the development of an efficient method of colchicine treatment very important. Because of less complex histological differentiation and ability to produce profuse multiple shoots in regeneration media, using excised immature embryos and chromosome doubling techniques may be efficient.

Although cultures of excised immature embryos treated with colchicine had heavy microbial contamination, some embryos did survive colchicine pretreatment for 2 to 3 days. One plantlet generated from a colchicinetreated immature embryos showed a white stripe along one side of the leaf margin. Seeds germinated in colchicine solution showed a markedly short stubby coleoptile and inhibited root growth,

but those that continued to survive in culture media showed good recovery.

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TISSUE CULTURE IN SEA WATER INCREASES SALT TOLERANCE OF RICE PLANTS

Yasuyuki Yamada, Masahiro Ogawa,
and Shinichi Yano

The use of tissue culture techniques for crop improvement has become increasingly popular (Chaleff 1980). Recent notable successes include maize resistant to pathotoxin (Gengenbach et al 1977), tobacco plants tolerant of salt (Nabors et al 1980), and tobacco plants resistant to amino acid analogs (Widholm 1976). The basic methodology that produced these successes has been to select cells with natural resistance to disease toxins, salts, and amino acid analogs, then to regenerate resistant plants.

Theoretically, because cultured plant cells are totipotent, there should be no problem in regenerating plants. In fact, regenerating plants from cells, especially from cereal plant cells, has proven difficult. So far, no one has discovered why this is so. But regeneration is a necessary step when tissue culture methods are used for crop improvement.

Cultures of wheat cells induced from immature embryos showed high regeneration frequencies (Shimada and Yamada 1979). Similar results have been reported for Sorghum (Dunstan et al 1978). Therefore, in our experiments with rice we used immature embryos for the induction of callus. Oryza sativa cv. IR36 was chosen as the experimental material because indica rice is eaten throughout Southeast Asia. However, indica strains are more difficult to grow in cell culture systems than are less popular japonica varieties (Chen and Lin 1976).

One improvement needed in rice crops is plants that can grow in high concentrations of $MgC1_2$, $CaSO_4$, and KC1, as well as in NaC1. To select cells tolerant of salt, we added seawater to the culture medium. Salt—tolerant cells selected from rice cells cultured in seawater have much stronger and more specific characteristics than cells grown only in a NaCl solution.

Rice plants regenerated from selected cells growing in the seawater medium were grown in a culture medium that also contained seawater. They produced seeds, control plants did

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not.

EXPERIMENTS AND RESULTS

The plant material used was Oryza sativa L. cv IR36. The husk of each spikelet was marked with its date of flowering. Spikelets were harvested at different stages of seed development. Harvested spikelets were sterilized for 10 minutes in 70% alcohol, immersed for 50 minutes in 3% sodium hypochlorite solution, then transferred to sterile petri dishes. After several rinses with sterilized deionized water, the husks were removed and the seeds hulled. Hulled seeds were sterilized by submerging for 2 minutes in 3% sodium hypochlorite solution, then rinsing several times with sterilized deionized water.

The pericarps were excised and immature embryos separated from the endosperms. Five-embryo lots were transferred to agar slants containing 8 ml of Linsmaier-Skoog (LS) medium supplemented with 3% sucrose, 0.1% casein hydrolysate, 0.1% casein hydrolysate, 0.1% yeast extract, 1% agar, and 10^{-5}M, 2,4-D. Incubation was under continuous fluorescent light at 28°C for 4 weeks.

Callus formed after 1 week of incubation (Table 1).

Table 1. Callus induction from immature embryos of rice grain harvested at different developmental stages and cultured on LS medium containing 10 µM, 2,4-D.

Days after flowering	Embryo size (mm)	Embryos inoculated (No.)	Callus induced (No.)	Callus induced (%)
5-6	1	20	20	100
6-9	1	55	54	98
9-11	1-1.2	50	50	100
11-14	1.2-1.3	65	57	88

The frequency of callus formed from immature embryos was nearly 100% at each stage of seed development except for samples taken 11 to 14 days after flowering. Callus could be induced best from immature embryos taken 6 to 11 days after flowering.

When callus is first induced, it consists almost entirely of heterogeneous cells (Fox 1963). We found that the heterogeneity of cells is greater in early subcultures than in subsequent subcultures (Yamada et a1 1978, Watanabe et a1 1981). Therefore, selection of cells tolerant of seawater should be made when callus is first induced.

The number of subcultures on a medium containing seawater needed to obtain salt-tolerant cells depended on the concentration of seawater and the culture method used. Based.

on the survival rate of cells, with our method four sub—cultures were more than enough to produce salt—tolerant cells.

Callus was induced from immature rice embryos on media containing different concentrations of seawater. Small groups of cell aggregates were separated from the parent callus and subcultured on media that also contained dif—

Table 2. Composition of seawater in media used to select salt—tolerant rice cells.

	NaCl	$MgSO_4$	$MgBr_2$	$MgC1_2$	$CaSO_4$	KCl
Composition of seawater (% w/w)	2.669	0.210	0.008	0.328	0.138	0.072
Medium salinity 0%	0	0	0	0	0	0
17.5%	0.467	0.037	0.0014	0.057	0.024	0.013
27.5%	0.734	0.058	0.0022	0.090	0.038	0.020
37.5%	1.001	0.079	0.0030	0.123	0.052	0.027
47.5%	1.268	0.100	0.0038	0.156	0.066	0.034
57.5%	1.535	0.121	0.0046	0.189	0.079	0.041
67.5%	1.802	0.142	0.0054	0.221	0.093	0.049

ferent concentrations of seawater (Table 2).

Seawater concentrations higher than 47.5% inhibited callus induction (Table 3). At 67.5% concentration, almost all embryos died. Many of the calli induced on media con—taining 57.5% and higher concentrations of seawater died when subcultured on media containing the same concentration of seawater as their induction medium. This suggests that these rice cells accumulated salts during subculture and that the concentration of salts became greater than the tolerance level of the cells.

After four subcultures with seawater, live rice cells were transferred to a regeneration medium without auxin but containing 10^{-7} M kinetin. When seawater was added to this medium, no plantlets were regenerated.

But, when selected salt—tolerant cells were transferred to a medium for regeneration under light, several green spots appeared on cells subcultured on media with salt concentra—tions of 17.5%, 27.5%, 37.5% and 47.5% (Table 4). At 57.5% and 67.5% seawater, no green spots appeared.

Six plantlets were obtained from salt—tolerant cells cultured in 37.5% seawater. Two plantlets that had 3 leaves were cultured in Kimura B solution containing 37.5% seawater. Although control plantlets died immediately after transfer to this culture solution, the 2 salt—tolerant plantlets grew for 10 days before dying.

Because the 35.5% concentration of seawater was too high for these plantlets, 2 more salt—tolerant plantlets

Table 3. Callus induction from immature rice embryos at different developmental stages on LS medium containing seawater[a].

Days after flowering	Callus (No.) at different salt concentrations											
	17.5%		27.5%		37.5%		47.5%		57.5%		67.5%	
	Induced	Healthy	Induced	Healthy	Induced	Healthy	Induced	Healthy	Induced	Healthy	Induced	Healthy
6—9	5	5	5	5	5	5	5	4	4	3	4	1
9—11	5	5	5	5	5	5	5	2	5	3	5	1

a) Five embryos from each developmental stage were placed on LS medium containing 10μM, 2,4-D and salt.

Table 4. Regeneration of rice plantlets from cells cultured on media containing salt[a].

Salt concentration (%) in the selection medium	Callus induction with seawater[b]	Growth of cultured cells in 4 subcultures with seawater	Regenerated plantlets (No.)[c]
0	+	+	3
17.5	+	+	0
27.5	+	+	1
37.5	+	+	6
47.5	+	+	0
57.5	+	±	-
67.5	±	±	-

a) + = positive, - = negative, ± = sometimes positive, sometimes negative, b) Calli induced and subcultured on media containing 10 μM 2,4-D and different concentrations of salt. c) Plantlets regenerated on a medium containing 0.1 μM kinetin and no seawater after subculture 4 on the selection medium. A dash indicates no test done.

were cultured in Kimura B solution with 17.5% seawater. Two other plantlets believed to be salt tolerant were cultured without seawater.

When 17.5% seawater was added to the culture solution, the rice plants regenerated from cells selected from sub—cultures containing 37.5% seawater (callus induction plus four subcultures) grew well and headed. Female panicles developed and the spikelets of these plants ripened. The control plantlets (one grown from normal rice seed, the other a plant regenerated from cells subcultured without seawater) did not flower.

DISCUSSION

Chromosome numbers of the regenerated rice plants were count—ed by the squash method. All were diploid (2n = 24). Regen—erated rice piants tolerant of salt have not shown chro—mosomal aberrations in any of our studies.

Control plants did not survive for even 1 day in 37.5% seawater, but the plants regenerated from cells selected from cultures containing 37.5% seawater survived for 10 days. Plants regenerated from cells selected from cultures con—taining 37.5% seawater grew well in a solution of 17.5% seawater. These plants ripened and produced good grain, unlike normal rice plants which did not flower.

Harvested grain was analyzed to determine the mineral content of the plants. The Na—Kratio was large for plants grown in 17.5% seawater—about5 for the leaves and about

Table 5. Mineral content of rice plants regenerated from salt—tolerant cells.

Cells selected 5 times	Culture of regenerated plants	Plant part	Na[a] (mg/dry	K[a] wt)	Na/K	Mg[b] (mg/dry	Ca[b] wt)
With 37.5% seawater		leaves	43.13	7.40	5.83	7.06	2.62
		root	21.58	15.98	1.35	15.86	3.11
Without seawater (control)	Kimura B solution containing 17.5% seawater	leaves	57.06	8.88	6.43	9.67	4.00
		root	24.53	16.40	1.50	14.12	3.79
Normal grain (control'		leaves	46.12	9.73	4.74	6.74	3.37
		root	24.65	14.90	1.65	12.78	2.54
With 37.5% seawater		leaves	10.56	24.68	0.43	1.73	3.20
		root	11.46	16.86	0.68	16.95	4.74
Normal grain (control)	Kimura B solution	leaves	5.76	20.23	0.28	2.40	2.94
		root	7.44	19.79	0.38	15.15	3.59

[a] Na, K: EKO flame photometer, FLD type.
[b] Mg, Ca: Hitachi atomic absorption photometer.

1.5 for the roots (Table 5). Plants grown in Kimura B culture solution usually have Na—Kratio of about 0.5 (rice selectively absorbs more potassium than sodium). This means that salt—tolerant rice plants absorb more Na than K from seawater and that much of this Na is transported to the leaves. This characteristic was found in both normal and. salt—tolerant plants.

Two explanations for the salt tolerance mechanism found in plants are possible. A salt—tolerant plant may have a defense system that protects it from absorbing excessive salt or it may absorb excess salt but detoxify itself physiologically or by extruding the extra salt.

The salt—tolerant rice plants showed distribution patterns for minerals similar to those of plants that have absorbed excess minerals (Table 5). Sometimes many salt crystals were found on the leaf sheath. These may have been extruded from the vascular bundle. This may be evidence that rice plants regenerated from cells tolerant of seawater possess a saltextrusion system.

Seed obtained from salt—tolerant rice plants germinated and this generation of plants is now growing in 17.5% seawater. Further studies on the characteristics and salt tolerance of these rice plants are in progress.

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INDUCTION AND SELECTION OF SALT-TOLERANT MUTANT RICES BY TISSUE CULTURE — RECENT PROGRESS AT IRRI

Shouichi Yoshida, Masahiro Ogawa, Kazuhiro Suenaga, and He Chun Ye

High soil salinity is one of the most important soil constraints to rice production in developing countries. In South and Southeast Asia, an estimated 54 million hectares are saline soils; about 50% of them coastal saline soils (Akbar and Ponnamperuma 1982). Increased salt tolerance of rice could increase and stabilize rice yields in the salt-affected areas now being cultivated, such as coastal regions where inundation of seawater frequently occurs and canal-irrigated areas. It also would permit the expansion of rice cultivation into marginal cropping land.

Screening existing material for salt tolerance has met some success (Ikehashi and Ponnamperuma 1978). However, the need is to increase the salt tolerance of rice beyond the maximum levels now possible. Salt tolerance appears to be much lower in rice than in other grain crops, such as in barley and wheat (Maas and Hoffman 1977).

Tissue culture, recognized as a novel means to generate genetic variability (Larkin and Scowcroft 1981), is possibly a new tool for improving salt tolerance in crop species.

Several scientists have attempted to increase the salt tolerance of some plant species, both at the cellular and the whole plant level, by tissue culture techniques (Table 1). At the moment, more knowledge is available at the cellular level than at the whole-plant level. Selection of salt-tolerant mutant plants is limited to only two species, Nicotiana tabacum and Oryza sativa. Of these, the work of Nabors et al on Nicotiana tabacum is most convincing. They produced salt-tolerant mutant cell lines by exposing cells to a salt-enriched liquid medium, then regenerated salt-tolerant mutant plants. They also demonstrated that salt tolerance is inheritable, at least to the F_2 generation. In rice, Oono and Sakaguchi (1978, 1980) have shown that some mutant plants regenerated from calli grown on a salt-enriched medium are tolerant of high sa-

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Table 1. Summary of attempts to increase the salt tolerance of plants through tissue and cell culture.

Species	Type of culture	Mutagen treatment	NaCl concentration (g/liter)	Salt tolerance of regenerated plants	Inheritance	References
Capsicum annuum	Callus and suspension	–	7, 10, 30	No regeneration	–	Dix and Street (1975)
Citrus sinensis	Callus	x—ray	5	Not confirmed	–	Kochba et al (1980)
Medicago sativa	Callus	–	10	No regeneration	–	Croughan et al (1978)
Medicago sativa	Callus	–	10	Not confirmed	–	Stavarek et al (1980)
Nicotiana sylvestris	Callus and suspension	–	0—20	No regeneration	–	Zenk (1974)
Nicotiana sylvestris	Callus and suspension	–	7, 10, 30	No regeneration	–	Dix (1975)
Nicotiana sylvestris	Callus and suspension	–	7, 10, 30	Not confirmed	–	Dix (1980)
Nicotiana tabacum	Suspension	EMS [a)]	1.6	No regeneration	–	Nabors et al (1975)
Nicotiana tabacum	Suspension	EMS [a)]	1.6	Confirmed	Heritable	Nabors et al (1980)
Oryza sativa	Callus	–	10	Confirmed	Heritable	Oono and Sakaguchi (1978, 1980)
Oryza sativa	Callus	–	10	Confirmed	–	Kucherenko (1980)

[a)] EMS = ethyl methane sulfonate.

linity and that the salt tolerance is probably inheritable.

IRRI's tissue culture work for induction and selection of salt—tolerant mutant rices was initiated in December 1980. We feel that tissue culture will be a useful breeding tool if it can be used to generate a wider genetic variability in the salt tolerance of rice and if selection can be made at the cellular level.

SEED-DERIVED CALLUS CULTURE SYSTEM

Either anther culture or somatic cell culture can be used for induction and selection of mutant plants. But in the anther culture of rice, the capacity to regenerate plants from callus diminishes so rapidly that it is totally lost by the second passage in subculture (Oono 1975). This rapid loss of morphogenetic capacity severely restricts the use of anther culture for the induction and selection of mutant plants. This limitation is particularly a problem if mutation is induced by a repeated cycle of subculture under a given stress and if selection is made at the cellular level. For this reason, we have chosen to use somatic—cell culture in our efforts to increase rice's salt tolerance. Seeds provide an excellent material to start with because a rigorous surface sterilization can be applied to seeds which results in a minimal contamination of the resultant callus culture.

Our seed—derived callus culture system has six steps (Fig. 1):

1) Callus is induced from surface—sterilized brown rice on a modified Murashige—Skoog (MS) (1962) medium(3 weeks).
2) The callus is subcultured using the same modified MS medium for callus proliferation (4 weeks).
3) The proliferated callus is divided into small pieces (about 50 mg each) and pieces are transferred separately to the modified MS media with or without NaC1. Salt—tolerant callus lines may be developed when NaC1—enriched medium is used during the subculturing. This step is repeated 3 to 6 times (4 weeks each passage).
4) Callus is finally transferred to a regeneration medium for plant regeneration (2 to 3 months). Regenerated plants are transferred from test tubes to plastic pots containing soil and fertilizer. Roots of the regenerated plants are dipped in 0.1% nicotineamide aqueous solution for 1 hour before transplanting to stimulate rooting. Pots are placed in a plastic chamber where almost 100% relative humidity is maintained. The chamber is lighted by fluorescent lamps. A week later, pots are transferred to a glasshouse maintained at 29/21°C (day/night).

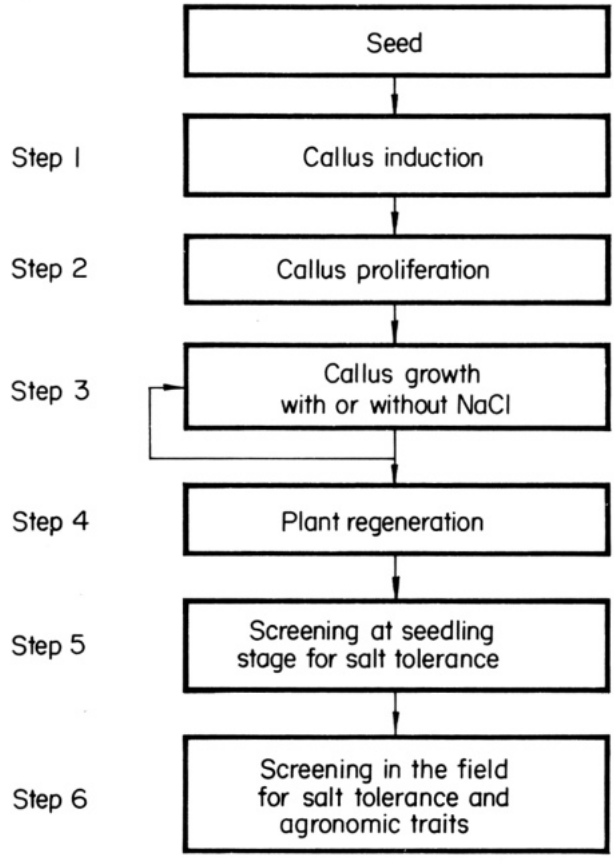

Fig. 1. Seed—derivedcallus culture system.

5) Seeds from regenerated plants are grown to the 3—leaf stage and screened for salt tolerance using a solution culture technique in an artificially—lighted growth cabinet. With this technique, 540 seedlings per cabinet can be screened every 3 to 5 weeks, depending on the degree of salt tolerance. Screening for a higher degree of salt tolerance takes longer. Salt—tolerant mutant plants are transferred to a normal culture solution, then to potted soil.
6) Seeds from the mutant plants are grown in the field and are screened for salt tolerance and agronomic traits.

The three kinds of culture media used in the seed—derived callus culture system (Table 2) are modifications of MS medium. Medium I and Medium II contain 2 mg 2,4-D/liter to induce callus. Although many varieties can develop callus without yeast extract, some varieties (particularly

Table 2. Media used for callus induction, for screening NaCl—tolerant calli, and for plant regeneration.

Medium I (Callus induction)		Medium II (Selection)		Medium III (Regeneration)	
Ms medium[a]		MS medium		MS medium	
2,4-D[b]	2 mg/liter	2,4-D	2 mg/liter	NAA[c]	2×10^{-6}-M
				Kinetin	5×10^{-5} M
Sucrose	30 g/liter	Sucrose	30 g/liter	Sucrose	70 g/liter
		NaCl	5—30 g/liter		
With or without yeast extract	3 g/liter	With or without yeast extract	3 g/liter	Yeast extract	3 g/liter
				Casein hydrolysate	3 g/liter
Agar	8 g/liter	Agar	8 g/liter	Agar	8 g/liter
pH	5.8	pH	5.8	pH	5.8

[a] Murashige—Skoog medium. [b] 2,4-D Dichlorophenoxy acetic acid.

[c] a - Napthalene acetic acid.

Table 3. Varietal differences in the formation of callus from mature rice seeds.

Variety	Seeds inoculated (No.)	Callus formation (%)
Fujisaka 5	168	71
Koshihikari	132	67
Norin 20	159	76
Reiho	119	85
Taichung 65	158	73
Giza 170	164	5
Giza 173	235	29
Mahsuri (Acc. No. 44452)	225	12
Mahsuri (Acc. No. 10929)	239	11
CICA 4	198	44
OS4	240	43
ADT 2 8	140	41
Kalarata	137	64
Nona Bokra	159	64
SR 26B	96	54
Pokkali	214	48
IR8	147	54
IR26	152	43
IR28	98	68
IR30	132	32
IR36	42	57
IR42	131	53
IR50	138	40
IR52	177	31
IR54	152	46
IR4432—20—2	173	20
IR4432—38—6—5—2	121	12
IR4595—4—1	82	62
IR4630—22—2	69	30
IR4763—73—1	86	58
IR9852—19—2	142	23
IR9884—54—3	101	42
IR9884—54—3—1	115	46
IR9975—5—1	132	13

IR lines) require yeast extract to develop a healthy callus. Medium II may or may not contain NaC1. Medium III for plant regeneration contains a high concentration of sucrose, yeast extract, and casein hydrolysate (Oono 1975).

FACTORS AFFECTING CALLUS INDUCTION AND GROWTH IN SUBCULTURE

Variety Marked varietal differences in capacity to induce callus were observed when yeast extract was not added. Callus induction efficiency ranged from 5% for Giza 170 to 85% for Reiho, averaging about 45% (Table 3).

Incubation time Most varieties developed a healthy callus in 3 weeks of incubation. Extended incubation generally resulted in increased efficiency of callus induction (Table 4). The effect was particularly remarkable with Giza 170,

Table 4. Effect of incubation time on the efficiency of callus formation.

Variety	Callus formation (%)	
	3 weeks	7 weeks
IR4432—20—2	36	42
IR4432—38—6—5—2	41	—
IR4595—4—1	56	—
IR4595—4—1—13	38	46
IR4630—22—2	—	—
IR4630—22—2—5—1—3	34	55
IR4763—73—1	11	11
IR4763—73—1—11	32	59
IR9852—19—2	15	29
IR9884—54—3	41	72
IR9884—54—3—1	27	63
IR9975—5—1	11	17
Pokkali	42	49
IR50	22	30
IR52	26	39
IR54	31	40
Giza 173	16	97
Giza 170	8	48
CICA 4	33	56
OS4	57	77
Mahsuri (Acc. No. 44452)	6	15
Mahsuri (Acc. No. 10929)	2	13

Giza 173, IR9884—54—3, IR9884—54—3—1,and Mahsuri accessions.

Light Callus induction normally is done in the dark room.

However, light improved callus induction efficiency of some varieties, such as Mahsuri accessions, IR52, IR54, IR4763—73—1, and IR9852—19—2, to a considerable extent (Table 5).

Table 5. Effect of light on the efficiency of callus formation.

Variety	Callus formation (%)	
	dark	light
IR4432—20—2	20	28
IR4432—38—6—5—2	12	13
IR4595—4—1	62	42
IR4595—4—1—13	38	-
IR4630—22—2	30	36
IR4630—22—2—5—1—3	34	33
IR4763—73—1	58	80
IR4763—73—1—11	32	37
IR9852—19—2	23	46
IR9884—54—3	42	56
IR9884—54—3—1	46	56
IR9975—5—1	13	29
Pokkali	48	48
IR50	40	39
IR52	31	61
IR54	46	87
Giza 173	29	40
Giza 170	5	-
CICA 4	44	33
OS4	43	43
Mahsuri (Acc. No. 44452)	12	53
Mahsuri (Acc. No. 10929)	11	34

Yeast extract Adding yeast extract to the medium increased callus induction efficiency of 15 varieties tested to nearly 100% (Table 6). It also improved callus growth during subculture. Most IR lines and Pokkali produced compact yellow calli during the induction period. These calli became soft and slimy and eventually necrotic when yeast was not added. The necrosis increased with time in subculture. The addition of 3 g yeast extract/liter remarkably improved callus growth in the subculture. Use of casein hydrolysate in addition to yeast extract did not have any additional effect on callus growth during the subculture.

On the other hand, most japonicas and traditional indicas, such as Nona Bokra, Kalarata, and SR26B, maintained normal growth of callus in the subculture without yeast extract.

Mature vs immature seeds Immature seeds were tested to see if they could increase efficiency of callus induction and increase plant regeneration. Immature seed did not have a consistent effect on callus induction, either with

Table 6. Effect of yeast extract on the efficiency of callus formation.

Variety	Callus formation (%)	
	MS medium	MS medium + yeast extract
Fujisaka 5	70	98
Koshihikari	60	98
Norin 20	75	100
Reiho	84	100
Taichung 65	72	100
IR8	53	100
IR26	42	100
IR28	68	100
IR30	31	100
IR36	57	85
IR42	52	80
ADT 28	40	100
Kalarata	64	90
Nona Bokra	64	100
SR26B	53	95

respect to days after anthesis or relative to mature seed (Table 7). When yeast extract was added to the medium, mature seed gave higher callus induction efficiencies in almost all varieties tested.

Sodium chloride Sodium chloride was added to the medium at step 3 and examined for a range of from 5 g/liter to 30 g/liter. Callus growth was retarded at 10—15 g/liter and greatly impaired at 20 g/liter. At 30 g/liter, no callus growth was observed. This observation led to the selection of 15 g/liter as the concentration for selecting salt—tolerant mutant callus lines. Callus transferred to the sodium chloride selection medium usually turns dark brown, an indication of necrosis. However, some portions of the callus manage to grow. When step 3 is repeated more than 3 times, vigorously growing callus emerges from the necrotic callus. These salt tolerant callus lines continue improved growth in subsequent subculture.

Table 7. Developmental differences of callus formation in rice grains.

Variety	Callus formation (%)							
	10 days after anthesis		20 days after anthesis		30 days after anthesis		Stored mature seed	
	MS	MS + Y.C.[a)]	MS	MS + Y.C.[a)]	MS	MS + Y.C.[a)]	MS	MS + Y.C.[a)]
Fijisaka 5	46	72	8	53	66	65	70	93
Koshihikari	7	16	5	18	20	32	60	100
Norin 20	67	79	7	53	66	73	75	100
Reiho	81	87	36	52	94	89	84	100
Taichung 65	64	84	39	63	84	91	72	100
IR8	66	68	23	33	36	64	53	100
IR26	75	79	60	66	56	68	42	73
IR28	76	61	68	58	69	33	68	83
IR30	44	45	4	20	13	37	31	87
IR36	64	61	45	55	39	33	57	71
IR42	87	89	35	27	72	64	52	73
ADT 28	88	93	20	33	12	36	40	92
Kalarata	58	57	27	66	5	40	64	77
SR26B	20	18	11	16	5	7	53	95
Nona Bokra	85	71	70	91	63	80	64	96

[a)] Callus was induced on MS medium containing 0.2% yeast extract and 0.2% casein hydrolysate.

FACTORS AFFECTING PLANT REGENERATION

Variety The morphogenetic capacity of different varieties to regenerate plants was examined using 11—week—old (for mature seed) or 12—week—old (for immature seed) calli. Varieties differed greatly in their capacity to regenerate plants. When callus was induced from mature seed, plant regeneration efficiencies ranged from 0% for IR8, IR26, IR28, IR30, IR36, and Koshihikari to 62% for Taichung 65

Table 8. Plant regeneration from calli originating from seeds at different developmental stages.

Variety	Developmental stage of seeds (days after anthesis)	Calli inoculated[a] (No.)	Regeneration (%)
Fujisaka 5	10	39	61.5
	20	11	27.3
	30	22	59.1
	Mature[b]	82	17.0
Koshihikari	10	-	-
	20	-	-
	30	34	0.0
	Mature	61	0.0
Norin 20	10	40	37.5
	20	24	29.2
	30	34	70.6
	Mature	64	13.0
Reiho	10	39	56.4
	20	48	39.6
	30	20	80.0
	Mature	67	31.0
Taichung 65	10	87	52.9
	20	37	48.6
	30	34	61.8
	Mature	131	62.0
ADT 28	10	44	47.7
	20	34	38.2
	30	29	31.0
	Mature	38	16.0
Kalarata	10	17	51.2
	20	31	12.9
	30	16	18.8
	Mature	70	17.0

(Table 8. Continued)

Variety	Developmental stage of seeds (days after anthesis)	Calli inoculated[a)] (No.)	Regeneration (%)
SR 26B	10	35	14.3
	20	10	50.0
	30	34	5.9
	Mature	18	17.0
IR8	10	25	4.0
	20	39	7.7
	30	23	0.0
	Mature	12	0.0
IR26	10	26	11.5
	20	23	8.7
	30	47	0.0
	Mature	3	0.0
IR28	10	7	0.0
	20	12	16.7
	30	9	0.0
	Mature	4	0.0
IR30	10	15	6.7
	20	9	0.0
	30	7	0.0
	Mature	7	0.0
IR36	10	3	0.0
	20	15	6.7
	30	14	0.0
	Mature	6	0.0
IR42	10	8	0.0
	20	10	0.0
	30	35	0.0
	Mature	9	11.0

[a)] 11—week—old calli from mature seed and 12—week—old calli from immature seeds. [b)] Mature seed was obtained from stored seeds, no information on date of harvest.

(Table 8). Each regeneration produced an average of 8 green plants. Varieties also differed markedly in frequency of albino plant formation.

Age of callus Efficiency of plant regeneration declines with callus age. With 11-week-old callus and when sodium chloride was not added to the medium for callus growth

(step 3), efficiencies ranged from 13% for Norin 20 to 62% for Taichung 65. It declined to 6% for Norin 20 and to 33% for Taichung 65 27 weeks after callus induction culture was initiated (Table 9).

Table 9. Effect of NaCl on efficiency of plant regeneration from 11- to 27—week—cultured calli.

Variety	% plant regeneration				
	11 wk	15 wk	19 wk	23 wk	27 wk
Fujisaka 5					
0% NaCl	17.0	12.9	11.4	7.8	12.9
1.5% NaCl	43.8	37.5	20.0	42.9	11.5
Norin 20					
0% NaCl	12.5	6.0	17.6	7.5	6.0
1.5% NaCl	58.8	21.4	62.5	14.3	3.3
Reiho					
0% NaCl	31.3	21.7	25.4	14.2	14.6
1.5% NaCl	55.6	40.0	61.5	54.5	27.3
Taichung 65					
0% NaCl	61.8	50.8	38.8	25.5	32.9
1.5% NaCl	76.9	76.9	87.0	60.0	27.3
ADT 28					
0% NaCl	15.8	48.6	15.0	40.0	2.2
1.5% NaCl	66.7	0.0	-	-	0.0
Kalarata					
0% NaCl	18.3	13.8	29.6	16.7	15.3
1.5% NaCl	33.3	50.0	0.0	-	-
Nona Bokra					
0% NaCl	53.5	58.8	64.7	40.0	39.7
1.5% NaCl	61.5	45.2	9.1	0.0	0.0
SR 26B					
0% NaCl	16.7	15.4	16.7	0.0	0.0
1.5% NaCl	40.0	-	33.3	-	0.0

Sodium chloride When sodium chloride was added to the medium (step 3), efficiency of plant regeneration was much higher (Table 9). However, this higher regeneration efficiency was counterbalanced by the earlier decreased growth of callus. As a consequence, overall efficiency of plant regeneration per unit of callus initially used for subculture was lower when sodium chloride was added to the medium

Mature vs immature seeds Use of immature seeds improved efficiencies of plant regeneration of different varieties to different degrees (Table 8), but the improvement was somewhat counterbalanced by increased contamination during callus induction. Another problem with immature seed is that the developmental stage that gives the highest plant regeneration efficiency varies with variety. Low efficiency Of plant regeneration, particularly that of IR lines, is a major constraint to using tissue culture for the selection of desirable mutant plants.

SCREENING SEEDLINGS FOR SALT TOLERANCE

Screening mutant plants for salt tolerance appears to limit the entire system of the seed—derived callus culture for selecting salt—tolerant mutant plants.

The screening technique we are using currently is a modification of the method used by Flowers and Yeo (1981). Seedlings are grown in a specified concentration of NaCl under a controlled environment. The number of days after salinization when 50% of the seedlings have died is taken as a quantitative measure of salt tolerance. To examine 5 varieties for salt tolerance, Flowers and Yeo used 14—day—old seedlings and 50 mol m^{-3} NaCl in the nutrient solution. In our preliminary work, we found that 3—leaf stage seedlings (8—days—old)and 0.75% NaCl in the standard nutrient solution give satisfactory results for screening purposes (Yoshida et al 1972).

Our technique uses a deep enamel tray (33 x 26 x 11 cm) with a capacity of 7 liters to hold the standard culture solution plus 0.75% NaC1. Electric conductivity of the solution is about 16 m mhos/cm. A cover lid styrofoam sheet that has 60 holes at a spacing of 2 cm accommodates 60 seedlings. To assess the salt tolerance of a variety, 30 8—day—old(3—leaf stage) seedlings are used. A Koito KG growth cabinet that accommodates 9 trays is maintained at 29/21°C (day/night), about 70% relative humidity, 12 hours photoperiod, and 20 klx light intensity.

After salinization, the tips of the lower leaves begin to dry up. The drying of leaves moves upward and the entire plant turns brown. A dead plant is defined as one whose leafblades and leaf sheaths have lost green color. The number of dead plants is recorded daily.

Varieties differ markedly in salt tolerance (Fig. 2). For convenience, the lenght of time at which 50% of the seedlings are dead is termed D_{50}. The D_{50} is a quantitative measure of the salt tolerance of a given variety. The D_{50} is estimated at 10 days for IR26 and Taichung 65 and 22 days for Nona Bokra.

Regenerated plants (R1 plants) are first grown to maturity. The seeds of these plants are then used to grow

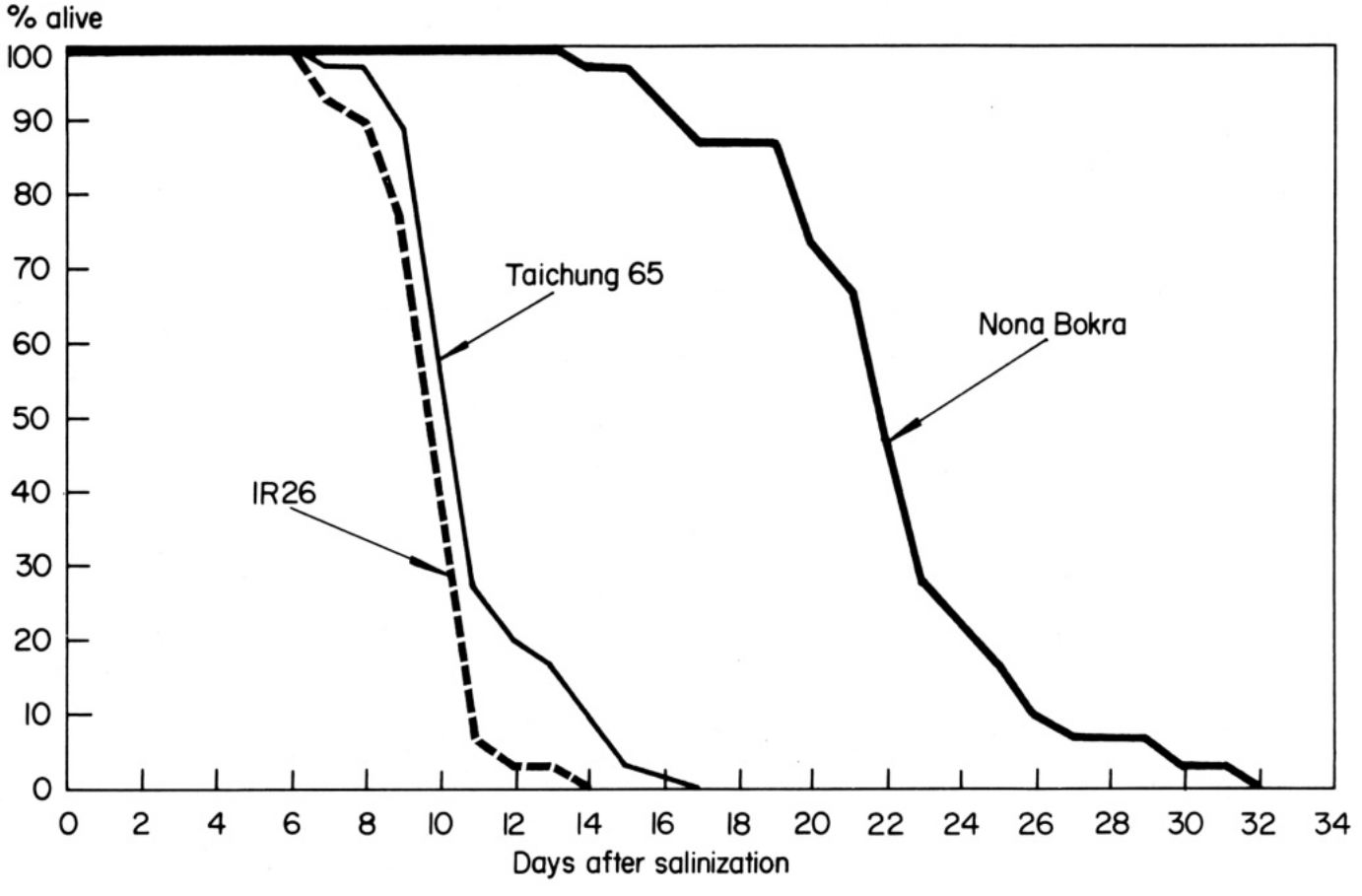

Fig. 2. Survival of 3 rice varieties following salinization with 0.75% sodium chloride.

seedlings (R2 plants) for screening. Salt—tolerant mutants are selected as the mutant plants that have survived after the death of the last plant of the mother variety. A massive screening is now underway on plants regenerated from calli grown with and without sodium chloride.

PERSPECTIVE

Because massive screening of regenerated plants for salt tolerance is still underway, it is too early to draw any conclusion out of our work. However, we point out several issues that require further examination.

Efficiency of plant regeneration

Efficiency of plant regeneration is low for IR lines, which have dwarf genes. Unless this is overcome, it is difficult to improve salt tolerance of improved high yielding rice varieties - IR lines and similar materials - by tissue culture techniques. Some traditional indica rices give high plant regeneration efficiencies, indicating that the problem is not a matter of indica or japonica varieties. It perhaps relates to a balance between different plant hormones.

Selection at the cellular level

Nabors et al's work on Nicotiana tabacum is an example of salt—tolerant plants produced from salt—tolerant callus. Selection was done at the cellular level. It is not clear from Oono and Sakaguchi's work on Oryza sativa if selec—tion was done at the cellular level. Because the mechanisms involved in salt tolerance of plants are complex (Mass and Nieman 1978) and because mutation may occur in a positive or a negative direction and both in shoot and in root tissues, salt—tolerant mutant callus may not necessarily produce salt—tolerant mutant plants.

If selection for salt tolerance is done only at the whole—plant level, the advantages of tissue culture tech—niques over radiation breeding or chemical mutagens have to be evaluated carefully. Thus, it is highly important that the relationship between salt—tolerant mutant calli and salt—tolerant mutant plants be established in rice.

Generation of wider genetic variability

As has been mentioned, the level of salt tolerance appears to be much lower in rice than in other grain crops. One of the expected advantages of tissue culture techniques is to generate wider genetic variability. But our preliminary

work suggests that a tissue culture cycle alone has a limited effect in generating a wider genetic variability in the salt tolerance of rice. Hence, some other means, such as combined use of chemical mutagens, should be examined to induce a wider, more useful genetic variability in rice.

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4-OXALYSINE-RESISTANT MUTANT FROM TOBACCO CALLUS

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Xu Zhu—yun and Li Wen—an

Cultured plant cells are useful tools for screening biochemical mutants. Since 1970, a few cell lines resistant to certain growth—inhibiting amino acid analogs — 5MT (5—methyltryptophan), p—fluorophenylalanine, amino—ethylcysteine, and Eth (ethionine) or valine — have been induced in cultured somatic cells of carrot, tobacco, and rice (Palmer and Widholm 1975, Chaleff and Carlson 1975, Widholm 1976, Bourgin 1978, Flick et al 1980).

Experiments on mutant plants regenerated from mutant cells and on certain biochemical traits transmitted to secondary generation calli have been done based on our previous work (Ho Chue—pui et al 1980.).

MATERIAL AND METHODS

A modified MS medium supplemented with B5 organic constituent and 2,4—D (1 mg/liter) was used throughout the experiments. Callus was derived from the leaf explant of Nicotiana tabacum L., Cultivar Gexin No. 5.

Subculturing through three passages generally stabilized the growth rate. Cell clumps (Ca. 5 mg) from 16—day—old cultures were used as inocula in an inhibiting medium (L—4—oxalysine, OL, added before autoclaving). After incubating at 25°C in the dark, resistant cell lines were selected.

Surviving calli were picked out of the OL (1 g/liter) medium and transferred to the same medium for about 3 weeks. After three passages in the inhibitory medium, cultures were subcultured in a medium without OL for six passages.

For mutagen treatment, small cell clumps of 13—day—old cultures were submerged in 2% EMS (ethylmethane sulphonate) for 2 hours, then rinsed several times to remove EMS. They were cultured on modified MS medium. Three weeks later, about half of the calli growing from surviving cells were transferred to an inhibiting medium

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containing 4 g OL/liter and cultured for 23 days. Surviving calli were divided into two portions. One was inoculated on a selection medium containing lg OL/liter and subcultured continuously (line W_{+1000}). The second was inoculated on a medium without the selection agent OL and subcultured (line W_0).

Three cultures – wild type, W_0, and W_{+100} - were grown on different OL concentrations to find their tolerance for or resistance to the growthinhibiting OL. The wild type. W_0, and W_+ (the first passage of W_{+1000} which was just returned back to the medium without OL) grown in media without OL were sampled and analyzed for fresh weight, lysine content, and peroxidase isoenzyme spectrum.

Random samples of 0.2-0.3 g fresh callus were homogenized by adding 4 ml, pH 9.4, 0.05 M pyrophosphate-HC1 buffer. The homogenate was centrifuged at 1600 × g for 10 minutes. An aliquot of the supernatant was used to determine lysine content (Felker et al 1978) and a second aliquot was used to estimate cytoplasmic protein content. Hot trichloroacetic acid was added to a final 5% concentration. The precipitated protein was dissolved with N NaOH and determined by Lowry's method.

For isoenzyme identification, 0.85% NaCl extract or 0.05 M pyrophosphate buffer extract was used for polyacrylamide gel electrophoretic analysis (Davis 1964). Staining with peroxidase isoenzyme was according to Liu's method (1973) and that with amylase isoenzyme according to Brewbaker's (1968).

EXPERIMENTAL RESULTS

Establishment of the mutant cell line

With this procedure, we succeeded in screening out a lysine analog—resistant line. When we started directly from EMS treatment without preliminary selection from a medium containing 1 g OL/liter for three passages, followed by six passages in a medium without the OL selective agent, no resistant line was produced.

Growth of the wild—type callus on the medium containing OL (0.1 g/liter) was completely inhibited. The variant W_0 was resistant. When the wild type, W_0, and W_{+1000} lines were compared by growing them simultaneously on different OL concentrations, the W_0 which had been subcultured through 6 passages of 4 months was less resistant than the W_{+1000}. However, even after 12 to 36 passages, the W_0 line remained resistant to OL. For 50% growth inhibition the resistant line W_0 required a concentration of OL 10 times that of the wild type (Figs. 1, 2).

Using either fresh weight or dry weight of each culture, the growth curves showed similar growth rates for

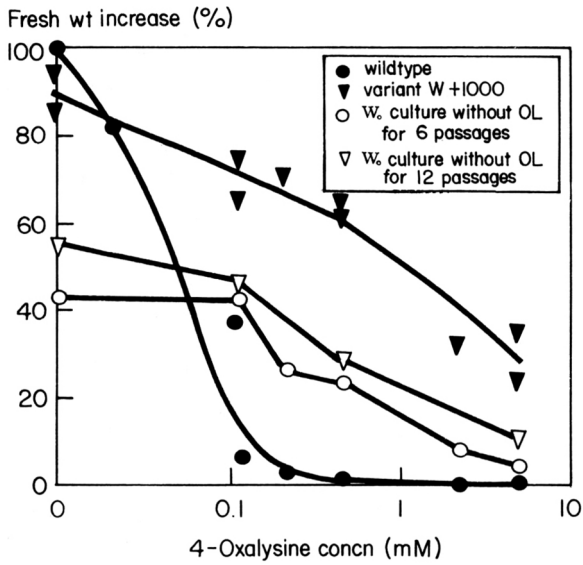

Fig. 1. Effect of 4—Oxalysineon growth of tobacco callus, ca. 30 mg tissue/inoculum. Each point represents the aver—age of 20—40cultures at 22 days in culture.

W_0 and W_+. Both were different from the wild type.

It has been reported that amino acid analog—resistant plant cell lines generally overproduce the corresponding amino acid (Palmer and Widholm 1975). Passages 11 and 12 of W_0 in all 5 samples accumulated twice as much lysine during cultivation than did the corresponding parent lines (Fig. 3). In addition, the cytoplasmic protein contents of W_0 were double those of the parent line.

In samples taken 7, 14, 21, and 33 days after inocula—tion from both the NaCl and the pyrophosphate buffer ex—tract, we found that the peroxidase isoenzyme spectrum on disc electrophoretic gel appeared in similar patterns. In the wild type, three slower bands migrated to the anode (denoted 1, 2, and 3). The slower band 1 was absent in passages 11 and 12 of W_0 and W_+. Both W_0 and W_+ were similar with two slower bands and a fast band (band 4) (Fig. 4). Band 4 was rather clear in W_0 cultured for 15 days. In the amylase isoenzyme test, we found two faster bands (1 and 2) in the wild type, but only one clear band in the W_0 and W_+.

Mutant plant regenerated from mutant cells

Passage 16 W_0 calli were transferred to the differentiation

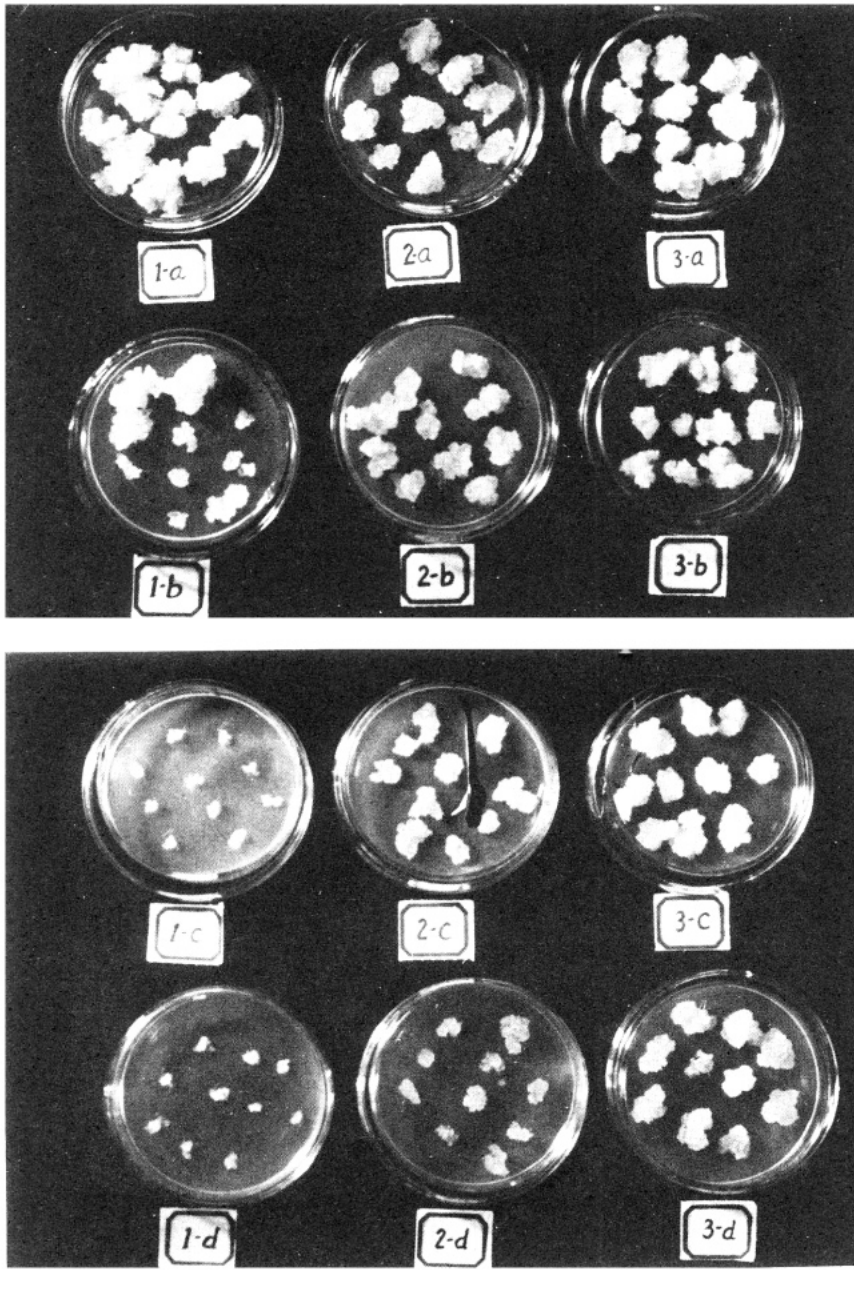

Fig. 2. Effect of OL concentration on growth of tobacco callus at 22 days of culture, ca. 0.7 g tissue inoculum/ petri dish. 1 = wild type 5T; 2 = W_0; 3 = W_{+100}. a = 0 mg OL/liter; b - 25 mg OL/liter (0.167 mM); c = 100 mg OL/ liter (0.666 mM);d = 1,000 mg OL/liter (6.66 mM).

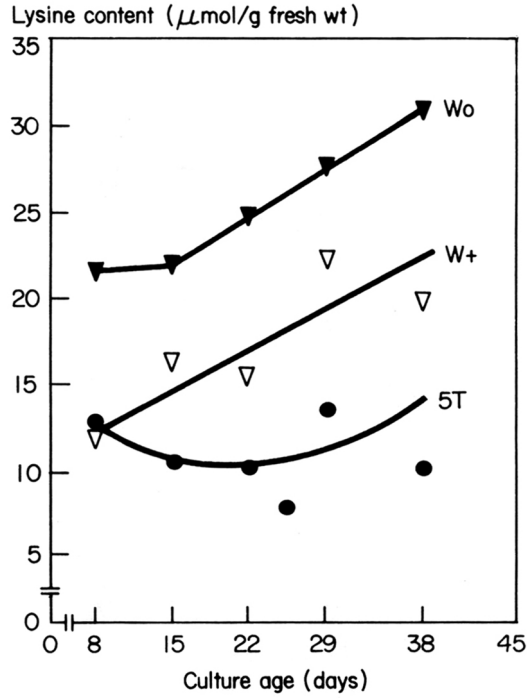

Fig. 3. Lysine content of wild type 5T, variant W_0, and variant W_+tobacco callus.

media. About 4,000 inocula and 82 treatments were tried. A few calli gave rise to green outgrowth (Ca. 3 mm long) which eventually produced many plantlets.

The detailed regeneration procedure included: W_0 calli transferred on medium B2 (MS + 6BA 2 mg/liter) for 75 days, subcultured another 53 days on the same medium, transferred to MS + coconut milk 10% (v/v) for 56 days, then inoculated on medium B2 for 31 days. A few calli with outgrowths were cultured on a solid MS medium for a month, on a liquid medium for a week, then on additional regeneration media under 12 hours daily illumination (Ca. 2 klux).

Two lines of regenerated rootless plantlets proliferated from two of the 8 media tested. Green outgrowth emerged from a light yellowish callus of a line cultured on s^3(MS + 0.1 mg IAA/liter + 2 mg NAA/liter) for 75 days. After transferral to MS medium, it produced buds and rooted into plantlets. This line is denoted 2—1—1/s^3.

It took 39 months and 51 passages for a leaf explant of an original wild type to develop into the regenerated

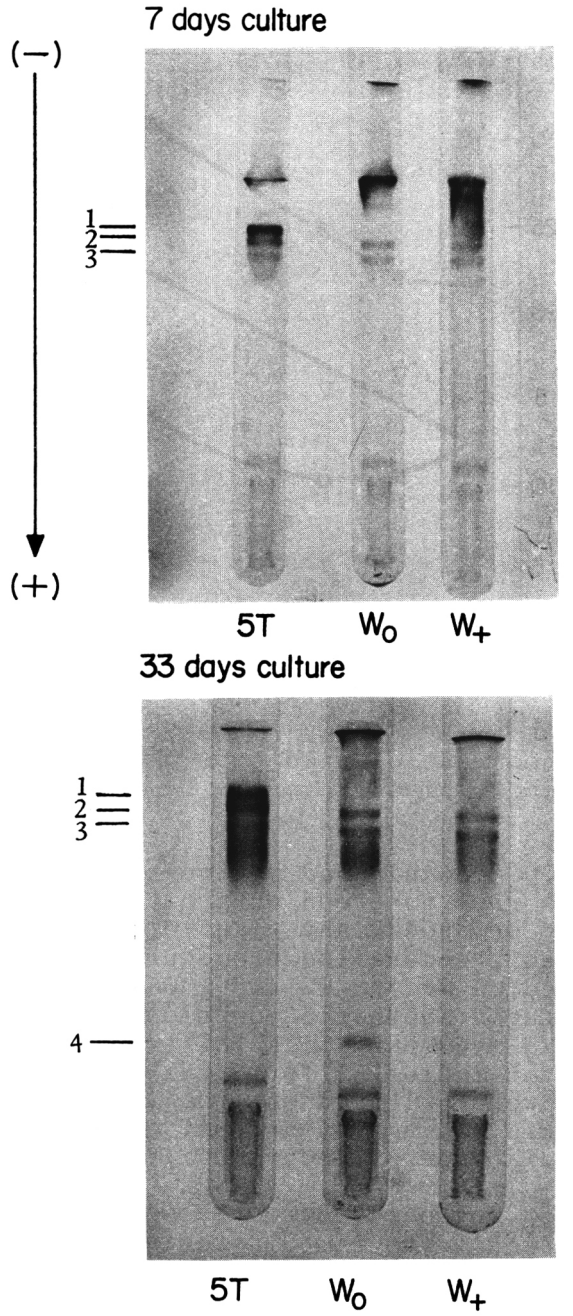

Fig. 4. Peroxidase isoenzyme spectrum of wild type 5T and variants W_0 and W_+ tobacco callus. Each gel—loaded protein = 20 µg.

plantlet. From W_0 culture to plantlet took 30 months and 35 passages. The 2—1—1/βs plantlets showed significant morphological variation which differed from the original wild type. The leaf blade became long and narrow, the corolla degenerated, a few plants had irregular flower structures with a particular flower formation ($\uparrow K_{(4)}C_{(2)}A_5G_1$), and bore capsules (Fig. 5).

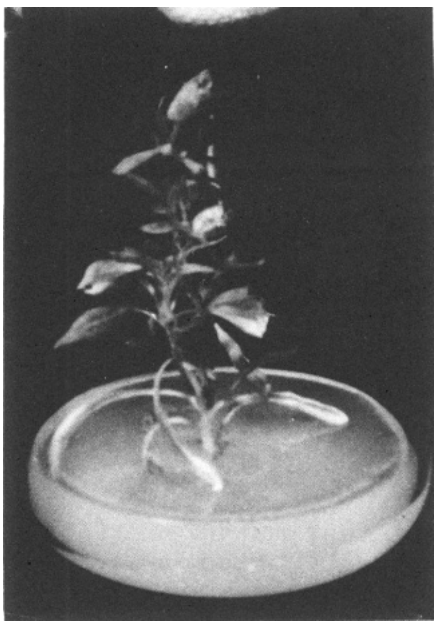

Fig. 5. Mutant plant with capsules regen— erated from OL—resistant tobacco callus W_0 on medium s^3.

Secondary generation callus derived from the mutant plant

Secondary callus line BCB was established from the leaves of the mutant plant of line 2—1—1/βs The OL resistance to growth inhibition (Fig. 6), the lysine content, and the peroxidase isoenzyme spectrum of line BCB were compared to those of line yw^+. We found that line BCB was similar to line yw^+ in all three tests. Line yw^+ was derived from the parent W_0 and was subcultured on a medium con— taining 1 g OL/liter for three passages.

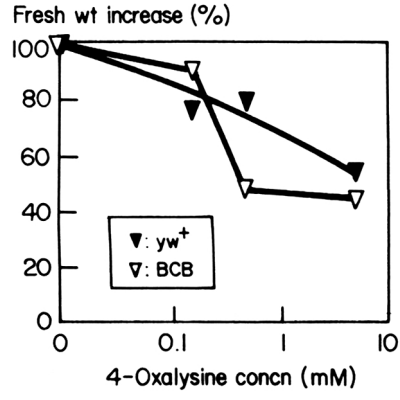

Fig. 6. Effect of OL on growth of callus of resistant tobacco lines, ca. 9 mg tissue/ inoculum. Each point represents the average of 30 cultures at 22 days in culture. Fresh weight increase of samples grown on no OL medium was calculated as 100%.

DISCUSSION

A lysine analog—resistant mutant cell line induced by 1-4-oxalysine has not been reported before. The W_0 obtained by mutagenic and selective agent described here shows once more that plant tissue culture is a useful technique for the isolation of biochemical mutants.

We wonder whether certain genetic or epigenetic variations were going on in W_0. Auxin and cytokinin habituation (Meins and Binns 1977) or whether the transient cycloheximide resistance (Gresshoff 1979) is an example of epigenetic variation. The W_0 phenotype appears to be different from these epigenetic variations. In our work, W_0 exhibited no cross resistance when cultured in the presence of inhibitory concentrations of ethionine or 5MT (Table 1). W_0 or BCB accumulated more lysine than did the wild type and its peroxidase isoenzyme spectrum appeared to be different from that of the wild type.

Line W_0 had undergone a fairly long process of treatment: successive subculturing on a medium containing 1 g OL/liter for 3 passages, on a medium without OL for 6 passages, EMS treatment, growth on a medium containing 4 g OL/liter, growth without OL for 36 passages, and yw^+ subculturing on a medium containing 1 g OL/liter for 3 passages. Throughout this long procedure, with three cycles

Table 1. Growth of wild type, variant W_0, and W_+ tobacco callus cultures with inhibitors[a].

Compounds added	Callus line		
	wild type	W_0W_+	
	Fresh weight mg/culture		
None	605	192	414
4—Oxalysine (6.66mM)	8	49	192
Ethionine (0.24 mM)	6	13	20
(0.49 mM)	7	12	12
5MT (0.092 mM)	11	15	15
(0.23 mM)	7	11	6

[a] Each value is an average of 30—40 individual cultures scored after 3 weeks culture.

of adding and withdrawing the selective pressure, it is probable that there is no chance of a surviving escapee, either from the wild type cell or from the epigenetic—alterated cells whose growth was favored in an OL medium mixed with resistant cells. Therefore, we think that the variant phenotype W_0 is a result of mutation transmissible through regeneration and expressed in the traits of OL resistance, lysine accumulation, and peroxidase isoenzyme spectrum at the secondary generation callus culture.

Further biochemical and genetic analyses of the mutant are under way. It seems that the OL—resistant cell line may be useful as a genetic marker in somatic hybridization.

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THE USE OF PHYTOTOXINS FOR IN VITRO SELECTION OF DISEASE-RESISTANT PLANTS

Elizabeth D. Earle

Improved disease—resistance is a major goal in the breeding programs of many plants, including cereals. Tissue culture techniques offer plant breeders a variety of new ways to identify, select, and transfer the genes involved in disease resistance (Brettell and Ingram ,1979, Earle and Gracen 1981). These include:

- use of cultured cells and protoplasts to study disease mechanisms.
- production of haploid tissues and/or plants in which recessive genes for disease resistance can be more readily detected.
- transferal of resistance via the wide crosses made possible by embryo culture, protoplast fusion, and other novel genetic manipulations.
- regeneration from culture of large populations of plants, some of which may show enhanced disease resistance.
- in vitro selection of material that can be regenerated into disease—resistant plants.

IN VITRO SELECTION OF DISEASE-RESISTANT MATERIAL

In vitro selection is an appealing strategy. It takes advantage of many of the most distinctive features of plant cell cultures -- growing large numbers of single (sometimes haploid) cells or cell clumps in a small space, exposing them to mutagens and selective agents, and regenerating plant populations from resistant material.

The production and complete analysis of disease—resistant plants by this method involves many steps:

1) preparing appropriate cultured material.
2) mutagenesis (optional).
3) exposing cultures to moderate or high concentrations

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of a toxin or other selective agent. If a sublethal concentration is used, the material growing most vigorous is transferred to increasingly higher concentrations.

4) subculturing surviving material on media with and without the selective agent.
5) retesting possibly resistant material after culture in the absence of the selective agent.
6) analyzing the biochemical basis of resistance expressed in the cultured material.
7) regenerating plants from resistant material.
8) assaying regenerated plants for resistance to toxin.
9) testing the resistance of plants to the pathogen.
10) analyzing the basis of resistance expressed at the plant level.
11) studying the inheritance of resistance in sexual crosses and/or the stability of resistance during asexual propagation.
12) studying growth, development, and agronomic traits of resistant plants.
13) incorporating resistant material into a breeding program.

All the steps in this ideal sequence are seldom completed.

This procedure selects for cells that survive exposure to a toxin or other selective agent. But effective disease resistance at the plant level often occurs via a hypersensitive response, such as rapid necrosis of localized areas which limits the spread of the pathogen and hence the disease damage. In vitro selection for resistance selects against cell types that are unusually sensitive. Therefore, it cannot be used to select for a type of resistance that may be of great practical importance. Replica plating or negative selection techniques might permit recovery of cells whose survival or division is inhibited by a toxin, but such approaches have not yet been used in this context. Moreover, there is no guarantee that plants regenerated from such cells would not show hypersensitivity rather than heightened susceptibility to disease.

Recovery of plants resistant to a particular disease requires heritable cellular variation, regeneration procedures, and a selective agent (step 3) active at the cell level which is also relevant to the resistance of whole plants to the pathogen. Variation and plant regeneration are not discussed here.

Substitution of different selective agents in step 3 makes the sequence applicable to selection for a wide range of useful agricultural traits, such as salt tolerance, overproduction of amino acids, resistance to heavy metals, and cold or heat tolerance. What sets disease resistance studies apart is the variety of agents that may be used for selection. Living pathogens, toxic but often poorly characterized

metabolites produced by pathogens, and chemicals whose structure is known are possible choices.

Types of selective agents

Pathogens. The use of pathogens as selective agents has the virtue of directness, eliminating concerns about the relevance of a toxin to a disease. Some promising studies with pathogen—tissue culture systems have been reported (Sacristan and Hoffman 1979, Helgeson et a1 1976). Unfortunately, the correlation between host—pathogen interactions in vitro and in vivo is often poor. Resistance evident at the plant level may not be expressed in culture (Brettell and Ingram 1979). Reasons for the incongruity often are not clear. Expression of resistance or susceptibility may require cell types, tissue arrangements, or physiological functions not present in the culture. Or, resistance may be suppressed because of the environmental or nutritional conditions used.

Differences between the responses of plant cells in vitro and in vivo can be a problem in all types of selections but the use of pathogens in such procedures faces additional serious obstacles. Uniform inoculation of cultured cells can be hard to achieve. Moreover, bacterial or fungal pathogens may overgrow plant cells on the culture medium, resulting in a confusing system in which results are difficult to interpret. The use of nonliving selective agents which can be readily sterilized and incorporated into a nutrient medium eliminates at least some of the difficulties of dealing with the pathogens themselves.

Chemicals of known composition. Carlson (1973) used a defined chemical to select for resistance to Pseudomonas tabaci, the bacterial pathogen that causes wildfire disease of Nicotiana tabacum L. P. tabaci produces a toxin (tabtoxin) that is responsible for some of the disease symptoms. Instead of using tabtoxin as a selective agent, Carlson used methionine sulfoximine (MSO), a methionine analog then thought to be an analog of the toxin as well. From calli derived from mutagenized haploid tobacco cells or leaf protoplasts, he recovered a number of variants resistant to MSO. Three cultures with stable resistance to MSO were diploidized, regenerated to plants, and tested for resistance to MSO and P. tabaci.

Preliminary genetic studies of F_2 progeny showed a complex inheritance of the ability of seedlings to grow on a medium with added MSO. Plants from two of the lines showed enhanced resistance to P. tabaci, although the pathogen still had more effect on them than on a naturally resistant variety. These mutants also had higher levels of free methionine in their leaves.

Interpretation of these results is complicated by ques—

tions about the structural and functional relationships of tabtoxin. MSO, and methionine (Strobel 1974). But the work certainly suggests that pathogens or their metabolites are not essential for successful selection. As understanding of the biochemical basis of susceptibility and resistance to disease increases, readily available chemicals may be used more widely to select for resistance to different aspects of pathogen action.

Phytotoxins. The problem of dealing with live pathogens and the scarcity of disease—related defined chemicals have directed attention to the use of phytotoxins, particularly host—specific ones, as selective agents (Earle 1978). A host—specific toxin is a pathogen—produced metabolite with the same specificity for plant material as the pathogen itself. Production of the toxin contributes significantly to pathogen virulence. Since such host—specific toxins probably are closely involved in disease development (Yoder 1980), resistance to the toxin is likely to provide at least partial resistance to the pathogen itself. Therefore, any host-specific toxins active at the cellular level are promising and convenient surrogates for pathogens in selection experiments.

In vitro selection using host—specific toxins

Selection for resistance to Helminthosporium maydis Race T.

Successful use of a host—specific toxin for in vitro selection is demonstrated by work with Zea mays L. and Helminthosporium maydis Race T (Gengenbach and Green 1975, Gengenbach et al 1977, 1981). This fungus damages maize with Texas (T) male—sterile cytoplasm much more severely than maize with normal male fertile (N) cytoplasm or with other male sterile cytoplasms. Race 0 of H. maydis, also a pathogen of maize, shows no specificity for T cytoplasm material. A toxin (HmT toxin) produced by H. maydis Race T appears to be responsible for the added virulence of that race on T cytoplasm. T mitochondria is probably a primary site of action for the toxin.

Gengenbach and Green used HmT toxin to select resistant material from T cytoplasm maize callus cultures. The toxin inhibited growth of T but not of N cytoplasm callus. Resistant material was obtained after the transfer of unmutagenized T cytoplasm callus from sublethal to increasingly higher levels of toxin. Callus selected for its ability to grow on concentrations lethal to control T callus retained resistance even after months of culture on a toxin—free medium. Mitochondria isolated from the selected resistant material were no longer sensitive to the toxin. Unfortunately, plants could not be recovered from the callus, so that steps 7—13 of the production—analysis sequence could not be done.

Gengenbach et al (1977) repeated the toxin selections

with callus derived from the scutellum of immature embryos. Plants could be regenerated from these cultures and a complete analysis was possible. Regenerated plants were resistant to HmT toxin in a leaf bioassay. Moreover, they were as resistant to H. maydis race T as plants with N cytoplasm. Thus, in vitro selection for resistance to HmT toxin yielded plants resistant to the pathogen itself. Sexual crosses showed that the resistance was maternally inherited. Mitochondria isolated from the selected resistant plants were resistant to HmT toxin.

The shift to toxin and pathogen resistance was accompanied by loss of T type male sterility. Regenerated plants were either male fertile or showed types of male sterility also seen in N cytoplasm plants derived from culture. The perturbations of tassel development appeared to be related to the culture procedure itself. In vitro selection failed to separate deleterious disease susceptibility from agronomically desirable T male sterility.

One interpretation of these results is that resistant callus and plants originated by selection of N mitochondrial genomes present in the original T cytoplasm material. Comparisons of mitochondrial DNA from N, T, and T resistant plants appear to rule out this possibility (Gengenbach et al 1981). Analysis of fragments obtained after treatment with restriction endonucleases reveals characteristic differences between the mtDNA of N and T cytoplasm maize. The mtDNA of the selected toxin—resistant plants was clearly different from that of N cytoplasm mtDNA and showed few or no differences from typical T cytoplasm mtDNA.

The origin of the selected resistant material and the molecular basis of the cytoplasmically—inherited shift to toxin resistance is still uncertain. However, it seems clear that the in vitro selection procedure did not select simply for already available N type of resistance but yielded something new.

These experiments fulfill most of the steps of the ideal selection procedure. Almost everything worked well. The toxin used was highly specific, giving a clear—cut distinction between resistant and sensitive material at both the cell and the plant level. Resistance to the toxin gave significant resistance to the pathogen. Selection was surprisingly easy, with no mutagenesis required. Resistant material was obtained from pieces of callus instead of from the cell suspensions more often used in selection experiments. Diploid material gave good results, perhaps because a cytoplasmic trait was involved.

The use of haploid cells might have permitted selection of nuclear traits influencing toxin sensitivity or male sterility. The observation of Brettell et al (1980) that spontaneous changes to toxin resistance frequently occur in culture may explain the ease of selection. Plant regenera—

tion not only succeeded but even gave fertile plants suitable for conventional genetic analysis.

The one failure, the inability to separate T type male sterility from disease susceptibility, limits the agricultural value of the work but does not represent a deficiency in the procedures used. Rather, it indicates that the two traits are very closely linked and perhaps even have an identical biological basis. This result does emphasize that selection for disease resistance is only a first step toward crop improvement via in vitro manipulations. Careful analysis of other agronomic features of selected material is always required.

Use of other host—specific toxins in selection. The maize—HmT toxin experiments provide an encouraging demonstration that in vitro selection for disease resistance is feasible. More than a dozen other host—specifictoxins have been identified (Yoder 1980). Resistance to these toxins and the pathogens that produce them is already known. It would be instructive to use these toxins in attempts to recover resistant plants and to see whether new modes of resistance can be obtained. It also would be interesting to determine whether in vitro selection for toxin resistance in susceptible but otherwise well—adapted genotypes could bypass some of the conventional breeding usually needed to transfer disease resistance.

Unfortunately, selection procedures based on the use of host—specific toxins have several major limitations. Most obviously, no such toxins are available for most diseases. Further work may identify some additional host—specific toxins, but there is no reason to expect that such toxins will be implicated in all or even most important plant diseases.

Even when host specific toxins are available, they may not be active against cultured cells and tissues. The toxin produced by Periconia circinata (Pringle and Scheffer 1963) has a clear—cut differential effect on sorghum, wilting leaves from susceptible plants but not affecting leaves from resistant plants. On the other hand, leaf mesophyll protoplasts from both types of plants show no obvious damage even after prolonged exposure to the toxin (Earle 1976, unpubl.). Toxin action or specificity may depend on cell types, organized structures, or physiological activities either not present in cultures or not essential for their survival. For example, toxins that affect chloroplasts, such as tentoxin from Alternaria tenuis (Steele et al 1976), are not likely to be good selective agents if the cultures are not green.

Possible failure of activity and specificity at the cell level is a general concern about in vitro selections that is relevant to all selective agents. The lack of host—specific toxins for most diseases requires that other types of selective agents be considered.

In vitro selection using non—specifictoxins

Nonspecific toxin refers to a pathogen—produced metabolite that damages material from the host and possibly from non—hosts as well. Relevance of the metabolite to disease devel—opment is less clear—cut than it is in the case of host—specific toxins. Many such toxic substances have been iso—lated (Strobe1 1974, 1977). Resistance to some of them might contribute to disease resistance, either qualitative—ly or quantitatively.

Selection for resistance to Phytophthora infestans. Work with Solanum tuberosum L. and Phytophthora infestans (the potato late blight pathogen) illustrates the use of a non—specific toxin as a selective agent. The liquid medium in which the fungus was cultured was filter—sterilized and tested for toxin activity against cells, callus, leaves, and shoots from a potato line sensitive to the pathogen (Behnke and Lönnendonker 1977). The degree of toxicity de—pended both on the fungal pathotype used and on the test system. Cells from dihaploid potato suspensions were killed by the addition of filtrate from pathotypes 1,2,3,4,5, and 7 at concentrations of about 2%. Ten percent of the filtrate from pathotype 2 was required for the same effect. Callus pieces (2—4 mm) and leaves and shoots were considerably less sensitive. They showed damage only at 40% concentra—tions of the more active filtrate. Callus from a variety of other higher plants also was sensitive. The toxin was not host—specific. Fractionation and purification of the toxin by gel filtration and thin layer chromatography revealed six different active substances, all of low molecular weight and aromatic structure.

Potato callus resistant to the culture filtrate was selected and regenerated into plants (Behnke 1979). Leaves and stems of plants regenerated from unselected callus were damaged by the toxin but those of plants from selected re—sistant callus werenot. Callus derived from toxin—resistant plants was resistant to the toxin. indicatingthatthe re—sistance was stable and was expressed both in vitro and at the plant level. No studies of inheritance of the toxin re—sistance were reported, perhaps because sexual crosses in potato are difficult.

The response of toxin—resistant material to the pathogen itself also was examined (Behnke 1980). Leaves of plants regenerated from control and toxin—resistant callus were inoculated with suspensions of P. infestans sporangia. Five days later, the average lesion size on leaves from toxin—resistant plants was significantly smaller (about 25% less) than it was on control leaves. On the other hand, sporula—tion was not significantly different on the two types of leaves.

The relevance of this type of altered resistance to long—term survival and yield of potato plants exposed to P.

infestans is not yet known. These results do suggest that in vitro selection for resistance to a nonspecific toxin may enhance some aspects of disease resistance. Identification and evaluation of such increased resistance will require careful and well-chosen quantitative assays of disease severity. Simply scoring plants as resistant or susceptible will not be adequate.

Use of other nonspecific toxins for selection. Publication of work using other nonspecific toxins as selective agents is limited and the work reported is much less advanced than the potato-Phytophthora studies. Most of it is still at the stage of demonstrating that a pathogen produces substances toxic to cultured material. For example, Bajaj et a1 (1980) reported that extracts from ears of Pennisetum americanum L. (pearl millet) infected with Claviceps fusiformis (ergot) strongly inhibited growth of pearl millet roots and callus.

STRATEGIES FOR IDENTIFYING NEW DISEASE-RELATED TOXINS

The isolation and testing of new toxins would make it possible to attempt in vitro screening for resistance to additional diseases. A collaborative project involving tissue culture and field programs at Cornell University is directed toward isolation of new toxins related to several fungal leaf blights of maize.

Toxins from Kabatiella zeae

One such effort focuses on Kabatiella zeae, which causes eyespot disease in maize. One reason for suspecting the involvement of a toxin is that lesion development is seen in advance of fungal growth. Also, toxins have been implicated in many leaf blights of cereals.

K. zeae obtained from infected leaves was grown first on potato dextrose agar, then in liquid Fries medium (Fox, Earle, and Gracen 1980, unpubl.).A filter—sterilized medium in which the fungus had been grown for several weeks provided a crude filtrate that possibly contained a phytotoxin. Because earlier work with HmT toxin showed that leaf protoplasts were a convenient and sensitive assay for toxin activity (Earle et al 1978, Gregory et a1 1980), we decided to test the filtrate against protoplasts from leaves of maize lines known from field rating studies to be either highly sensitive or relatively resistant to K. zeae.

Light microscopy showed that populations of protoplasts from susceptible plants usually were more severely damaged by culture filtrate (at 1/250 and 1/1000 dilutions) than were protoplasts from field—resistant plants (Table 1). The same dilutions of uninoculated Fries medium had little deleterious effect. Apparently something toxic and possibly

host specific was added to the medium during fungal growth.

If these observations are confirmed, we hope to increase any specific activity by fractionation and further assays. Then the concentrated toxin will be tested against protoplasts and plants from additional maize lines to measure the correlation between the field disease rating of plants and the toxin sensitivity of protoplasts and plants. Toxin from fungal isolates of differing virulence also will be assayed to see how toxin production relates to virulence Any promising toxins will be used to recover eyespot—resistant plants from regenerating callus cultures of W182BN, a highly susceptible inbred line of maize.

Cell and protoplast assays for toxin activity

Because selection of appropriate assays for new toxins is important, the choice of leaf protoplasts as the assay system for toxin activity in crude *K. zeae* culture filtrates requires some discussion. Protoplasts were used for several reasons:

- They had proved sensitive to very low concentrations of HmT toxin, which is also produced by a leaf blight fungus (Earle et al 1978, Gregory et al 1980).
- High yield and ease of preparation allow many assays with material from a single seedling.
- They can be obtained from any desired genotype of maize.

Because visible damage to protoplasts can be induced by many different agents, assays involving protoplast survival require no assumptions about mode of toxin action. Moreover, because any toxin that shows activity and host specificity at the protoplast level affects individual cells, it is likely to be useful for in vitro selection. The initial results of the *K. zeae* studies suggest that protoplasts provided a suitable assay for that system.

Protoplast and cell assays for new toxins also have some definite limitations. Negative results cannot be interpreted unambiguously. A culture filtrate may fail to affect any of the cells or protoplasts treated if the pathogen does not produce any toxins. On the other hand, culture conditions may simply have been suboptimal for toxin production, *so* that the toxin titer is extremely low. Or, a toxin which is present in a high concentration may be undetected because its action involves cell components, metabolic processes, or cell types not present in the treated population.

A toxin that induces vascular wilt by plugging the xylem night have no deleterious effects on isolated leaf mesophyll protoplasts. Leaf protoplasts unable to divide also will not reveal toxin—induced inhibition of division or proliferation. Cell cultures would be better assay material for such a toxin, but production of actively growing cultures

Table 1. Effects of *Kabatiella zeae* culture filtrate on leaf mesophyll protoplasts from maize inbreds resistant and susceptible to *K. zeae*.

Inbred	Field rating	Healthy protoplasts (no.)/3.2 µl after 3 days of culture[a]			
		Exp. 1		Exp. 2	
		0 filtrate	1/1000 filtrate	0 filtrate	1/1000 filtrate
Ay—191—71	Resistant	106	125	121	109
NY 821	Susceptible	103	17	92	4

[a] Spherical and turgid protoplasts with distinct disc—like chloroplasts were cultured in 0.5 M sorbitol + 10 mM $CaCl_2$ in darkness. Differential effects of filtrate on Ay—191—71 and NY 821 protoplasts were already apparent, although less clear—cut, on days 1 and 2. After 4 days, all NY 821 protoplasts exposed to filtrate were unhealthy and collapsed. Similar effects on protoplasts from susceptible inbred Co150 were seen in other experiments (Fox, Earle, and Gracen 1980, unpubl.).

from a variety of genotypes may be difficult and time—consuming. Futhermore, long—term cell cultures often lose or alter components such as chloroplast that may be important in leaf X toxin interactions. Sensitivity to a toxin also may be influenced by environmental or nutrient conditions or by the source or history of the cells used (Bronson and Scheffer 1977, Haberlach et al 1978).

A culture filtrate equally toxic to cells and to protoplasts from plants resistant and susceptible to a disease may simply contain a toxic fungal metabolite with little or no relevance to the disease. It also may contain a toxinthat is involved in disease development but that shows no specificity at the cell level. Host specificity may depend on the interaction of the toxin with multicellular tissues or on the initial behavior of the pathogen on the plant surface.

For these reasons, neither negative nor positive results with protoplast or cell assays should stand alone. Assays using other plant materials, such as seeds, leaf pieces, and plantlets, from resistant and sensitive lines may reveal toxic activity undetected at the protoplast level. Such assays also are needed to assess the role in disease of any toxins found active against protoplasts.

CONCLUSIONS

Generalizations about the role of phytotoxins in selection for disease resistance are of limited value because each host X pathogen interaction has distinctive features. However, several lines of research are likely to be useful:

- Known host—specific toxins could be used in further attempts to select for resistant material. Systematic attempts to compare effects on protoplasts, cultured cells, callus, organized tissues, and plants might give useful information on the mechanism of toxin action and on the frequency of shifts from sensitivity to resistance in different systems. It also would be interesting to see whether resistance obtained by in vitro selection has the same genetic and physiological basis as already known resistance. Such work could readily be done with cereal crops for which host—specific toxins are available (Earle 1978, Auriol et al 1978). Pathogens of cereals producing host—specific toxins include Helminthosporium victoriae (oats), H. carbonum Race 1, H. maydis Race T. Phyllosticta maydis (maize), Periconia circinata (sorghum), and Rhynchosporium secale (barley).
- Toxins affecting plants which generate well from culture could be tested in selective experiments. Many toxic substances produced by plant pathogens but not clearly host—specific have been isolated, assayed on

various plant parts, and sometimes partially purified (Strobel 1974). If sone plants regenerated from callus resistant to nonspecific toxins show enhanced disease resistance, attempts to utilize such toxins could be accelerated.

- A systematic search for additional toxins involved in economically important diseases could be made. The approach described in our studies with K. zeae may be generally applicable. Cultured cells or protoplasts may provide the most sensitive assays for toxins active at the cellular level, such as the ones that are most suitable as selective agents for in vitro studies. New toxins would be of great value in field screening for resistance and in studies of disease mechanisms, even if they prove to be un— suitable for in vitro selection.
- Studies of disease mechanisms could open new ap— proaches to in vitro selection. Identification of the chemical structure of a toxin may permit use of synthetic toxins or toxin analogs. Furthermore, clarification of the chemical difference between resistant and susceptible plants may suggest entirely new types of selection for resistance. These could be based not on differential survival or resistant and susceptible cells, but on separation and sorting of different cell types. For example, demonstrating that a particular membrane protein is related to resistance might allow labeling of resistant cells with flourescent antibodies and subsequent collection of these cells by fluorescence—activated cell sorting.
- Efforts to regenerate plants from protoplasts, cells, and callus of a wide range of cereals must be con— tinued. Without efficient techniques for regeneration, even the best selective systems cannot be fully ex— ploited.

Even with progress in these areas, tissue culture tech— niques will not displace conventional field approaches to breeding for disease resistance. However, they may con— tribute significantly to the agricultural improvement of some cereal crops.

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IMPROVED RICE PROTEINS IN PLANTS REGENERATED FROM S-AEC-RESISTANT CALLUS

G.W. Schaeffer and F.T. Sharpe, Jr.

It is clear that societies of the world must utilize all their resources in order to improve crop production and the protein quality of existing crops (Miflin 1975). Increasing populations and increasing demands for food require the exploitation of all available technologies for crop production, including in vitro techniques, tissue culture, and molecular genetics. Better understanding of genetic, biochemical, and developmental processes of plant growth will help to predictably modify plants for increased food and fiber production.

While the effects of full application of in vitro systems on improved food production cannot be predicted, it is clear that we need to understand the genetic, biochemical, and developmental aspects of plant growth in much greater detail. We also need to understand the phenotypes recovered from specific in vitro selections. The discipline of tissue culture already has made vast contributions to micropropagation and selection. But perhaps its greatest contribution will be in understanding basic regulatory phenomena of crop plant growth and the mechanisms by which plants change during evolution.

Biochemists and physiologists are becoming increasingly aware of the need for clear-cut mutations to prescribe the experimental limits of what plant scientists are doing. The challenge in the study of biochemical mutants is not only to recognize a mutation, but also to direct the selection process so that only a given and specific class of desirable phenotypes will be recovered.

One factor that has encouraged geneticists and plant breeders to try in vitro systems is the genetic and/or epigenetic variability of plants recovered from in vitro culture. This genetic variability, due in part to in vitro instability, may be a most useful characteristic. In vitro techniques may become one of the best methods for the recovery of truly unique genotypes from spontaneous mutation, from the rearrangement of genetic material within the chro-

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mosomes, and from an alteration of regulatory elements.

Heinz et al (1977) showed useful variations in Saccharum Oono (1978) described extensive morphological heterogeneity in Oryza progeny regenerated from callus. Variants from the tissue culture of Lolium interspecies hybrids have been reported and characterized (Ahloowalia 1978). There are many other examples of variability recovered with in vitro techniques (Schaeffer 1978, 1979; Schaeffer and Sharpe 1981; Thomas et al 1979; Weber and Lark 1979; Widholm 1978).

But in vitro systems must be defined and exploited. Regeneration of plants from the tissue culture of most major crop plants is still unpredictable and difficult. Progress in regeneration of crop plants also will be slow because the basic problem is not adequately researched. Limitations in the applications of recombinant DNA and in vitro discoveries will not reside with technical manipulations, but with the biology of plant regeneration, the integration of isolated genes into complex genomes, and the regulation of new gene expressionin a precise biochemical environment.

The release of useful rice and wheat cultivars reported in China several years ago prompted experiments to improve the nutritional quality of rice (Oryza sativa). Experiments were designed to recover rice resistant to S—aminoethylcystein (S—AEC), the analog of lysine, and to regenerate plants from resistant callus.

Halsall et al (1972) and Brock et al (1973) proposed a scheme for the overproduction of lysine. Cells insensitive to feedback inhibition by lysine and by lysine plus threonine were selected (Gengenbach et al 1978, Bryan et al 1970, Green and Philips 1974, Hibberb et al 1980). Bright et al (1979) used S—AEC as a selecting agent. Analogs of amino acids stereo—chemically close to the natural amino acid were found to function as supplemental or false feedback inhibitors (Carlson 1973, Chaleff and Carlson 1975).

The inhibitory effects of S—AEC can be overcome by lysine In this respect, S—AEC hypothetically qualifies as a true analog of lysine. In reality, S—AEC, besides being a competitive inhibitor of lysine in the cell, has other functions which may be more important in the recovery of specific phenotypes. S—AEC is incorporated into protein (Brock et al 1973, Green and Donovan 1980) and may act as an analog in components of protein synthesis.

The general pathway for lysine synthesis in microbial systems probably functions in rice as well. Because lysine inhibits the first enzyme in the pathway of lysine synthesis allosterically, it should be possible to select cells insensitive to lysine feedback inhibition or to recover cells resistant to S—AEC. The recovery of resistant cell lines has been accomplished with a number of cell types, including carrot (Matthews et al 1980, Widholm 1978). The enzyme activity levels of aspartokinase, dihydrodipicolinic acid synthetase, and homoserine dehydrogenase have been examined

(Matthews et al 1980). The levels of the two forms of aspartokinase in S-AEC resistant cells were different from the controls. Aspartokinase from resistant lines was lower. The lower rate also correlated with decreased sensitivity to feedback inhibition of aspartokinase by lysine in the presence of threonine. Halsall et al (1972) reported that aspartokinase from rice is sensitive to feedback inhibition by S—AEC.

Schaeffer and Sharpe (1981) reported the recovery of plants that had improved rice seed protein from S—AEC resistant rice callus.

EXPERIMENTAL SYSTEMS

The rice variety Assam 5 (Oryza sativa L. PI 353705) was grown in the greenhouse at 18 — 35°C. Supplemental flourescent light provided a 14—hour photoperiod. Anthers were excised and cultured as described by Schaeffer and Sharpe (1981) and Guha—Mukherjee (1973). The anther culture medium consisted of Blaydes (1966) inorganic salts and vitamins, 2 g yeast extract/liter, and 250 ml coconut milk (CCM)/liter with 2 mg 2,4-D/liter.

Developing calli were lifted from the anthers and placed on an increase medium (IC) consisting of Blaydes inorganic salts supplemented with 2 g yeast extract/liter, 150 ml CCM/liter, and 2 mg 2,4—D/liter. Media were routinely autoclaved 15 minutes at 15 psi for sterilization. Tissues were grown in 25 × 150 mm test tubes capped with cotton or plastic tops and were transferred routinely at 28 — 35days.

The recovery of cells resistant to S—AEC was accomplished by breaking the calli into small aggregates of approximately 2 mm^2 and spreading them evenly on a tissue increase medium with 10^{-3} M S—AEC added in 5—cm plastic petric dishes. Callus that grew in the presence of S—AEC was recultured by transferring it 3 times in a medium with 2 × 10^{-3} M S—AEC to assure resistance to the analog (Schaeffer and Sharpe 1981).

Plants were regenerated from callus by placing the tissues on a Murashige—Skoog (MS) inorganic salts and vitamin—supplemented medium containing 1 mg each of indole—3—acetic acid (IAA) and kinetin/liter. The MS medium also was supplemented routinely with 146 mg glutamine and 100 mg myo—inositol/liter.

Healthy regenerated plantlets, adequately rooted and 4 — 6cm tall, were taken from the test tubes and the roots washed to remove the agar. Seedlings were transplanted into

Jiffy Mix[1)].

The plants were conditioned for 5 days at 25°C under continuous cool white flourescent light at 2.0 µE, grown with a 14—hour photoperiod under 175 µE cool white flour—escent and incandescent light. After two weeks in the plant growth chamber, they were transplanted to soil for seed production in the greenhouse.

From 90 seeds recovered from 1 plant, 15 plants were grown and evaluated for seed number and weight, protein and amino acid determinations, height, time to flowering, and coloration. Some lines were selfed four times for advanced characterizations.

RESULTS AND DISCUSSION

Analysis of seed from the original plant regenerated from S—AEC resistant callus (M13) showed that seed protein and lysine content was a higher percentage of the total amino acids in protein hydrolysates in the mutant than in the control. Mutant seed size was smaller. One line of progeny flowered earlier and other lines flowered later than the control. Unexpected heterogeneity in the progeny included decreased fertility during the winter months; greater sen—sitivity to environmental stress, particularly cold; less fully extended panicles; enhancement of the bent node char—acter, and greater propensity toward floral structure dis—coloration.

Perhaps the most valuable and consistent changes re—covered from plants regenerated from S—AEC resistant callus were increased protein (Fig. 1) and lysine (Fig. 2). An increase in seed protein or lysine or both consistently showed in the third through the fifth generation progeny from the M13 progeny plants regenerated from S—AEC resistant callus (Table 1).

Although only one regenerated plant produced enough seed for progeny testing, there was interesting variability in the protein and lysine levels of progeny from that plant. M86 had significantly higher seed weight and protein in the seed than M99. But the histogram for seed number is more normal with M99 than with MS6. M99 flowered earlier than both M86 and the control (Fig. 3).

Lysine content, expressed as percentage of total amino acids in protein hydrolyzates, was 3—10% higher than the greenhouse—grown controls. The 10% more lysine content of

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[1)] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or IRRI and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

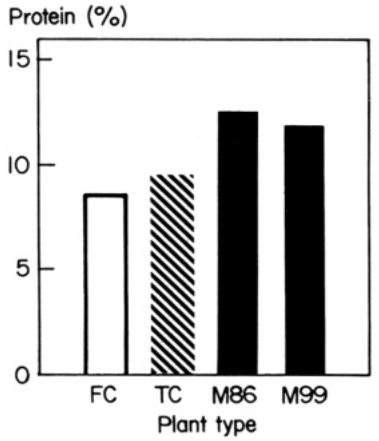

Fig. 1. Percent protein in greenhouse—grown rice grains representing field control (FC), internal tissue culture controls (TC), thirdgeneration progeny of plants regenerated from S—AECresistant callus (M86, M99). Histograms represent the average of a minimum of 15 determinations per treatment. Controls are significantly different from mutants at 0.05 probability level.

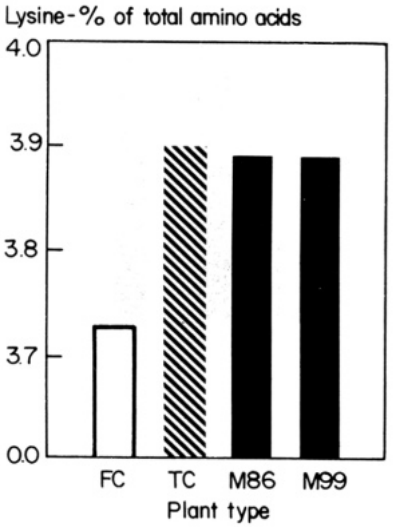

Fig. 2. Lysine level expressed as percentage of total amino acids in acid hydrolyzed protein. The numbers analyzed and probability levels are the same as in Figure 1. Field control (FC) is significantly lower than internal tissue culture controls (TC), and third—generation mutant progeny (M86, M99).

Table 1. Ratio of mutant/control (M86/FC) for three seed characteristics at different levels of selfing.

Seed generation	Plant (no.)	Ratio (M86/FC) Protein (mg/seed)	Lysine (% of total amino acid)	Seed weight (mg)
Original	1	1.03	1.10	0.84
Second	1	1.14	1.17	0.90
Third[a)]	5	1.09	1.05	1.02
Fourth[a)]	9	1.60	0.99	0.98
Fifth[a)]	20	1.54	1.05	1.03

[a)]Progeny means from single plants selected from previous generations for optimum protein, lysine, and seed number.

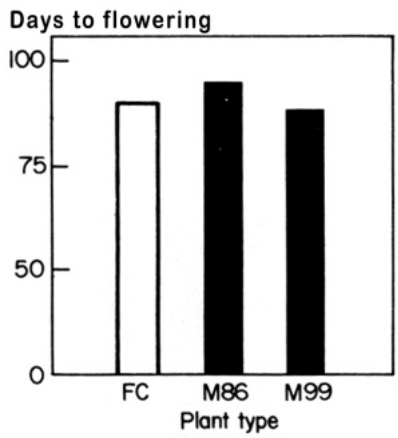

Fig.3. Rice plant growth rates and flowering dates of third generation mutant progeny (M86, M99) and field controls (FC) grown in the greenhouse.

seed from the initial plant may represent some carryover effects from the regenerated plant. Protein lysine increased 3-6% in advanced generations.

The combined nutritional benefit from the increased protein and lysine could make in vitro selection a valuable technique for the generation of new genotypes. The transmission under experimental conditions of useful characteristics through five generations shows that the elevated protein and lysine levels were transmitted through selfing. Protein levels

were improved in advanced progeny and lysine was decreased after the second selfing. The elevated lysine level over the first two generations could reflect some carryover effects from tissue culture. Seed weight also improved in progeny selected later. It is not possible at this time to define precisely the contribution of reduced seed number toincreased protein and lysine. The most recent experiments show mutant plants with seed yields equal to control but with 10% more seed protein content than control.

Table 2. Composition of brown and hand—milled rice of parental source PI 353705 and tissue—cultured M13 mutant [a)].

Property	Sample 1		Sample 2	
	PI 353705	M13	PI 353705	M13
Brown rice				
Weight (mg/grain)	20.5	12.5	17.6	16.5
Lysine (g/16.8 g N)	4.3	4.6	4.1	4.7
Calculated protein (% recovered N × 5.95)	8.1	10.9	9.7	12.7
Protein (mg/seed)	1.7	1.4	1.7	2.1
Lysine (mg/seed)	0.071	0.063	0.070	0.098
Milled rice				
Lysine (g/16.8 g N)	3.9	4.3	-	4.5
Calculated protein (% recovered N × 5.95)	6.8	10.6	-	11.1

[a)] Analytical work by B.O. Juliano, IRRI, Los Baños, Philippines.

The increase in lysine probably was not a generalized response, but may reflect an increase in a specific protein. Indirect evidence comes from protein and amino acid analyses of milled and brown rice (Table 2). Removal of the aleurone layer removed a greater percentage of the total protein from the control than from the tissue—culture mutant. Probably the M13 line had more protein in the endosperm. This may explain in part an observation that some good—yieldingplants also had excellent protein levels in the grain (data not shown), a quality that would be of nutritional significance in milled rice.

The negative correlation between protein level and lysine content normally observed in rice breeding (Beachell et al 1972) and other cereals (Singh and Axtell 1973) was

broken in the S—AEC resistant variants. The 10% increase in lysine did not produce a decrease in protein. The correlation coefficient between percent protein and lysine in percent of total amino acid in the controls was —0.8567, significant at the 0.0001 level of probability. But there was no significant correlation for four of the tissue—cultured cell types, including the internal controls. This implies that the negative correlations can be broken by anther/tissue culture techniques.

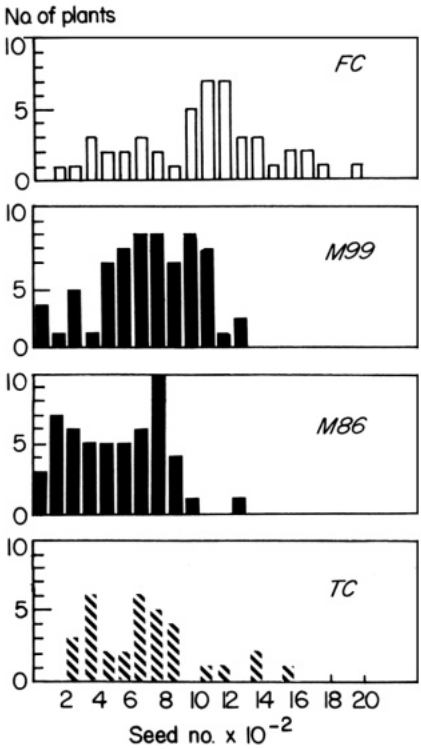

Fig. 4. Histogram of rice seed production by greenhouse—grown third generation mutant progeny (M86, M99) and field controls (FC) and tissue—cultured controls (TC). The mean seed number for FC is 906; for M99, 668; for M86, 568, and for TC, 777.

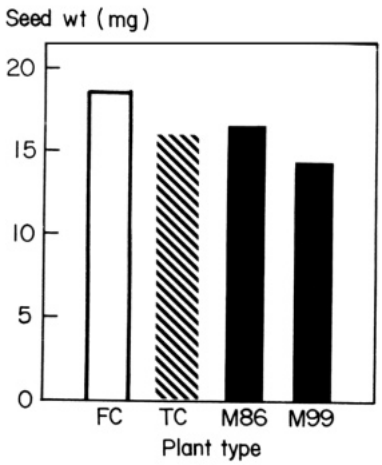

Fig. 5. Weight of rice grains (mg/seed) from greenhouse—grown mutant progeny (M86, M99) and field control (FC) and tissue—cultured control (TC).

One difficulty in interpreting the original data was loss of fertility during winter experiments. The loss was

greater for tissue culture—derived plants than for greenhouse and field controls (Fig. 4). Apparently, tissue culture variants are more temperature sensitive.

The percent of protein in the tissue culture controls (Fig. 1) did not increase significantly over the field controls, even though seed number was reduced in the tissue culture controls (Fig. 4). This implies that S—AEC selection is required for the increased seed protein observed in the resistant cell types. Seed from 136 plants with S—AEC heritage had a significantly greater mean for protein that either internal or field controls.

One valuable parameter of the nutritional quality of rice is seed lysine content. Figure 2 shows the high levels of lysine in two (M99 and M86) of the selected cell lines and an internal control (TC). The value represents contributions from increased seed protein as well as an increase of percent lysine in the protein. However, Figure 5 includes data from several experiments in which greenhouse temperatures were lower than ideal. The data also include some early generations in which residual effects from in vitro culture produced low seed numbers and increased lysine and protein. For example, Table 1 shows what we think are carryover effects during the first and second generations of tissue culture. The third, fourth, and fifth generations reflect progeny selected in part for high seed number.

Growth rates of the M13 series and controls were similar (Fig. 6), even though flowering dates were shifted in some of the M13 types. One type required 95 days to flower.

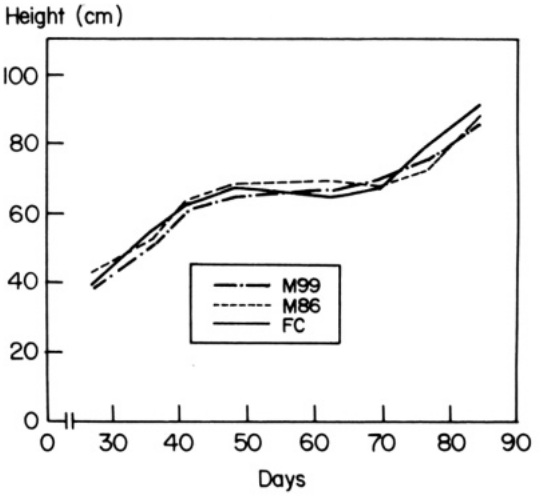

Fig. 6. Growth rate of field control (FC) and mutant progeny M86 and M99 grown in the greenhouse. Height represents the distance from the crown of the plant to the fully extended leaf tip.

Others flowered 5 days before the control. Considerable heterogeneity in size, color, and tillering in the tissue culture material was not evident in the controls. Under seven environmental stress, particularly cold, abnormal panicles were found. Some germination of embryos on the panicles was observed during and after cold stress. These irregularities were more intense with the M13 series than with the controls

Our conclusion is that in vitro techniques can be utilized for the recovery of useful genetic variability, including increased levels of seed protein and lysine. But researchers must guard against undesirable heterogeneity induced or enhanced by in vitro techniques.

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STIMULATING POLLEN HAPLOID CULTURE MUTATION IN ORYZA SATIVA subsp. KENG (JAPONICA)

Hu Zhong

Melchers and Bergmann pointed out the advantages of using haploid plant tissue culture for mutation experiments as early as 1958. Haploid mutants can express their phenotypes immediately and are convenient for screening. The doubling of the chromosomes in the regenerated plant leads directly to a homozygous plant.

The success of anther culture in tobacco plants in the 1960s gave great impetus to experiments on haploid culture mutation. During the 1970s, several auxotrophic and resistance mutants were selected from haploid somatic cell and protoplast cultures of tobacco (Carlson 1973, see review by Zenk 1974, Maliga 1978). A white—flowered mutant of tobacco was obtained by treating cultured anthers with a chemical mutagen (Kitsch 1972).

However, until now no mutant with economic value has been reported. For rice, there are a few reports on muta— tion in haploid somatic tissue culture as well as in anther culture. It is desirable to accumulate more experimental data to evaluate these techniques for rice improvement.

Our research group has worked on this problem since 1975. The approach is to lay down a suitable culture system for mutation experiments, to observe the effects of mu— tagens on a culture system and the variations in visible characters of regenerated plants, and to examine the genetic nature and application of the mutants obtained. The results of mutation experiments on- microspores in anther culture of O. sativa subsp. keng (japonica) are reported here.

IMPROVING THE ANTHER CULTURE PROCEDURE

Floating the anther

An improved culture procedure was worked out in 1977 (Hu et al 1978a). Anthers at the late uninucleate stage are floated on a liquid medium of N_6 basic components (Chu et al 1975) with 3% sucrose and 1—2 mg 2,4—D/liter and kept at 8°C for 4 days. The medium is replaced by the same

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Table 1. Influence of chemical mutagens at early stage of anther culture on the induction of pollen plantlets (Hu et al 1980).

Rice variety	Mutagen and dosage	Treatment conditions		Anthers cultured (No.)	Callus formation (%)	Callus differentiation (%)		Albinos (%)
		temp. (°C)	duration (hr.)			green	albino	
8126	EMS(ml/1) [a]							
	0	26	12	510	12.1	36.0	3.0	7.6
	2.0	26	12	510	9.8	26.1	32.7	57.5
	4.0	26	12	660	9.6	13.2	23.0	63.5
77-32	MNNG(mg/1) [a]							
	0	10	15	573	41.2	6.3	8.4	57.1
	25	10	15	711	23.9	2.4	8.2	77.8
	100	10	15	643	10.7	1.5	14.5	90.6
768	EI(ml/1) [a]							
	0	26	20	373	59.2	15.8	13.6	46.3
	0.5	26	20	375	72.5	12.1	15.4	56.0
	1.0	26	20	514	18.7	12.5	20.8	62.5

[a] EMS = ethylmethane sulphonate, MNNG = 1-methyl-3-nitro-1-nitrosuguanidine, EI = ethylene imide.

medium with tested substances added (Table 1). Anthers may still float on the surface or may sink in the liquid medium with 0.05% Tween 80.

Anthers are incubated for the desirable durations at specified temperatures. Then, they are washed, transferred to a solid medium with NAA 2 + kinetin (0.2—0.5 mg/liter), and incubated at 26° for callus induction. The calli dif— ferentiate into plantlets on a solid medium with NAA 0.5 + kinetin (2 mg/liter).

This procedure is convenient for testing the effects of chemical substances on the development of pollen at the early stage of culture and gives a higher induction fre— quency of pollen plantlets, especially improving the dif— ferentiation of callus in the case of injured anthers (Hu et a1 1978b).

Plating a pollen suspension

Many pollen in a single anther may develop into calli. But when an anther is cultured on a solid medium, calli growing out of the anther wall cannot be separated from each other. When this happens, a transferable callus may be of multi— pollen origin. When anthers are incubated floating on a liquid medium, calli are easy to separate. Several calli of single pollen origin may be retrieved.

To avoid shortcomings in both cases, a new procedure has been suggested (Hu and Liang 1979). Anthers floated on a liquid medium are incubated at 26°C for about nine days, when the pollen have developed into multicellular globules but before pollen walls rupture. Then pollen is separated from dehiscent anthers by stirring. The dehiscence can be regulated by changing the concentration of sucrose in the medium. The prepared pollen suspension is cultured on agar and multicellular pollen are incubated to give rise to calli. In this case, each callus will be of a single pollen origin.

The technique gives a culture pressure for the selec— tion of certain calli. An efficiency of 1.5 calli per anther can be attained. This figure is higher than for a conventional procedure, but still much lower than optimal. A single cultured anther may have 100 multicellular pollens. Moreover, most regenerated plantlets are albinos. Further improvement is needed.

IRRADIATING PANICLES WITH g—RAY

Devreux and Saccardo (1971) observed 50% aberrant phenotypes among pollen plants produced by anther culture of flower buds irradiated with X—ray at a dosage of 1 kR.

Panicles at the uninucleate stage were irradiated with cobalt source g - ray at 0.5—5.0 kR and anthers were dis—

sected from panicles for culture. The frequency of callus formation was different from that of the control. At a dosage of less than 1.0 kR, calli were stimulated but at more than 3.0 kR callus formation decreased rapidly. The differentiation frequency of callus and the formation of albino plantlets were not influenced significantly in the tested dosage ranges.

No increased formation of pollen plants with abnormal leaf color was observed. In about 120 strains of H_2 pollen plants from 6 varieties examined, drastic variations in visible characters were observed in 3 strains from 2 varieties. These variations were stable and heritable in H_3 plants. One strain with early maturity and short culm was selected for yield trials but failed because of lodg—ing.

These results show that this technique is not efficient for inducing variability.

TREATING ANTHERS WITH COLCHICINE

Pollen with large nuclei have been observed in anthers treated with colchicine (Hu and Liang 1979).

Colchicine was incorporated into a liquid medium on which anthers were incubated at 26°C for 2 days. Treatment with colchicine at 50—250 mg/liter increased the induction of green pollen plantlets. The proportion of diploids in the pollen plants was 75%, compared to 50% in the control. Colchicine at more than 500 mg/liter decreased the induc—tion of pollen plants and increased the formation of albinos. These results are similar to those obtained in tobacco (Nitsch 1977). Colchicine in the liquid medium does induce doubling of the chromosomes of pollen in floating anthers, despite the obstruction of the anther wall.

The technique is a possibility for obtaining more fertile pollen plants in anther culture.

TREATING ANTHERS WITH CHEMICAL MUTAGENS

Chemical mutagen stimulation of plant induction

Chemical mutagen stimulants ethylmethane sulphonate (EMS), ethylene imide (EI), and 1–methyl–3–nitro–1–nitrosuguanidine (MNNG) were incorporated into a liquid medium to treat anthers at the early stage of culture. Partial results are presented in Table 1 (Hu et al 1980). Chemical mutagens at lower dosages slightly stimulated callus induction. However, the induction frequency of callus decreased rapidly with increased dosages and the proportion of albino plantlets increased remarkably. Total differentiation frequency of callus was not changed.

This means that chemical mutagens are effective factors in inducing albinism in pollen plants. Albinism also can be induced in anther culture by a higher temperature at the early stage of culture, a higher concentration of 2,4—D in the medium (Wang et al 1978), and a higher temperature at the booting stage of anther donor plants (Hu et al 1978b).

However, even in the case of increased formation of albino plants, variation in characters of induced pollen plants was not observed. It is assumed that albinism in anther culture could be a mutation which is more easily induced than mutations of other characters. The fact that a chemical mutagen easily induces formation of albinos is a problem in mutation experiments with anther culture be—cause the number of green plants is reduced.

Stable variability induced by chemical mutagen

Table 2 gives a general survey of induced character varia—tions in 151 strains of H_2 pollen plants produced from cultured anthers of rice varieties treated with EMS and EI at the early stage of culture. These strains, all grown in the field during the normal growth season, were compared with H_2 strains of pollen plants from anthers receiving no mutagen treatment. Six strains showed drastic variation in visible characters, such as short culm, early heading, more tillers, and good fertility. These variations are uniform in plants within a strain and heritable (Zhuang et al unpubl) This was because mutation is induced in a hap—loid cell and plants are regenerated from the spontaneous doubling of the mutant cell. However, none of these mutant lines are of economic value because their comprehensive agronomic characters are not better than those of the original varieties.

Unstable variability induced by chemical mutagen

Segregation in visible characters occurred in two strains (Table 2). The pattern of segregation in the strain from variety 8126 is shown in Figure 1. Anthers incubated at 26°C for 2 days were treated with 0.25% EMS at 26°C for 8 hours. Of the regenerated plantlets, 34.9% were albinos, compared to 6.7% in the control. No plant with variegated leaves was observed. In one H_2 strain, 19% of the plants were yellow—green. This is a stable mutant.

The F_1 hybrid plants of a mutant × normal variety cross were all green. In the F_2 of the hybrid, green and yellow—green plants segregated at 3:1. This indicates that the mutant is due to a single nucleus recessive gene. All pollen plantlets produced by anther culture from the mutant were yellow—green or albinos. The plantlets produced by anther culture from the F_1 hybrids of a mutant × normal variety

Table 2. Variation in visual characters of rice pollen plants from anthers treated with chemical mutagens at early stage of anther culture.

Original variety	Mutagen	H_2 strains (No.)	H_2 strains showing drastic variation (No.)		Main characters	Segregation in H_3 strains
			stable	unstable		
04-G84	E1	20	1	0	earlier maturing (15 days) and short culm	no
04-110	EMS	30	3	0	1. shorter culm (27 cm)	no
					2. later maturing (10 days)	no
					3. more tillering	no
Southwest 175	EMS	30	1	0	earlier maturing (20 days)	no
768	EMS	31	1	0	higher fertility, less grain weight	no
8126	EMS	30	0	1	19% yellow-green plants	short culm, early maturing plant
Mountain	EMS	1	0	1	awnless	20% of plants with purple glume

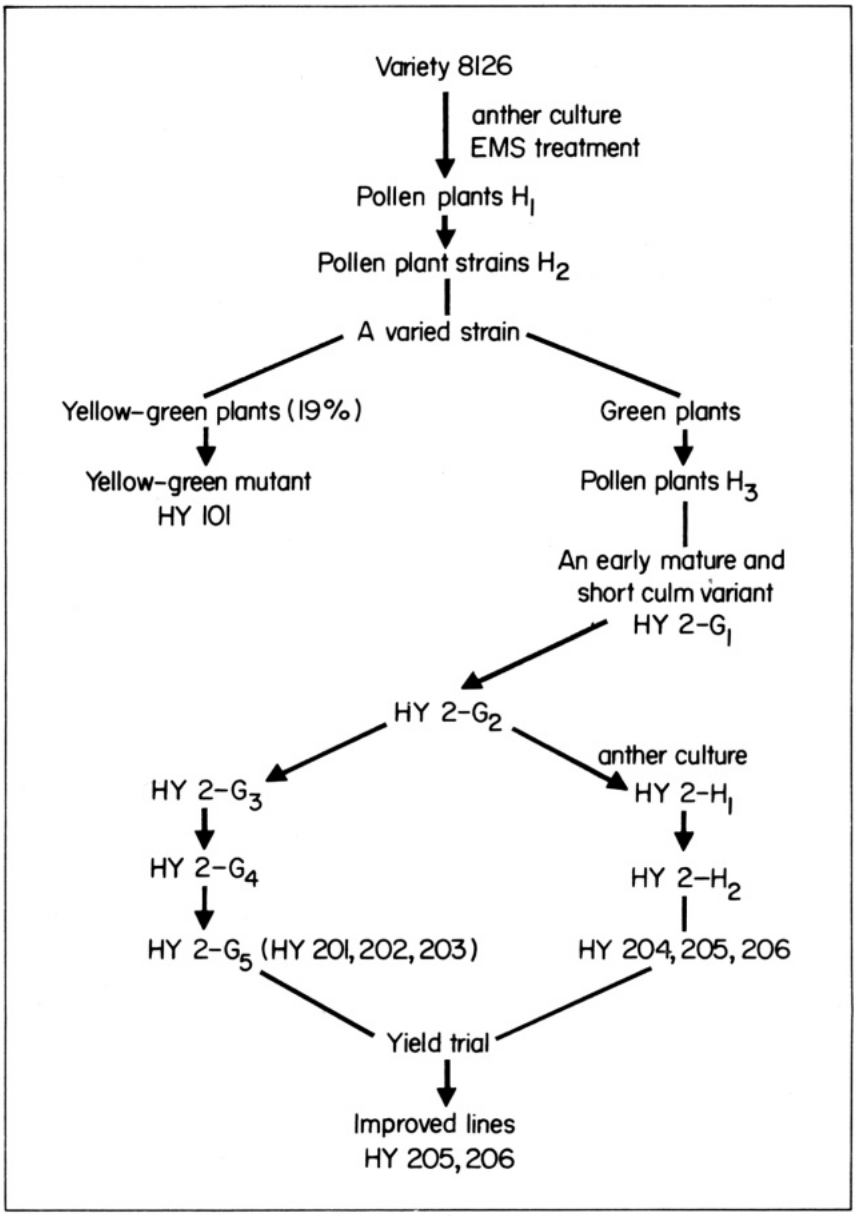

Fig. 1. Pattern of segregation of rice pollen plants produced by anther culture, leading to improved lines HY205 and HY206.

cross segregated into green and yellow—green at 1:1, except for the albinos (Hu et al 1981a). This figure coincides with the possible distribution of one pair of allele in the gametes. These results imply that there is no competi—tion among the two types of pollen and that no anther cul—

ture plantlets originated from the somatic tissue of anthers.

The photosynthetic characteristics of the mutant were studied (Hu et al 1981b). Its chloroplasts were deficient in light harvesting chl a/b protein complex and grana stacking. However, on the basis of chlorophyll, the photoreduction activity of chloroplasts and the CO_2 assimilation rate of leaves were much higher in the mutant than in a normal variety.

A short—culmed and early—maturing plant segregated in H_3 plants. The next generation of this variant showed intense segregation in plant height, heading date, and grain weight.

To stabilize the variations and to select the superior characters, further anther culture as well as a conventional selfing procedure were used. In this respect, anther culture has the advantage because numerous stable strains with various phenotypes can be obtained more quickly. There were 92 strains of H_2 pollen plants and 60 strains of selfing progeny available for selection. Stable lines with early maturity and short culm were easy to get (Table 3),

Table 3. Characters of selected mutant lines (HY 201—206) and original variety (8126).

Name	Plant height (cm)	Heading date (month/day)	Fertility (%)	Grain weight 100 (g)	Blast susceptibility
8126	122	7/28	67.2	2.38	yes
HY 201	105	7/3	75.6	2.75	yes
HY 202	106	7/21	72.5	3.20	yes
HY 203	106	7/7	77.5	2.92	yes
HY 204	90	7/18	78.3	2.80	yes
HY 205	112	7/19	76.0	2.35	no
HY 206	115	7/25	83.0	2.85	no

but their resistances were not better than the original variety. Two lines with better fertility and blast resistance were selected. They are of economic value, as the original variety 8126 is one of the most popular varieties in Yunnan province.

Segregation of posterity also has been recorded in pollen plants from hybrids of rice (Chen and Li 1978). In

our observations, Pollen Plants showing segregation of posterity occurred less than 2% of the time in pollen plants from varieties of keng (japonica), 2—8% from hybrids between keng rice, and more than 8% from hybrids between keng and hsien (indica) rice by conventional anther culture methods. The explanation of this phenomenon may be that a mutation is induced in a pollen after the doubling of its chromosomes. However, this may not be the sole explanation. The induction of unstable variations in pollen plants by the use of chemical mutagens seems contrary to the advantage of using haploids in mutation experiments. However, it provides more variants from which to select.

CONCLUSION

Even looking at only the drastic variations in visible characters, these results indicate that the use of chemical mutagens can induce mutations of pollen in cultured anthers of rice which will develop into varied plants. Most of these plants will be homozygous with stable and heritable characters.

The serious problem with this technique is that too few variants can be obtained. This originates from difficulties related to pollen haploid culture. First, the induction fequency of pollen plants in rice anther culture is very low. Second, the increased formation of albino plantlets in mutagen treatment further reduces the number of green plants. Third, the haploid plant is not valuable for the selection of visible characters. Usually, numerous haploid plants are discarded. The selection is made in H_2 plants because H_1 plants cannot express their phenotypes completely and normally.

Enhancement of the induction frequency of pollen plants is badly needed. Before it can be attained, it seems acceptable to induce more unstable variability because it can provide more variants for selection. To conduct selection at the pollen stage, callus culture could be considered for the improvement of certain characters, such as resistance. Haploid somatic tissue culture initiated from young haploid panicle fragments could be an alternative culture system for mutation experiments (see Hu Zhong abstract, this vol.).

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USE OF TISSUE CULTURE FOR BREEDING HERBICIDE-TOLERANT VARIETIES

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Herbicides have become a significant part of modern agricultural practices during the past 20 years. Because of the shortage of farm labor and energy resources, herbicides are gradually replacing hand hoeing and mechanical cultivation for weed control. But the development of new herbicides has become much more expensive with additional chemical regulation, more stringent toxicology standards, and increased concern for the environment.

It is easier to obtain permission to use a registered herbicide on undesignated crops than to develop and register a new herbicide for a specific crop. With the rapid development of genetic technology, it is faster and more economical to breed a new crop variety that is tolerant of a specific herbicide than to develop a new herbicide for a specific crop. For these reasons, selection for herbicide tolerant varieties will be an important and active area in plant breeding in the near future.

ADVANTAGES OF TOLERANT CROP VARIETIES

Herbicide tolerant varieties have several obvious advantages. First, even including the time needed to breed a new variety with tolerance of registered herbicides, the expense is less than that needed to develop a new herbicide, especially when modern technologies such as tissue culture and genetic engineering are used. Second, because crop tolerance is genetically controlled, the tolerance tends to be more stable in different environments. Third, by accumulating tolerant genes in a variety, it is possible to use higher rates of herbicides to eliminate hard-to-controlweeds. Fourth, a well-established variety with physiogenetic tolerance provides for more flexible use of herbicides and results in a saving of manpower.

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BREEDING HERBICIDE TOLERANT VARIETIES

Differential tolerance of herbicides has been noted in several crops as well as in weeds. In some cases, the genetic basis of tolerance has been analyzed. This indicates a great possibility for success in breeding herbicide tolerant varieties.

Tolerant genotypes in natural germplasms

Resistance to or tolerance of herbicides can occur at different levels by such mechanisms as uptake, translocation, and detoxification. Considering that many herbicides are selective and affect one plant species but not another, we can expect that tolerant genes and biotypes exist in the natural germplasm as a preadaptation form. Differential crop tolerance of various herbicides can be found in germplasms (Table 1).

Table 1. Genetic tolerance of herbicides in some crops.

Herbicide	Crop	Germplasm
Paraquat	Ryegrass	Strains
MCPA	Flax	Varieties
2,4-D	Carrot	Biotypes
	Sugarcane	Clones
Dalapon	Sugarcane	Clones
Propachlor	Sorghum	Varieties
Alachlor	Maize	Inbreds
Diuron	Sugarcane	Clones
	Barley	Biotypes
Bentazone	Soybean	Varieties
Simazine	Mustard	Varieties
	Wheat	Varieties
Atrazine	Soybean	Biotypes
Propazine	Sorghum	Varieties
Trifuralin	Maize	Inbreds

Genetic basis of tolerance

A few analyses of genetic herbicide tolerance have been reported. Siduron tolerance in *Hordeum* is inherited by three dominant genes (Schooler et al 1972). Atrazine tolerance in *Linum* is quantitatively inherited (Comstock and Anderson 1968) and in maize is controlled by a single recessive nuclear gene (Grogan et al 1963). In *Brassica campestris*, the same trait is maternally inherited in the chloroplast (Souza—Machado et al 1978). A picloram tolerant strain of *Nicotiana* was traced to a single dominant nuclear gene

(Chaleff and Parsons 1978). Bentazon and phenmedipham tolerant lines from haploid tobacco were found to be controlled by one or two recessive genes (Radin and Carlson 1978).

STRATEGIES FOR TOLERANCE BREEDING

The breeding of herbicide tolerant varieties can be approached in several ways.

Screening tolerant genes from germplasms

Many cases of differential tolerance of herbicides have indicated that tolerance is controlled by a rather simple genetic mechanism. Genes that now respond to new man-made chemicals were neutral in the past. These genes could easily be preserved in natural populations or germplasm collections. Flax and soybeans tolerant of atrazine were found by screening several thousand germplasms (Anderson and Behren 1967).

Mutation induction of tolerant genes

When a tolerant gene is not available in the germplasm or population, mutation breeding offers a possibility for inducing tolerance. Pinthus et al (1972) indicated that mutation induction and repeated selection increased the resistance of tomato to diphenamid and of wheat to terbutryn.

Selecting and introducing tolerant genes

The introduction of herbicide tolerant genes into susceptible varieties is very similar to breeding for pest resistance. Repeated backcrossing and delicate selection are necessary. Providing an adequate and uniform herbicide environment for selection is the most important and difficult part of the process.

High selection pressure takes a large population of plants and a substantial field area to select for complete resistance in a sensitive crop. If a 99.9% kill is accomplished when 10^6 plants are screened, you are left with seed from 10^3 plants to be screened the following season. Clearly this is not easy.

In vitro cell culture technologies offer a good alternative for overcoming these problems in field selection.

CELL CULTURE FOR BREEDING HERBICIDE TOLERANCE

Cell culture has been seriously considered for obtaining a better selection pressure. There are several reasons to

prefer cell culture over the field selection or classical breeding that use whole plants. Selection pressure and its effectiveness in the field rarely can exceed 99.9% kill because of the difficulty of uniform herbicide application.

It is not unusual to obtain wide variation in herbicide effects in a field (as much as 50% from site to site) because of soil and weather variability and different equipment management. Therefore, the plants selected can be of three classes: (1) escapees, (2) those from areas where the selection pressure is lower than anticipated, giving the appearance of a more tolerant plant, and (3) tolerant plants.

Increasing the herbicide dosage can effectively reduce the problems that exist when herbicide rates are lower than expected. However, with the extreme variation in field screening, the selection pressure also may be increased far beyond the tolerance level of truly tolerant plants, damaging the plant physiologically so that seed set does not occur or so that plants are totally eliminated. Also, increasing dosage may not solve the problem of selecting escapees instead of tolerant plants, but may in fact lead to the selection of escapees only.

The in vitro system of screening effectively reduces these environmental and dosage variation problems. The greater uniformity of herbicide application in a culture medium reduces the number of escapees and, because selection in cell culture is basically a mutation system, increases the efficiency of mutation selection.

Furthermore, if we start the herbicide/culture screening system with a certain genotype, upon selection and regeneration we should obtain plants of the same genetic background with tolerant genes. If tolerant genes are recessive, no additional backcrossing is necessary. If tolerant genes are dominant, only a few selfing-segregation-selection generations are needed to obtain homozygous plants.

Because cultured plant cells are basically unicellular organisms, a number of well-established techniques in microbial genetics can be applied to plant cell culture systems. Populations of cultured plant cells generally contain more genetic variability than whole plants because of higher spontaneous mutation, somatic crossover, chromosomal changes, and differentiation of genetic vegetation. Many studies have suggested that it is not necessary to use mutagens to increase genetic variability for the selection of mutants.

But cells and plants are different in many aspects. For example, most cell cultures do not photosynthesize nor translocate. If the mode of action of herbicide tolerance is based on the recovery of photosynthesis damage, a tolerant mutant may not be selected. If the mode of action of herbicide tolerance is based on the inhibition of translocation of the herbicide from organ to organ, selection may not work in cell culture. Cultured cells and whole plants are different developmental states characterized by dif-

ferent patterns of gene expression and regulation. A useful mutant must carry genes that can express themselves in both single cells and in whole plants.

Approaches

Several systems have been used in this area.

Diploid cell selection. Dominant mutated genes are relatively easy to select in diploid cell populations. Recessive mutants theoretically are difficult. However, there are indications that recessive mutations appear in cultured diploid cells at a much higher frequency than might ordinarily be expected. Recessive homozygotes may be produced by a high rate of somatic recombination. In fact, several variants or mutants have been selected from diploid cell populations. Figure 1 is the schema of this system.

Haploid cell selection. There is a good reason to use haploid cell populations to select recessive tolerant mutants. The probability of selecting recessive mutants from the haploid is much higher than it is from the diploid. However, it is not easy to establish and to maintain a stable haploid cell line. Haploid cell lines are usually weak, slow growing, and easily overtaken by mutated diploid or polyploid cells.

Haploid cells can be used in two ways. Haploid callus might be induced from anther or pollen in many crops. These materials could be used as a selection source, as was mentioned in diploid cell systems. Figure 2 is the schema.

A novel haploid plant selection system developed by Radin and Carlson (1978) is able to bypass the disadvantages of weakness and instability of cultured haploid cells. First, young haploid leaves were treated with mutagens. Then, leaves were treated with a herbicide for selection of mutant haploid cells. Green islands which indicated a population of tolerant cells were isolated in vitro and haploid plants were regenerated from these clones. After diploidization, crossing experiments were carried out for genetic analysis. Figure 3 is the schema.

Cell fusion for transferring the cytoplasmic factor. The use of somatic hybridization by protoplast fusion may be worth considering, especially when the desired trait cannot be transfered because of incompatibility or because it is cytoplasmic. One case might be the plastid—inherited atrazine resistance in Brassica campestris noted by Souza—Machado et a1 (1978). It is conceivable that this cytoplasmic genome may be transferred to various Brassica crops without deleterious effects, conferring S—triazine resistance. Figure 4 is the schema.

Recombinant DNA for transferring tolerant genes. It might be

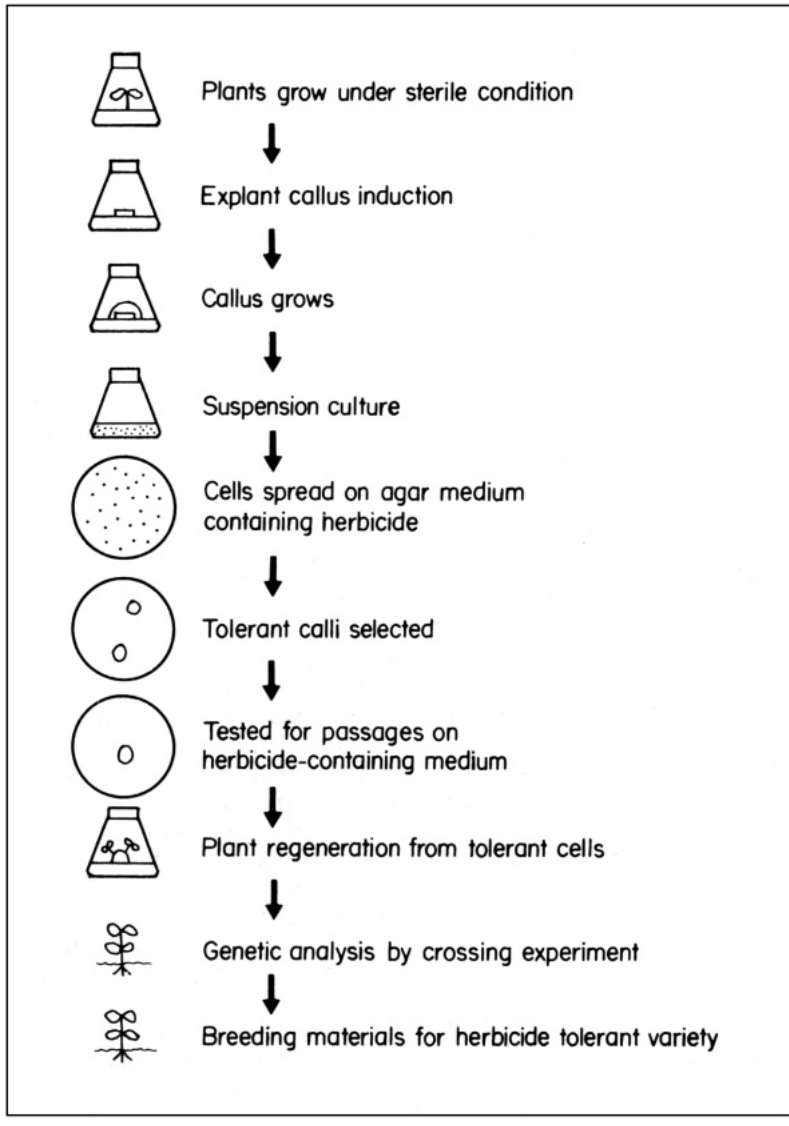

Fig. 1. Procedure for selecting herbicide tolerant mutants from diploid somatic cells.

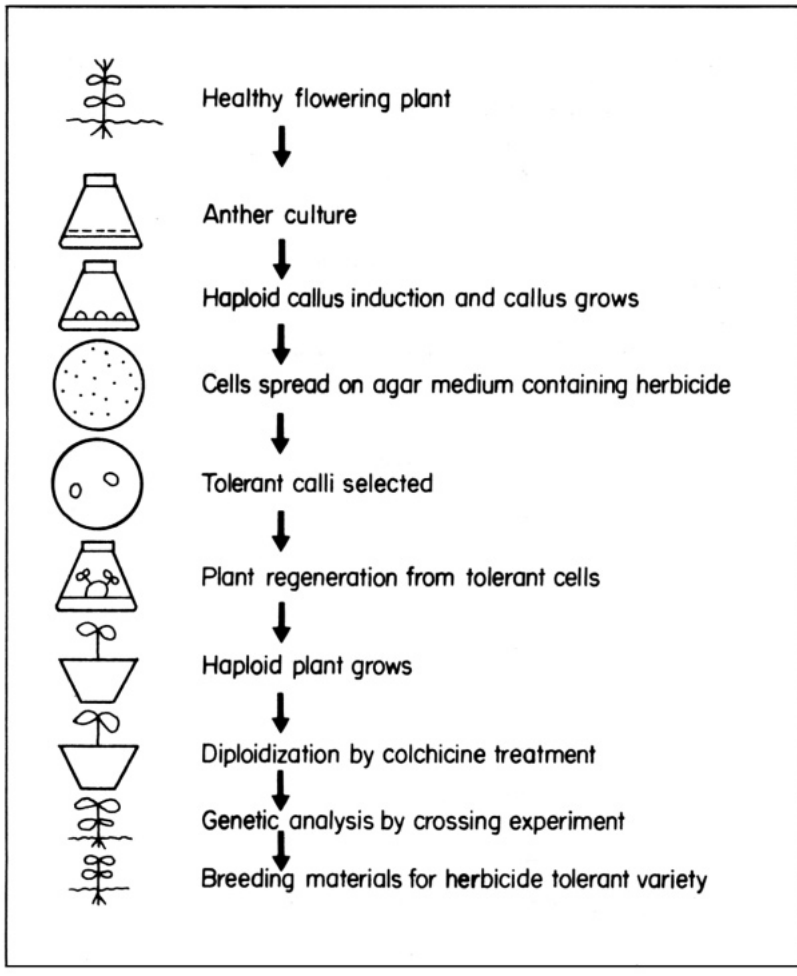

Fig. 2. Procedure for selecting herbicide tolerant mutants from anther cultured haploid cells.

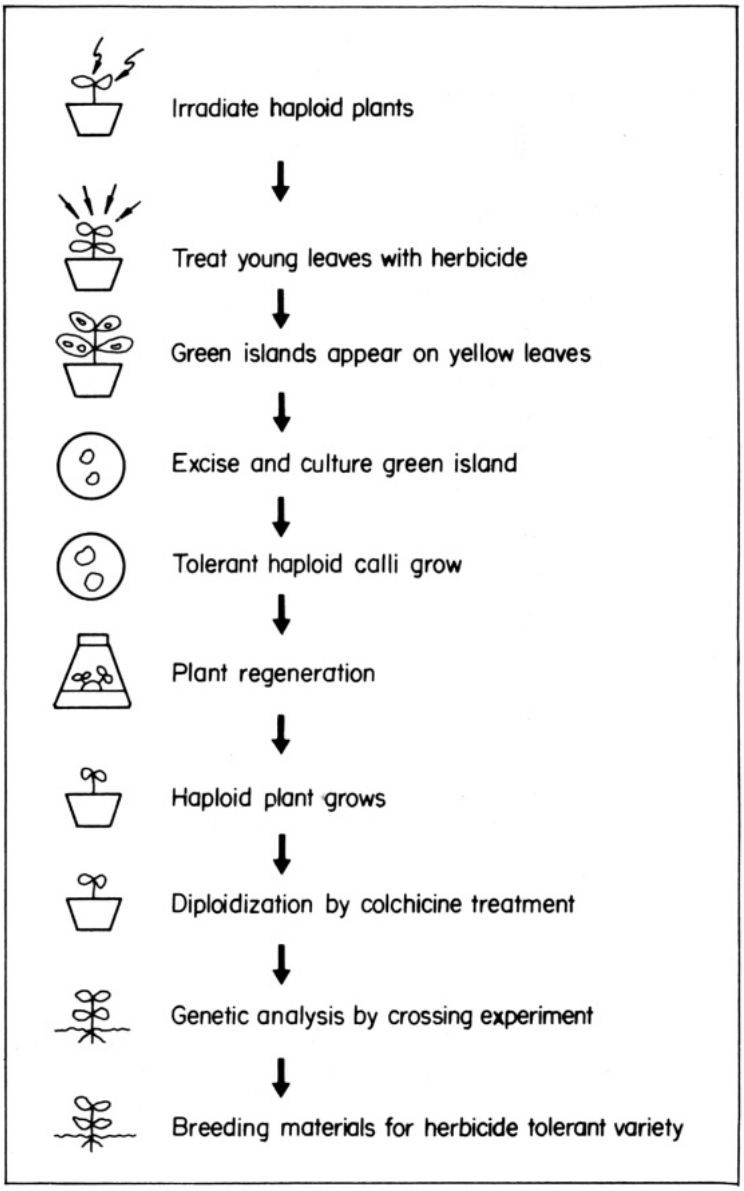

Fig. 3. Procedure for selecting herbicide tolerant mutants from haploid plants.

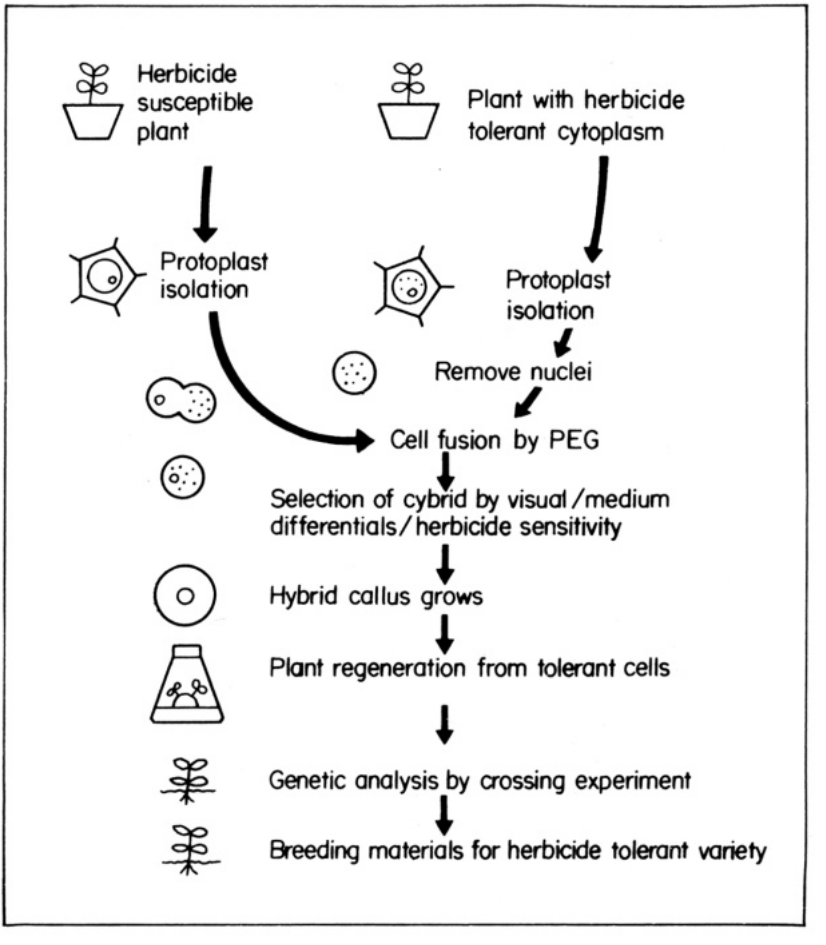

Fig. 4. Procedure for transferring herbicide tolerant cytoplasm by cell fusion.

too early to suggest this as a practical method. However, predictions are that the first successful transfer of genes by recombinant DNA in plants are likely to be in herbicide tolerance breeding, since the genetic basis for herbicide tolerance is relatively more simple than it is for other characters. The tremendous commercial advantage for agrochemical companies might be an additional factor which will develop this area. Figure 5 is the schema.

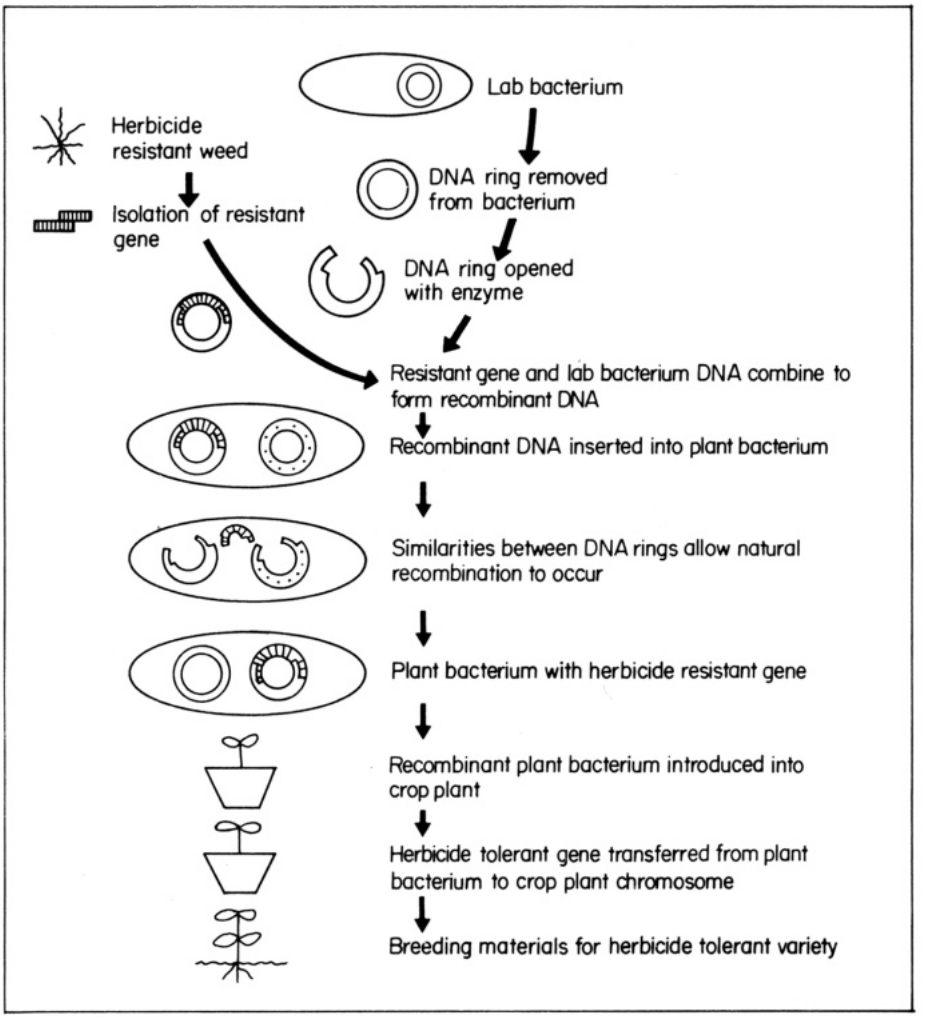

Fig. 5. Procedure for transferring herbicide tolerant genes by DNA recombination.

Type of tolerance

Reports to date indicate that some variant lines that tolerate normally lethal concentrations of different herbicides have been isolated. The tolerant materials, cells, or plants produced from in vitro cell culture systems may be categorized (Meredith and Carlson 1981, Miller and Hughes 1980):

- Selected cells are tolerant but lose their tolerance when grown away from the herbicide. Cell lines of this type have probably not undergone any genetic change but instead have made a biochemical adaptation to the presence of the herbicide. Also, there may be a mixed population of susceptible and tolerant cells.
- Selected cells are tolerant even after passage through culture away from the herbicide. The stability of the tolerance from generation to generation is suggestive of genetic change.
- Selected cells are tolerant but lose their tolerance when a plant is regenerated from the cell. The expression of tolerant genes is affected by the developmental stage and/or tissue differentiation in the plant.
- Tolerance lost in regenerated plants is regained in cell cultures derived from regenerated plants. The mutated tolerant genes can express themselves only in cell culture.
- Tolerance is expressed in a regenerated plant and in cell cultures derived from the regenerated plants. The tolerant genes are stable and can express themselves in both in vitro and in vivo conditions. The expression of mutated genes is not affected by plant or cell differentiation.
- Tolerance is stable in cells and plants but is lost in their progeny. The types of genes that are retained in in the plant but not transmitted genetically to the next generation may be useful for breeding vegetatively propagated crops.
- Tolerance is transmitted to the progeny of regenerated plants and its genetic basis is clear. This is the material that will be useful for plant breeding. From crossing experiments, the genetic basis can be studied to classify whether it is monogenic, polygenic, dominant, recessive, nuclear, or cytoplasmic. After confirming the genetic bases, genes can be incorporated into breeding programs to develop herbicide tolerant crop varieties.

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GERMPLASM PRESERVATION THROUGH TISSUE CULTURE: AN OVERVIEW

Lydsey A. Withers

Man's needs for food, fuel, medicines, fiber, and construction materials are rapidly outstripping the available supplies throughout the world. Increasing the efficiency of traditional agricultural methods could increase productivity to meet part of these demands. But it is becoming clear that more radical measures also are necessary to reduce the gap between demand and supply. In recognition of this reality, attention is turning to the possibility of applying novel in vitro tissue culture techniques to crop breeding and production.

These novel techniques, by eliminating pathogens from crops, may lead to improved productivity. Genetic engineering could result in the development of new, high-yielding varieties that have increased disease resistance. In vitro propagation may then be used for rapid clonal multiplication of these superior varieties. The danger is that this approach may be embraced too eagerly. Older, traditional varieties may be superseded and eventually become part of the growing list of extinct germplasm. A plural approach is needed, utilizing the best of the old, welcoming the best of the new, and conserving as wide a range of germplasm as possible for application in future breeding programs.

Traditional approaches to plant genetic conservation involve the storage of seeds and vegetative propagules and the maintenance of plantations. Problems are encountered in all of these areas, especially when long-term storage is required. A major problem in seed storage is deterioration. This is most acute for recalcitrant seed species (King and Roberts 1979, Roberts 1975). Bulky vegetative propagules require large amounts of storage space and are more prone to deterioration. Plantation storage overcomes some problems of deterioration but the material is open to environmental threats, to pathogen attack, and to failures in maintenance and renewal.

International exchange of germplasm is an important adjunct to conservation. The exchange of seeds may not be especially problematic. But vegetative material is highly prone to deterioration in transit and poses greater quarantine problems.

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The application of tissue culture techniques to crop species may reveal some new areas of difficulty, particularly where genetic manipulation is involved. At the early stages, potentially valuable genotypes may be represented only as protoplasts, cells, or calli. They will require storage until methods can be developed to culture them to the whole plant stage. Even then, the plants may be sterile and may carry all the problems now encountered with existing vegetatively propagated crops.

Perhaps it is fortunate that tissue culture itself may offer a new departure in genetic conservation technology. Interest is now concentrating on the possibility of storing preexisting genotypes in the form of tissue cultures, using methods that circumvent many of the long-standing problems involved in germplasm storage and exchange.

Tissue culture involves a wise range of systems, from the structurally simple protoplast or pollen grain to the complex somatic embryo or multiple-shoot culture. Culture methods are equally diverse. It would be surprising if one categorical method of culture storage were possible. The technology is still in its infancy. We have a great deal to learn about the storage requirements of the various culture systems. However, methodological principles are gradually being established and the prospects are good for the development of a comprehensive range of storage techniques soon.

Procedures for interchanging material between the separate phases of independent plant growth, clonal propagation in vitro, and storage are essential. The independent plant growth stage connects the scheme with all aspects of germplasm utilization. The in vitro propagation stage provides techniques for eradicating pathogens and carrying out other special manipulations. The storage makes possible the exchange of germplasm in a sequestered form which can be certified pathogen-free, easing passage through quarantine.

Both long-and short-term storage methods are desirable. For certain applications, we are talking in terms of periods ranging from months to 1 or 2 years; for others, decades to centuries. In the case of the conservation of germplasm from the field, the time involved should be at least as long as the regeneration cycle of the plant. In the laboratory, other practical criteria may be involved.

STORAGE TECHNIQUES

The storage of cultures in the growing state

Standard culture maintenance procedures. The familiar procedure to initiate and maintain a culture involves introducing a piece of plant material into aseptic culture, stimulating growth and/or development, and periodically transfering the culture to fresh medium. For many cultures, the time

between a transfer or subculturing can be divided into a lag—phase, during which no increase in biomass is detected; a rapid, possibly exponential, growth phase; and a station—ary phase brought about by one or more limiting factors in the culture medium. Timely subculturing minimizes the sta—tionary phase and avoids the risks of stress induced by prolonged exposure to suboptimal culture conditions. Adjust—ment of the ratios of medium constituents and the use of a larger volume of medium may forestall the onset of the sta—tionary phase.

Under normal culture conditions, the subculturing inter—val may be as short as a few days or as long as several months, depending on the type of culture and species. Simply main—taining a species in continuous growth may provide adequate short—term storage. However, this approach cannot meet all requirements. It is necessary to seek means of either slow—ing growth or suspending it entirely.

Maintenance of cultures in limited growth. Methods to retard culture growth can be divided into five categories:

- Reduction of growth temperature. The temperature should be chosen to compromise between a satisfactory limitation of growth and the onset of chilling or freezing injury. Storage temperatures in the 4—12°C range are common, although some species -- including tropicals -- may need higher temperatures (ca. 20°C).
- Atmospheric modification, such as growth at low pressure or low partial pressure of oxygen. Overlay—ing a culture on a semisolid medium also will reduce the supply of oxygen to the culture.
- Partial dessication.
- Application of osmotic retardants such as mannitol and sorbitol.
- Application of retardants such as abscisic acid and cycocel (CCC).

Because osmotic stress has been found to lead to the accumulation of endogenous abscisic acid, it is possible that chemical means of growth limitation have fundamental similarities to the application of osmotic retardants and desiccation.

The further possibility of reducing the supply of es—sential nutrients such as sucrose or mineral salts is avail—able, but it carries the risk of actually reducing the subculture interval rather than extending it.

However much growth rates may be reduced by any of these methods, it is essential that some growth be occur—ring. Otherwise, degenerative processes take over and the interval between subculturings would be determined by the need to rescue the culture rather than to supply it with the nutrients to sustain retarded growth. Sequential sub—culturing eventually would become impossible.

When a long—term storage method requiring no periodic

renewal is sought, a completely different approach must be taken. All metabolism must be suspended, effectively suspending time. Several approaches may be available for some biological systems. But for plant tissue cultures, there appears to be only one -- cryopreservation.

Cryopreservation

A moderately substantial technology is attached to preservation in the frozen state. The transfer of a tissue culture to and from storage temperature imposes severe stresses which must be alleviated by appropriate treatments. The cryopreservation process can be divided into seven stages: pregrowth, cryoprotection, freezing, storage, thawing, viability estimation, and recovery. These have been described in detail by Ashwood—Smith and Farrant (1980), Finkle et a1 (this volume), Meryman (1966), Meryman and Williams (1981), and Withers (1980a, b, c; 1981a).

Pregrowth. The culture conditions to which a specimen is exposed before cryopreservation can seriously affect survival There is a definite relationship between growth cycle stage and freeze tolerance, especially for cell cultures. For best results, the culture should be harvested in the early exponential stage. For somatic embryos developed from cell cultures and from pollen, the early globular—and heart—shaped stages are the most freeze tolerant. For shoot—tip cultures, the optimum time appears to be soon after excision (ca. 2 days). Pregrowth in a medium containing additional mannitol, proline, or dimethyl sulfoxide has been found to improve the freeze tolerance of some cell and shoot—tip cultures. (A number of the pregrowth effects relate to morphology. It is generally possible to say that the smaller the cell, tissue, or organ, the higher its freeze tolerance.)

Cryoprotection. Several compounds have been found to have cryoprotectant activity. Dimethyl sulfoxide and glycerol are most commonly used. Cryoprotectant mixtures which also incorporate some sugars, sugar alcohols, amino acids, and high molecular weight polymers are widely used. With some types of culture, a single cryoprotectant (proline or dimethyl sulfoxide) may be effective, but this should be considered exceptional. Typically, final cryoprotectant concentrations fall in the 0.5 —— 2molar range.

A suitable quantity of culture is harvested and, if necessary, transferred to liquid medium (as would be the case for a callus culture). The culture is cooled on ice. An equal volume of cryoprotectant solution is prepared at double the final concentration required and chilled. The culture is added slowly. The cryoprotected culture is dispensed into a polypropylene ampul a glass ampul (less satisfactory), or a foil envelope. Some specimens supported on a hypodermic

Table 1. Summary of successful cryopreservation of plant tissue cultures, highest survival and regeneration data for each species.

Species	Culture system	Freezing program[a]	Survival (%) Post-thaw viability[b]	Survival (%) Recover growth[c]	Plant regeneration[d]	Reference
Acer pseudoplatanus	Cell suspension	Slow/stepwise	90	+		Nag and Street 1975a,b; Sugawara and Sakai 1974; Withers and King 1980; Withers and Street 1977
Acer saccharum	Cell suspension	Slow (to −40°C)	20			Towill and Mazur 1974
Arachis hypogaea	Shoot tip[e]	Slow/rapid		31	+	Bajaj 1979
Asparagus officinalis	Shoot tip	Rapid		38	+	Seibert 1977
Atropa belladonna	Cell suspension	Slow	40	+		Nag and Street 1975a,b
Atropa belladonna	Anther	Rapid		+	+	Bajai 1978a
Atropa belladonna	Pollen embryos	Slow	31	+		Bajaj 1977a; 1978a,b
Bromus inermis	Protoplast	Stepwise	68			Mazur and Hartmann 1979
Capsicum annuum	Cell suspension	Slow	25			Withers and Street 1977
Capsicum annuum	Cell suspension	Slow (to −30°C)	50	+		Withers and Street 1977

(Table 1. Continued)

Chrysanthemum morifolium	Callus	Stepwise (to −3.5°c)		50		Bannier and Steponkus 1972
Cicer arietinum	Shoot tip	Slow/rapid		40	+	Bajaj 1979
Datura stramonium	Cell suspension	Slow	40			Bajaj 1976
Daucus carota	Cell suspension	Slow	65	+	+	Dougall and Wetherell 1974, Nag and Street 1973, Withers and Street 1977
Daucus carota	Callus	Stepwise (to −80°c)		+		Nitzsche 1978
Daucus carota	Somatic embryo/ clonal plantlet	Slow		100	+	Withers 1979
Daucus carota	Protoplast	Slow/stepwise	90	+	+	Mazur and Hartmann 1979, Takeuchi et a1 1980, Withers 1980b
Dianthus caryophyllus	Shoot tip	Slow/stepwise/rapid		100	+	Anderson 1979; Seibert 1976, 1977; Seibert and Wetherbee 1977; Uemura and Sakai 1980
Dianthus caryophyllus	Cell suspension	Slow		50		Anderson 1979
Fragaria × ananassa	Shoot tip	Slow/stepwise		95	+	Kartha et a1 1980, Sakai et a1 1978
Glycine max	Cell suspension	Slow	52			Baiai 1976

(Table 1. Continued)

Haplopappus ravenii	Cell suspension	Slow (to —20°C)		+		Hollen and Blakely 1975
Hyoscyamus muticus	Cell suspension	Stepwise	30	+	+	Withers and King 1980
Ipomoea sp.	Cell suspension	Slow (to —40°C)		+		Latta 1971
Lactuca sativa	Shoot tip	Rapid		67	+	Seibert 1977
Linum Usitatissimum	Cell suspension	Slow (to —50°C)	14			Quatrano 1968
Lolium sp.	Seedling	Rapid		30	+	Dale and Withers (see Withers 198b)
Lycopersicon esculentum	Seedling	Rapid		45	+	Grout et a1 1978
Manihot sp.	Shoot tip	Rapid		13	+	Bajaj 1977b, Henshaw et a1 1980a, Stamp 1978
Medicago sativa	Callus	Slow		+		Finkle et a1 1979
Nicotiana sylvestris	Cell suspension	Slow	15			Shillito 1978
Nicotiana tabacum	Cell suspension	Slow	60	10	+	Bajaj 1976
Nicotiana tabacum	Anther	Slow		7	+	Bajaj 1978a
Nicotiana tabacum	Pollen embryo	Slow	31	+	+	Bajaj 1977a; 1978a,b
Oryza sativa	Cell suspension	Slow	65	+		Cella et a1 1978, Sala et a1 1979
Oryza sativa	Callus	Slow		+		Finkle et a1 1979

(Table 1. Continued)

Oryza sativa	Anther	Slow		0.5	+	Bajaj 1980
Petunia hybrida	Anther	Slow		5	+	Bajaj 1978a
Phoenix dactylifera	Callus	Slow		+	+	Finkle et a1 1979, Tisserat et a1 1981
Pisum sativum	Shoot tip	Slow		88		Kartha et a1 1979
Pisum sativum	Zygotic embryo Meristem	Rapid		30	+	Grout 1979
Populus euramericana	Callus	Stepwise		+		Sakai and Sugawara 1973
Rosa sp.	Cell suspension	Stepwise	70	+		Withers and King 1980 Withers 1981a
Saccharum sp.	Cell suspension	Slow	30			Chen et a1 1979
Saccharum sp.	Cell suspension	Slow		+		Ulrich et al 1979, Finkle and Ulrich 1979
Saccharum sp.	Cell suspension	Slow (to —23°C)		+	+	Ulrich et al 1979
Sambucus racemosa	Callus	Not state		+		Yoshida (see Sakai and Nishiyama 1978)
Solanum geniocalyx	Shoot tip	Rapid		60	+	Grout and Henshaw 1978; Hensahw et a1 1980a,b
Solanum tuberosum	Shoot tip	Slow/rapid		36	+	Bajaj 1978c; Henshaw et a1 1980a,b; Towill 1979

(Table 1. Continued)

Sorghum bicolor	Cell suspension	Stepwise	25	+		Withers and King 1980
Zea mays	Cell suspension	Stepwise	80	+		Withers 1980d; Withers and King 1979, 1980
Zea mays	Callus	Slow		+	+	Withers 1978, 1980b
Zea mays	Zygotic embryo	Slow		+	+	Withers 1978, 1980b

[a] Storage in liquid nitrogen unless indicated in parentheses. [b] Viability test data. [c] + = Recovery growth reported with no value given. [d] Plant regeneration may be possible in systems other than those reported. [e] Shoot tip includes specimen described as shoot apices and meristems.

needle or glass cover slip can be frozen by direct exposure to the coolant.

Freezing, storing, and thawing. The choice of cooling rate is probably one of the most demanding aspects of the cryopreservation procedure. In many cases, a slow rate of cooling (0.5-5°C/min) is most suitable. Protective dehydration occurs during the early stages of cooling. However, if cells are cooled too slowly, they overdehydrate and suffer an excessive concentration of intracellular solutes. Protective dehydration may be induced by stepwise cooling, with the specimen held at a sub-zero temperature for a certain period of time before further cooling.

Rapid freezing may be suitable for some shoot-tip cultures. Cooling rates in excess of 50°C/min may be used. This method carries the risk of intracellular ice formation. This can be minimized by adding adequate cryoprotectants and by using as rapid a cooling rate as possible. We are not yet in a position to give exact guidelines for the most suitable rates for cooling individual specimens, but it is usually possible to choose between the two basic options of slow or rapid freezing (Table 1).

Storage must be at a suitably low temperature, ideally below -100°C. Over the medium and long term, deterioration occurs if specimens are stored at too high a temperature. This is thought to be due to physical damage by ice crystallization.

This phenomenon also must be considered when choosing thawing rates. Rapidly frozen specimen in which there is a large amount of intracellular ice must be thawed very rapidly to prevent recrystallization. In theory, slowly frozen material may be thawed slowly. But in practice, it is usually thawed rapidly in warm water (ca. 30-40°C) as a precaution against recrystallization of any residual intracellular ice.

Viability estimation. It is useful to estimate relatively rapidly the condition of a freshly thawed specimen. For protoplast and cell cultures, fluorescein diacetate staining is suitable. The TTC test (reduction of 2,3,5-triphenyl tetrazolium chloride) is more suitable for bulkier specimens such as pieces of callus or shoot tips. It also can be used for cells. (The definitive success indicator is recovery of growth. This can be monitored by physiological assay, biomass estimation, electron microscopy, and continued viability testing).

Recovery. Recovery growth is initiated by returning the specimen to culture. Post-thaw washing is not always necessary and may even be injurious. Recent findings suggest that improvements can be made in washing procedures (Finkle et al, this vol.). Recovery growth can be carried out using

the same culture method as for pregrowth. However, there is evidence that some specimens pregrown on a liquid medium recover best on a semisolid medium.

There is considerable scope for improving recovery rates by modifying the culture medium constituents or the culture environment. For example, the addition of gibberelin or activated charcoal to the culture medium may promote organized development. Lower light levels may be beneficial during the early stages of recovery.

Apparatus. Existing culture facilities may be adequate for the maintenance of cultures in the growing state. However, when growth is to be limited by temperature reduction or other means incompatible with the maintenance of standard growth conditions, additional facilities will be required.

The adoption of cryopreservation as a storage method inevitably demands a certain amount of special equipment, but it is not necessarily complex nor costly. For slow and stepwise freezing, it is possible to purchase programmable freezing units cooled by liquid nitrogen (LN) which offer a wider range of freezing rates and holding temperatures. These require electricity. For slow, nonlinear cooling, the specimen may be suspended in the cold atmosphere of LN vapor in the neck of a vacuum flask or refrigerator containing LN. A simple slow or stepwise freezing unit consisting of an alcohol bath cooled by an electrically powered cooling coil has been used successfully by Withers and King (1980). Other improvised methods are described by Withers (1980a, b).

No special apparatus is necessary for rapid freezing. The specimen is transferred directly from the temperature at which the cryoprotectant is added to a container of LN. Alcohol cooled with solid CO_2 also may be used, but this will not cool specimens as rapidly.

A storage refrigerator that maintains a suitably low temperature is essential. Ideally, it should be cooled with LN to give a storage temperature ca. —150°C in the gas phase and ca. —196°C in the liquid phase. It is possible to obtain such refrigerators, which will hold several thousand ampuls organized in stacks of drawers. Refrigerators cooled electrically or by solid CO_2 are suitable only for short-term storage (days or weeks).

No special apparatus is required for thawing. A warm water bath running at 30—40°C, or even a beaker of warm water, will suffice. It may be advantageous to use sterile water as a precaution against microbial contamination. Where slow thawing is required, the specimen may simply be left to warm at room temperature.

APPLICATION OF STORAGE METHODS — THE CURRENT SITUATION

Two sources of information are available. First, there is a

reasonably large literature on the subject, particularly in relation to cryopreservation. Second, the International Board

Table 2. Summary of successful attempts to carry out in vitro propaga— tion, storage, and germplasm exchange in crops (Survey sponsored by the International Board for Plant Genetic Resources; reported by Withers 1981a,b).

Crop species	Propagation	Storage in growing state[a)]	Cryopreserva— tion[b)]	Germplasm exchange
Agave spp.	+			
Ananas spp.	+	+		
Arachis hypogaea	+			
Artocarpus spp.				
Carica papaya	+	+		+
Cicer arietinum	+		+	
Cinchona spp.	+			
Citrus spp.	+	+	+	
Cocos nucifera	+	+		
Coffea spp.	+	+		
Colocasia esculenta	+	+		+
Dioscorea spp.	+	+		+
Eleais guineensis	+		+	+
Ficus spp.	+	+		+
Hevea brasiliensis	+			+
Ipomoea spp.	+	+		+
Mangifera indica	+			
Manihot sp.	+	+	+	+
Maranta arundinacea				
Musa spp.	+	+		+
Other palms				
Passiflora caerulea	+	+		
Persea americana	+			
Phoenix dactylifera	+	+	+	+
Piper nigrum	+			
Saccharum sp.	+	+	+	+
Solanum tuberosum	+	+	+	+
Other Solanum spp.	+	+	+	+
Thea sinensis	+			
Theobromo cacao		+		
Vitis spp.	+	+		+
Xanthosomoa saggitifolium	+	+		+

[a)] + indicates storage for at least 10 weeks before subculturing.

[b)] + does not indicate that plant regeneration was achieved after storage nor that viability levels were high and/or reproducible.

for Plant Genetic Resources (IBPGR) recently commissioned a survey to determine the extent to which tissue culture meth— ods are being applied to the propagation, conservation, and

germplasm exchange of certain crop plants (Table 2). Preliminary reports have been made (Withers 1981 b, c), and information is being added continuously.

Table 3. Storage of shoot tip and similar cultures by growth at reduced temperatures.

Species	Growthtemperature (°C)		References
	Normal	Reduced	
Beta vulgaris	20	12	Hussey and Hepher 1978
Dactylis sp.	a)	2-4	Dale 1978
Festuca sp.	a)	2-4	Dale 1978
Fragaria sp.	a)	1-4	Mullin and Schlegel 1976
Ipomoea batatas	28	22	Alan 1979
Lolium multiflorum	25	2—4	Dale 1980
Malus domestica	26	1-4	Lundergen and Janick 1979
Manihot esculentum	30/25[b]	20	CIAT 1978
Medicago sativa	25	2-6	Cheyne and Dale 1980
Phleum sp.	a/	2—4	Dale 1978
Solanum spp.[c]	22	6	Henshaw et al 1980a,b
Trifolium repens	25	2-6	Cheyne and Dale 1980
Vitis rupestris	20	9	Galzy 1969

a) No value quoted. b) Alternating day and night temperatures.

c) Several genotypes.

Storing cultures in the growing state

In all, more than 50 species have been stored in the growing state, although the technical approaches, the degree of success, and the detail available vary. Growth at a reduced temperature has been most widely reported as a means for growth limitation (Table 3). Storage for at least 1 year between subculturings is feasible for all the shoot—tip cultures listed. Storage could last up to 6 years for Fragaria with only the periodic addition of drops of fresh medium to replace that lost by evaporation and metabolism. Some genotypes of Solanum survived 18 months of storage at 6°C, yielding satisfactory viability levels of 60—70% (Henshaw et al 1980a, Westcott et al 1977). But others declined more rapidly, demanding more frequent transfer.

Storage under other limiting conditions has been reported for five species (Table 4). A wider range of culture systems is involved but protoplasts, cells, and anthers are not included. In general, these studies have been less comprehensive than those involving low—temperature growth.

Attempts to use continued growth as a storage method are more widespread than the literature would suggest. This approach was indicated for 19 of the subjects in the IBPGR survey, taking the ability to extend the subculture interval

Table 4. Storage of tissue cultures under limiting conditions other than low-temperature growth.

Species	Culture system	Limiting factor	References
Chrysanthemum morifolium	Callus, plantlets	Low pressure/ low oxygen	Bridgen and Staby 1981
Daucus carota	Callus	Desiccation	Nitzsche 1978
Daucus carota	Callus	Mineral oil overlay	Caplin 1959
Daucus carota	Somatic embryos	Partial desiccation/ low Sucrose	Jones 1974
Lycopersicon esculentum	Cultured roots	Low pressure/low oxygen	M.P. Bridgen, Department of Horticulture, Ohio State University, personal communication
Nicotiana tabacum	Callus, plantlets	Low pressure/low oxygen	Bridgen and Staby 1981
Solanum sp.	shoot-tip cultures	Hormonal/osmotic retardants	Henshaw et al 1980a, b

to at least 10 weeks as the criterion of success. While this would not be satisfactory for long—term storage, in many cases it would greatly relieve the practical demands of cul— ture maintenance. With further investigation, storage methods probably could be improved to compare favorably with those already reported.

In addition to the species listed in Table 2, survey respondents said that 20 more species could be stored in the growing state, usually at reduced temperatures. In assessing all storage attempts, respondents considered 50% to have yielded reproducible results. Problems were related to fail— ure to maintain a healthy condition in limited growth, mi— crobial contamination, and loss of totipotency by callus cultures. These problems indicate a continuing need for re— search on basic techniques. The loss of totipotency underlines the fact that storage in the growing state is likely to be most suitable for organized cultures that are genetically stable, such as shoot tips.

Cryopreservation

Investigations into cryopreservation are relatively difficult to carry out on a casual basis. The centers of research activity usually have used model systems which are relatively unimportant in conservation. However, there are some notable exceptions and interest in cryopreservation of species with a conservation problem is growing.

To date, the storage in LN of more than 30 species has been reported, with varying degrees of success (Table 1). Several other species have been frozen to intermediate tem— peratures, suggesting some freeze tolerance but with no in— dication that survival could be achieved after exposure to LN. It is clear that the survival indicated by an initial viability test is no guarantee of recovery growth nor of plant regeneration. Some studies did not even attempt re— covery growth or plant regeneration. Although all culture systems are represented, the most effort has been concen— trated on cell cultures and meristem/shoot—tip cultures. No clear methodological trends can be drawn from the data, largely because few comparative studies involving more than one species or more than one culture system within a species have been done. It is not clear which differences in meth— odology and response are due to morphological variation and which are due to taxonomic or other factors.

The IBPGR survey lists eight successes in cryopreserva— tion (Table 2). adding only two to the list: zygotic embryos of Eleais guineensis and an unspecified culture of Citrus sp. Approximately 50% of the storage attempts are considered reproducible. Problems encountered include failure to regen— erate organized structures after thawing and low or erratic levels of viability. Failure to regenerate probably is due to massive structural damage to the specimen rather than to

loss of totipotency, in the strict sense.

Linking storage to propagation and germplasm exchange

Tissue culture storage should not be considered in isolation, It is essential to develop long— and short—term storage methods compatible with clonal propagation, germplasm ex—change, and eventual utilization of the germplasm.

Clonal propagation in vitro and storing the in vitro propagules in the growing state and by cryopreservation ap—pear possible only for Fragaria. Fragaria eventually can be returned to growth in the field. (Germplasm exchange has not been attempted for this subject but it can be assumed to be feasible). All other species investigated had deficiencies, either in propagation or in storage technology, due to dif—ficulties or neglect.

The most complete picture emerges for Manihot and Solanum. These are undoubtedly more demanding subjects than Fragaria, both in behayior in culture and in the range of genotypes that have to be cultured. Current studies with these species provide the best examples of the comprehensive application of in vitro methodology to the genetic conserva—tion of crop plants. At the Centro Internacional de Agricul—tura Tropical (CIAT) in Colombia, about 300 cultivars of cassava have been freed of pathogens using combined ther—motherapy and meristem culture. More than 600 other cultivars can be propagated in vitro. Storage by growth at low tempera-tures is being investigated. To date, many cultures have survived 3 to 6 months in storage, some more than 2 years. Successful international exchange of cultures has been achieved, with material dispatched as far as Southeast Asia. Cooperative projects with other institutions also are inves—tigating cryopreservation, but so far no technique that can be readily adopted has emerged.

At the Centro Internacional de la Papa (CIP) in Peru, a similar program is under way for its many thousand potato genotypes. CIP is attempting to develop laboratory germplasm storage methods to replace the current field method involving the annual renewal of plantations. Tissue culture techniques are being used to establish a collection of pathogentested American and European cultivars, a collection certified free of seed—borne viruses, and a collection of triploid and pentaploid cultivars that cannot be conserved as seeds. A range of methods is being used, including serial subculturing every 2 months, growth limited by the application of abscisic acid and temperature reduction (annual subculture), and growth limited by the application of mannitol (subculture twice yearly at the most). Mannitol application has given the best survival levels with the fewest practical problems.

Cryopreservation techniques in cooperating institutions have achieved reproducibly good results only with meristems/ shoot tips isolated from seedlings. Success has yet to be

achieved with in vitro—propagated material. The methods cannot be applied to genotypes cloned in vitro to material that has been stored as cultures in limited growth.

The international exchange of cultures from CIP is routine. Experiences with cassava and potato would indicate that there are no insurmountable problems involved in exchanging growing cultures. High success rates can be achieved, given reasonable care in packaging, no serious delays in transit, and sufficient training of the receiving laboratory's staff. Note that respondents to the IBPGR survey have successfully exchanged tissue cultures of 15 of the listed crops (Table 2).

To summarize the situation with Manihot and Solanum:

- Propagation (and pathogen elimination) techniques are virtually routine.
- Germplasm storage by growth limitation is under investigation and reproducible methods are emerging.
- Germplasm exchange can be carried out without difficulty.

However, cryopreservation is still presenting serious problems.

It appears that foundations have been laid for similar comprehensive schemes for some other crops. Propagation in vitro is the least problematic but storage techniques, particularly cryopreservation, are less well developed. This is due largely to insufficient work, so that we are not yet able to define the impediments to progress, let alone attempt to overcome them. It is hoped that this situation will improve as interest and activity in cryopreservation increase.

THE APPLICATION OF TISSUE CULTURE STORAGE FOR GERMPLASM PRESERVATION

Choice of storage method

Although some workers wishing to adopt tissue culture methods for preserving germplasm may find a procedure in the literature which meets their requirements, usually this will not be the case. A certain amount of work will be needed to develop a suitable method. One of the first decisions concerns the choice of basic storage method. Within the two main options (storage in the growing state or cryopreservation), further choices must be made. The different technical approaches have practical and biological advantages and disadvantages.

Level of technical development. Maintenance of cultures in the growing state, particularly at a normal rate of growth, has a relatively well—developed technology in media requirements, subculture routines, and environmental factors. Its drawbacks become evident when other aspects are considered. Storage under limited growth has been used fairly widely for

organized cultures growing on a semisolid medium or fed with a liquid medium by paper wicks. But there has been very little success in cultures submerged in a liquid medium. Serious problems of anoxia are likely if they are rendered static during storage. However, some cell and other types of culture normally cultured in a liquid medium can be transferred to a semisolid medium, where growth limitation could be attempted.

At the present level of technical development, it seems most sound to recommend temperature reduction and/or the application of osmotic stress to retard growth. Too low a temperature or too high a concentration of retardant should be avoided. Periods of growth limitation can be alternated with periods of convalescence under normal growth conditions. These recommendations, based on work with shoot-tip cultures, may apply to callus, cell, anther, and plantlet cultures growing on a semisolid medium. They do not apply to protoplasts.

Cryopreservation technology is less well developed for plant-tissue cultures than for many other biological systems. However, the subject is moving forward at a relatively rapid rate. For many cell cultures, we can recommend suitable storage protocols detailing cryoprotectants, slow or stepwise freezing programs, and pregrowth and recovery procedures. But this is not the case for callus, protoplast, and anther cultures. For organized cultures, including meristems, shoot tips, embryos, and plantlets/seedlings, at least one approach to freezing (rapid or slow) may be suitable, but it is not yet clear whether a single, generally applicable method will emerge.

Technical expertise. Tissue culture itself requires a certain amount of technical expertise, dexterity, and meticulous work. For the maintenance of cultures in the growing state, these skills are still required, plus the ability to recognize the symptoms of culture deterioration to facilitate timely subculturing. It is inevitable that some new skills will be required to carry out cryopreservation, but these should be no more demanding than other aspects of tissue culture work. Expert instruction in cryopreservation techniques would be an advantage, but should not be considered essential.

Cost. Routine culture maintenance is relatively expensive. Large quantities of chemicals are consumed in medium preparation. The increasing tendency to use disposable containers also is costly and wasteful of resources. Reusable containers require cleaning and re-sterilizing and they have a finite life. Maintenance of a stable culture environment requires special equipment and consumes a lot of energy. Many man-hours are involved in monitoring and subculturing.

Limiting the growth rate of cultures can reduce costs

if retardants and environmental facilities are not too expensive. But when additional refrigeration equipment is involved, compare costs carefully.

Because cryopreservation is a departure from standard culture maintenance procedures, some equipment investment will be inevitable. The cost of a freezing unit depends on the type of freezing regime used and the degree of sophistication and automation. An expensive commercial unit is not essential. But a refrigerator cooled by LN is necessary. Facilities to obtain and store the coolant should be available. LN is relatively inexpensive and its wide use in medicine, animal husbandry, and industry ensures good distribution networks. LN also can be produced locally using a compression unit. Depending on the refrigerator design, LN consumption will range from 100 to 1,000 liters a year. But no power supply is required and only simple, periodic maintenance checks are necessary.

The relative costs of storing cultures in the growing state and by cryopreservation will change over time. The initial investment for cryopreservation is greater, but recurrent costs are minimal.

Reproducibility of techniques and stability in storage. The material committed to storage will be unique in exact genotype and culture history. Therefore, storage procedures must be reproducible and the material must be stable in storage. Little information is available on reproducible procedures, although respondents to the IBPGR survey indicated that storage in the growing state and by cryopreservation gave similar levels of reproducibility (in the region of 50%). This indicates that there is both little difference between the two approaches and considerable scope for improvement.

Stability in storage can be considered both quantitatively and qualitatively. Maintenance of viability levels is important and material recovered from storage should not be altered in phenotype and genotype. Cryopreserved material can be considered indefinitely as quantitatively stable. Quantitative deterioration is an inevitable risk for material stored in the growing state and subculture routines must be adjusted to accommodate this. The steady growth of the cultures, leading to some replacement, only partially compensates for this risk. Some loss due to accident, error, and microbial contamination also must be anticipated.

Genetic instability may involve genotype modification and selection from within heterogeneous populations. Certain types of culture, notably cells and calli, have a reputation for instability. This is not always justified. Most genetic changes are the result of abnormal processes during growth and cell division. Storage in the growing state carries the greatest risk, except for highly stable shoot tip and similar cultures. The slower the rate of growth, the less the risk. But the possibility of selection may be increased by the

stresses imposed on cultures in limited growth.

Growth—related phenomena are eliminated by cryopreserv tion. This eliminates the possibility of genetic change and suspends time in ephemeral systems where continued develop— ment may be undesirable, such as in protoplasts. There re— mains the slight possibility of genetic change as a result of background radiation. Any damage to the DNA would not be reversible during storage, since normal repair mechanisms would be suspended. However, investigations into the cumu— lative effect of radiation upon other biological systems (Whittingham et al 1977) indicate that storage can be en— tirely satisfactory for decades and centuries. And, there are techniques for radiation screening.

Maximum storage period. Renewal times for material stored i the growing state will be determined by the normal subcul— ture routine. Where satisfactory growth limitation has been achieved, storage periods of 1 year can be anticipated. Within the constraints discussed, theoretically there is no time limit on storage by cryopreservation.

Recovery, utilization, and exchange of cultures. Recovery from limited growth appears to be relatively easy, because cultures need only be transferred to standard growth condi— tions. Recovery from cryopreservation requires more care during the post—thaw period. There is no evidence for either method of long—term impediments to growth after storage. However, cryopreservation involves the storage of relatively small specimens which may mean that time will be required to grow adequate quantities from recovered cultures.

No serious problems are involved in the international exchange of growing cultures. The exchange of frozen culture has not been explored, but there are precedents in the area of animal husbandry. The necessary equipment and handling procedures appear to be available. Transporting frozen cul— tures would eliminate the risks related to fluctuating en— vironmental conditions and would permit transferring materials to storage at its destination without discontinuity in storage conditions.

At present, storage in the growing state has advantage particularly where the relatively short—term storage of sho tip and similar cultures is concerned, if there are no serious limitations on space or man—hours, if the number of genotypes to be handled is not excessive, and if there is a reasonably active turnover of material. But cryopreservation is more suitable for protoplast, cell, and callus cultures where it is more important to suspend all metabolism to ensure stability. As expertise in the cryopreservation of organized cultures increases, cryopreservation is likely to be the chosenlong—term storage method for shoot tips and embryos because of its advantages of lower cost and greater convenience and stability.

Research priorities

The overwhelming deficiency in relevant research is the general level of activity. It is important that appropriate studies be encouraged. Where satisfactory methods are available now, workers should be encouraged to use them rather than to waste valuable time and resources on routine culture maintenance. Developmental work should concentrate on species and culture systems of interest for application in agriculture rather than on model systems. In this work, collaboration between those requiring and those developing storage techniques should be continued in order to solve the most pressing problems.

In future germplasm storage programs, there will be a role for storage in continued, limited growth and for cryopreservation. Efforts should be made to develop storage methods that permit the exchange of material between the two as well as between storage and normal rapid growth.

In developing storage methods based on growth limitation, attention should be directed toward determining the optimum storage temperatures and the concentrations of additives for a wide range of species. Most current knowledge relates to cassava and potato, which do not represent a broad taxonomic base. New retardant chemicals may be found and there is scope for further use of storage routines based on alternating limiting and normal conditions.

Cryopreservation work is needed on all culture systems, but particular emphasis should be placed on the development of procedures for protoplasts and organized structures such as shoot tips and embryos. Protoplasts are especially important in genetic engineering programs but they lack a suitable storage method. Shoot tips and embryos often suffer massive structural damage using existing cryopreservation methods.

Most progress in cryopreservation probably will be brought about by refining pregrowth, cryoprotection, and recovery growth procedures rather than by refining the freezing and thawing stages. Efforts should be made to understand the physiological phenomena underlying pregrowth increases in freeze tolerance and in post-thaw injury and recovery.

There are sound grounds for assuming that material stored by cryopreservation will be entirely stable, but it is important that this be verified for plant tissue cultures. The possibility of exchanging plant tissue cultures in the frozen state also should be investigated.

Concluding remarks

Culture maintenance has always been a familiar but tedious part of tissue culture laboratory routines. With the new storage methods described, we may look forward to the time

when each laboratory has its own tissue culture gene—bank of slowly growing and/or frozen material to preserve new gen—otypes and to provide uniform culture stocks. This will release valuable time and facilities for more essential tasks.

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CRYOPRESERVATION OF RICE CELLS

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and Frank Sharpe, Jr.

The areas of the world with crop plant genetic diversity are important gene reservoirs for making crosses between cultivars within a specific plant species (Fig. 1). But diverse varieties are rapidly disappearing. A similar loss is occurring among species. Many species that could have future utility occur widely, including in deep jungle areas, where many have not yet been identified. But, through man's encroachments, they are vanishing from the face of the earth at an increasing rate.

These little known relatives of our food plants might offer new sources of food, new sources of genes for hybridization, or even sources of characteristics for genetic engineering. But the immediate question is: "How do we keep them?" We need to preserve as many as possible, in a practicable, stable, and economic way.

Deep-cold temperature (cryogenic) storage offers substantial promise for fulfilling this objective. Plant genotypes and their tissue often can be preserved indefinitely, although we cannot be sure in advance how long a given tissue can be stored. Nor do we understand in detail the impact of cryogenic freezing on cells or by what means harmful impacts can be avoided. We also do not know if any genetically transmissible damage takes place in the cells that survive freezing-thawing manipulations.

The remarkable finding is that any living, water-turgid tissues at all can survive freezing to the extreme temperature of liquid nitrogen, -196°C. But this has been accomplished for many species. Some tissues of cereal plants--particularly rice, maize, and wheat -- recently have been shown to survive such low temperatures. Very small changes in treatment techniques can make the difference in whether cells will or will not survive cryogenic freezing.

HISTORICAL CONCEPTS

Although cryobiology may be said to have started with the

Western Regional Research Center, ARS, U.S. Department of Agriculture, Berkeley, California, 94710, and Cell Culture and Nitrogen Fixation Laboratory, ARS, U.S. Department of Agriculture, Beltsville, Maryland, 20705, U.S.A.

Fig. 1. Origin of the world's basic food plants. Source: Wilkes (1977). Each of the world's basic food plants originated in a relatively con— fined geographic region. The regions overlap for a number of crops, but nine major and three minor centers in the Old and New World have been identified as being the areas which account for the origin and diversity of the vast majority of cultivated plants in our world. Known as Vavilov centers, after a Russian plant breeder and genet— icist, these valuable reservoirs of crop plant germplasm are now disap— pearing.

(Fig. 1. Continued)

OLD WORLD CENTERS

1. ETHIOPIA
   - Banana (endemic)
   - Barley
   - Castor bean
   - Coffee
   - Flax
   - Khat
   - Okra
   - Onion
   - Sesame
   - Sorghum
   - Wheat

2. MEDITERRANEAN
   - Asparagus
   - Beets
   - Cabbage
   - Carob
   - Chicory
   - Hops
   - Lettuce
   - Oats
   - Olive
   - Parsnip
   - Rhubarb
   - Wheat
   - Turnips
   - Wheat

3. ASIA MINOR
   - Alfalfa
   - Almond (wild)
   - Apricot (secondary)
   - Barley
   - Beets (secondary)
   - Cabbage
   - Cherry
   - Date palm
   - Carrots
   - Fig
   - Flax
   - Grapes
   - Lentils
   - Oats
   - Onions (secondary)
   - Opium poppy
   - Pea
   - Pear
   - Pistachio
   - Pomegranate
   - Rye
   - Wheat

4. CENTRAL ASIATIC (Afghanistan—Turkestan)
   - Almond
   - Apple (wild)
   - Apricot
   - Broad bean
   - Cantaloupe
   - Carrots
   - Chick pea
   - Cotton (*G. herbaceum*)
   - Flax
   - Grapes (*V. vinifera*)
   - Hemp
   - Lentils
   - Mustard
   - Onion
   - Pea
   - Pear (wild)
   - Sesame
   - Spinach

5. INDO—BURMA
   - Amaranths
   - Betel nut
   - Betel pepper
   - Chick pea
   - Cotton (*G. arboreum*)
   - Cowpea
   - Cucumber
   - Egg plant
   - Hemp
   - Jute
   - Lemon
   - Mango
   - Millets
   - Orange
   - Pepper (black)
   - Rice
   - Sugar cane (wild)
   - Taro
   - Yam

6. SIAM, MALAYA, JAVA
   - Banana
   - Betel palm
   - Breadfruit
   - Coconut
   - Ginger
   - Grapefruit
   - Sugar cane (wild)
   - Tung
   - Yam

7. CHINA
   - Adzuki bean
   - Apricot
   - Buckwheat
   - Chinese cabbage
   - Cowpea (secondary)
   - Kaoliang (sorghum)
   - Millets
   - Oats (secondary)
   - Orange (secondary)
   - Paper mulberry
   - Peach
   - Radish
   - Rhubarb
   - Soybean
   - Sugar cane (endemic)
   - Tea

(Fig. 1. Continued)

NEW WORLD CENTERS

8. MEXICO—GUATEMALA
- Amaranths
- Bean (P. vulgaris)
- Bean (P. multiflorus)
- Bean (P. lunatus)
- Bean (P. acutifolius)
- Corn
- Cacao
- Cashew
- Cotton (G. hirsutum)
- Guava
- Papaya
- Pepper (red)
- Sapodilla
- Sisal
- Squash
- Sweet potato
- Tobacco (N. rustica)
- Tomato

9. PERU-ECUADOR-BOLIVIA
- Bean (P. vulgaris)
- Bean (P. lunatus)
- Cacao
- Corn (secondary)
- Cotton
- Edible roots (oca, ullucu, arracacha, añu)
- Guava
- Papaya
- Pepper (red)
- Potato (many species)
- Quinine
- Quinoa
- Squash (C. maxima)
- Tobacco (N. tabacum)
- Tomato

Minor Centers

10. SOUTHERN CHILE
- Potato
- Strawberry (Chilean

11. BRAZIL—PARAGUAY
- Brazil nut
- Cacao (secondary
- Cashew
- Cassava
- Maté
- Pararubber
- Peanut
- Pineapple

12. UNITED STATES
- Sunflower
- Blueberry
- Cranberry
- Jerusalem artichoke

complex freeze hardening process of cold climate plants in nature, Maximow (1912) carried out the first significant cryogenic manipulations. He showed that an extract from cold—hardened red cabbage leaves enabled cells of tropical Tradescantia discolor leaves to survive several degrees of freezing. He showed similar effects on plant tissue to which he had added solutions of sugars and other compounds. More recently, Polge et al (1949) found glycerol to be an excel—lent cryoprotective agent for sperm cells. Lovelock and Bishop (1959) discovered the remarkable cryoprotective prop—erties of dimethylsulfoxide (DMSO) on red blood cells.

We are not sure about the manner in which these com—pounds operate, but they are cell—penetrating compounds that probably mitigate freeze damage by affecting the in—ternal structure and physical—chemical makeup of the cell. Other cryoprotective compounds -- sucrose and polymers such as polyethylene glycol -- do not penetrate well. Such com—pounds exert an osmotic dehydrating effect. The removal of water shrinks the cells and diminishes freeze damage. But this shrinkage (plasmolysis) may itself be harmful to some cells (Fig. 2). When sugarcane cells were treated with a cryoprotective and plasmolyzing solution of glucose and DMSO which was later washed out, a large proportion of the cells

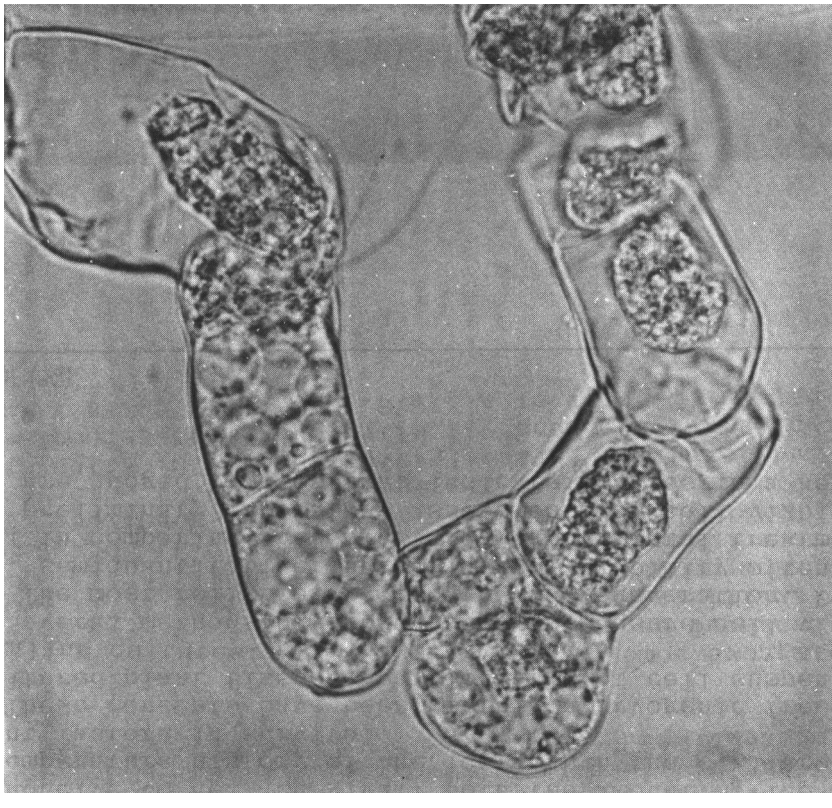


Fig. 2. Photomicrograph of unfrozen sugarcane cells exposed to glucose—DMSO (8% w/v—12%) at near 0°C, then washed with simplified nutrient solution. Source: Finkle and Ulrich (1979).

placed in an isotonic solution displayed permanent plasmol—ysis, or death (Finkle and Ulrich 1979).

Two other manipulations that can have large effects on cell survival are the rates of cooling and thawing. Figure 3 (Mazur 1965) shows that the rate of cooling affects the size (water content) of yeast cells. Given a slow rate of freezing of about 1°C/min, water migrates outward and fre—ezes outside the cell. Less water in the cell means that less intracellular ice is formed, which appears to minimize cell damage. Half of the small yeast cells survived when frozen at a relatively slow rate (about 5°C/min). Such a rate can realistically be achieved for multicellular plant tissues.

Figure 4 shows the effects of the rate of rewarming on survival of frozen sections of mulberry twig (Sakai and Yoshida 1967). During thawing, a minimum time in the actively—recrystallizing temperature zone of −30° to —40°C

is conducive to cell survival. Both the amount of ice and the temperature history of the ice within the cell are im—portant factors in survival.

These concepts have been useful in cryogenic freezing of cultured plant tissues, particularly of cell suspensions and callus cultures.

Several methods have been used to measure cell viabil—ity. The most reliable are growth and regeneration. But rapid, semiquantitative indications of viability often are useful in comparing the effects of experimental treatments or as preliminary laboratory assessments of cell viability. These are judged by dyes that indicate changes in a key

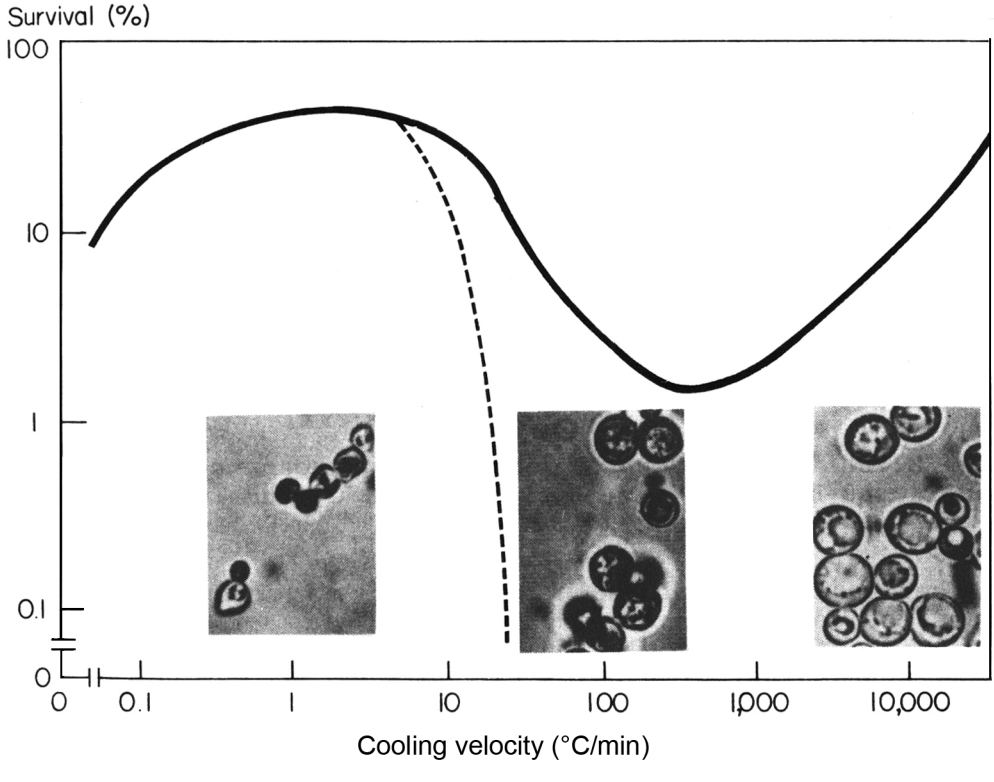


Fig. 3. Survival and morphology of yeast cells (Saccharomyces cerevisiae) suspended in distilled water, cooled at varying rates to −30°C or below, and rewarmed either rapidly(———)or slowly(— — — —)Left and center micrographs are of cells substi tuted with cold ethanol after slow and rapid freezing. Right micrograph is of untreated cells. (Ultra—rapidly cooled yeast appear morphologically normal when examined by electron mi—croscope.) Source: Mazur (1965).

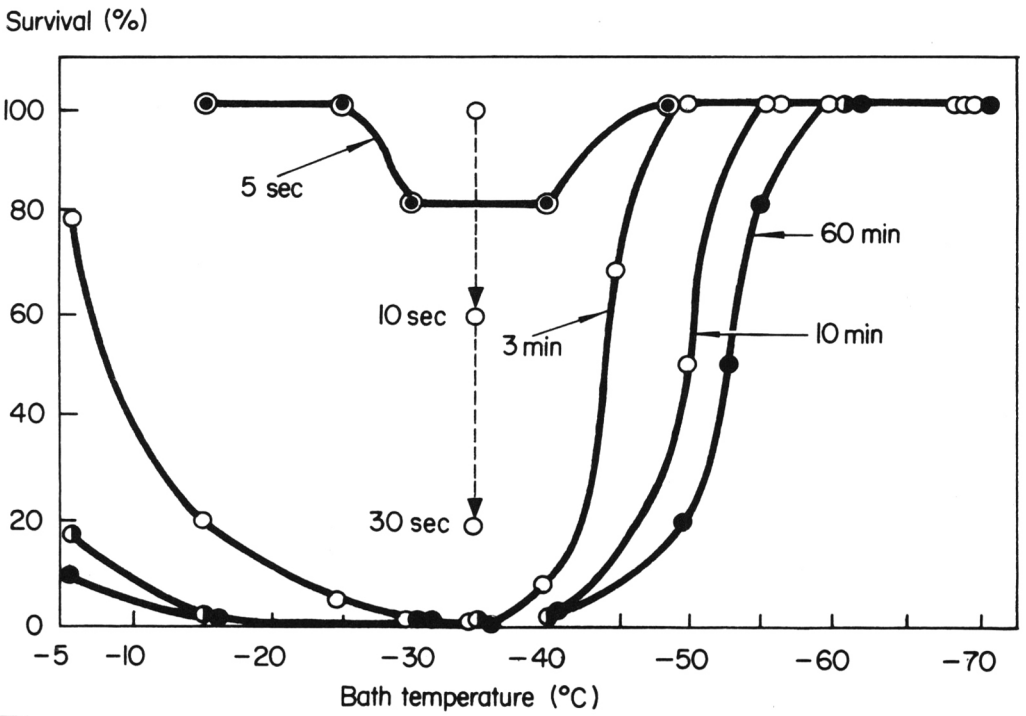


Fig. 4. Survival of prefrozen cells kept at different temperatures for different periods after removal from liquid nitrogen. Tissue sections prefrozen at —10°C for 5 minutes were immersed in liquid nitrogen. After removal from liquid nitrogen, cells were kept for 3 seconds and 3, 10, and 60 minutes in isopentane baths maintained at varying temperatures. They were rewarmed rapidly in 30°C water. Source: Sakai and Yoshida (1967).

metabolic system by color. One example is a decrease in the enzymatic reduction of triphenyltetrazolium chloride (TTC) to the extractable red formazan dye (Withers 1980b). Changes in membrane integrity modify the admittance of indicator dyes such as fluorescein diacetate or Evans blue into the cell (Withers 1980b).

The values given by these indicators are often inexact or subjective and depend on culture conditions and other factors (Finkle and Ulrich 1979, Sala et al 1979, Withers 1980b). The methods usually measure only one phase of a cell's maintenance system. These auxilliary methods are useful but their quantitative results often are inconsistent in absolute values of control data from experiment to experiment (Finkle and Ulrich 1979). Growth capability can be difficult to interpret, even within the same culture line (Sala et a1 1979).

DEVELOPMENT AND ASSESSMENT OF CRYOGENIC METHODOLOGY

The success of methods to achieve survival of frozen cells depends on the type of plant material used. A slow-freezing methodology (cooling rates of up to a few degrees per minute) based on the earlier studies of Sakai and Yoshida (1967) was useful for carrot suspensions (Nag and Street 1975), rice suspensions (Sala et al 1979), and callus. On the other hand, Bajaj (1977a) and Grout and Henshaw (1978) with potato shoot tips, Seibert and Wetherbee (1977) with carnation apices, Bajaj (1977b) with cassava buds, and Tisserat, Ulrich, and Finkle (U.S. Department of Agriculture, pers. comm.) with date palm pollen attained survival by an ultrarapid plunge of the tissue into liquid nitrogen. It may be significant that this group primarily consisted of compact organized tissues for which it appears that very rapid freezing may give greater survival than slow freezing.

A set of procedures we have found useful in recent studies, including rice experiments, is shown in Figure 5.

Cryoprotective mixture

We used a three—component mixture of cryoprotective agents--polyethylene glycol (P), glucose (G), and dimethylsulfoxide (D) -- in the proportions shown. Without cryoprotectant, sugarcane cells did not survive even moderate freezing (Fig. 6, Curve A). The added cryoprotective compounds can foster a considerably increased survival of freezing at low temperatures (Fig. 6, curve B). But, many useful cryoprotectants (added, then washed out before the cell assays) are toxic to the cells (evaluated at 0°C in Fig. 6) and are increasingly toxic with increasing concentrations (Fig. 6, curves C and D) (Finkle and Ulrich 1979). The specifics of this toxic effect are not well defined. The cryoprotective compounds

PROCEDURE

Cryoprotective additions:
PEG-Glucose-DMSO (P-G-D, 10—8—10% w/v)
added to cells

Freezing:
3°C/min to -4°C
seeding at -4°C
1°C/min to -30°C
Then into L.N. (—-196°C)

Thawing:
Swirl in +40°C bath
Wash with 3% sucrose medium at R.T.

Determining survival:
Transfer to growth medium:
Or, add triphenyltetrazolium chloride (TTC)
to determine Viability Index
(530 nm absorbance)

Fig. 5. Procedure for cryogenic treatment and evaluation of survival.

tested were a mixture of G and D. The concentrations of the compounds indicated are B: 4—6%w/v, G: 8—12%, and D:12-18%. The highest concentration (Curve D), 3.0 M total, shows the greatest toxicity and goes beyond the optimum concentration for effective cryoprotection under these circumstances.

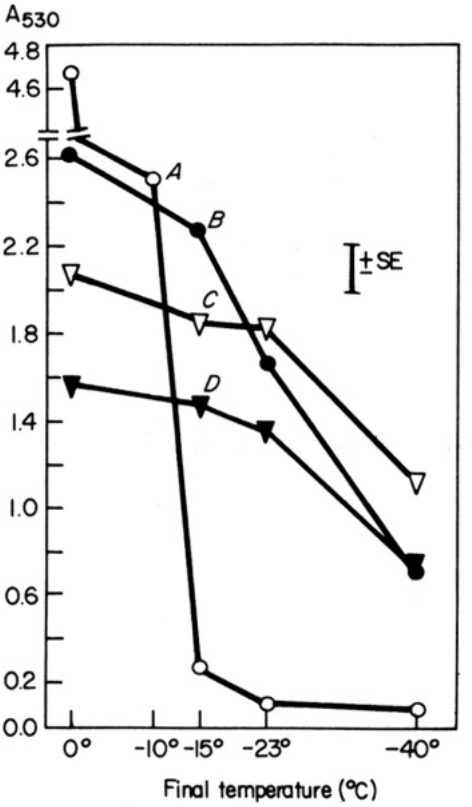


Fig. 6. Effects of cryoprotectant concentration on sugarcane cell survival in freezing. Curve A, no additions; Curve B, 4%—6% glucose—DMSO; Curve C, 8%—12%; Curve D, 12—18%. Source: Finkle and Ulrich (1979).

A mixture of two cryoprotective compounds can be more effective than any one alone (Fig. 7). There is an optimum

ratio of about 8% w/v in G and 10% in D, with a molar ratio in G:D of about 1:3 (Finkle and Ulrich 1979).

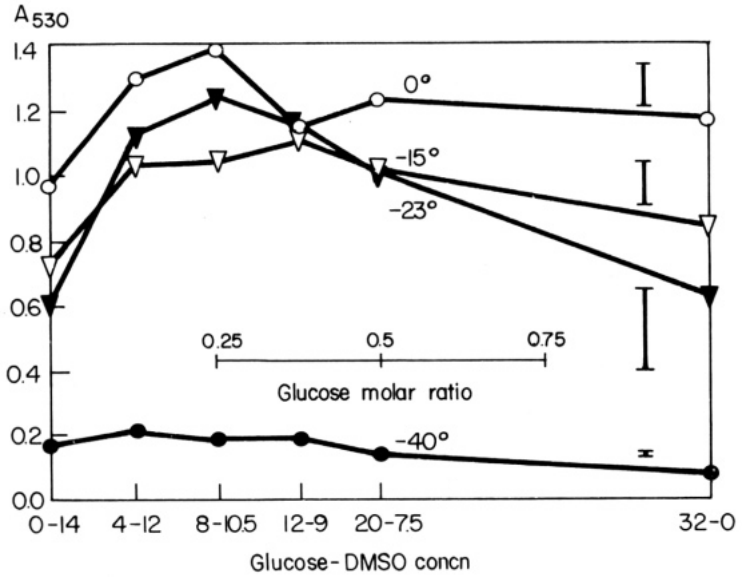
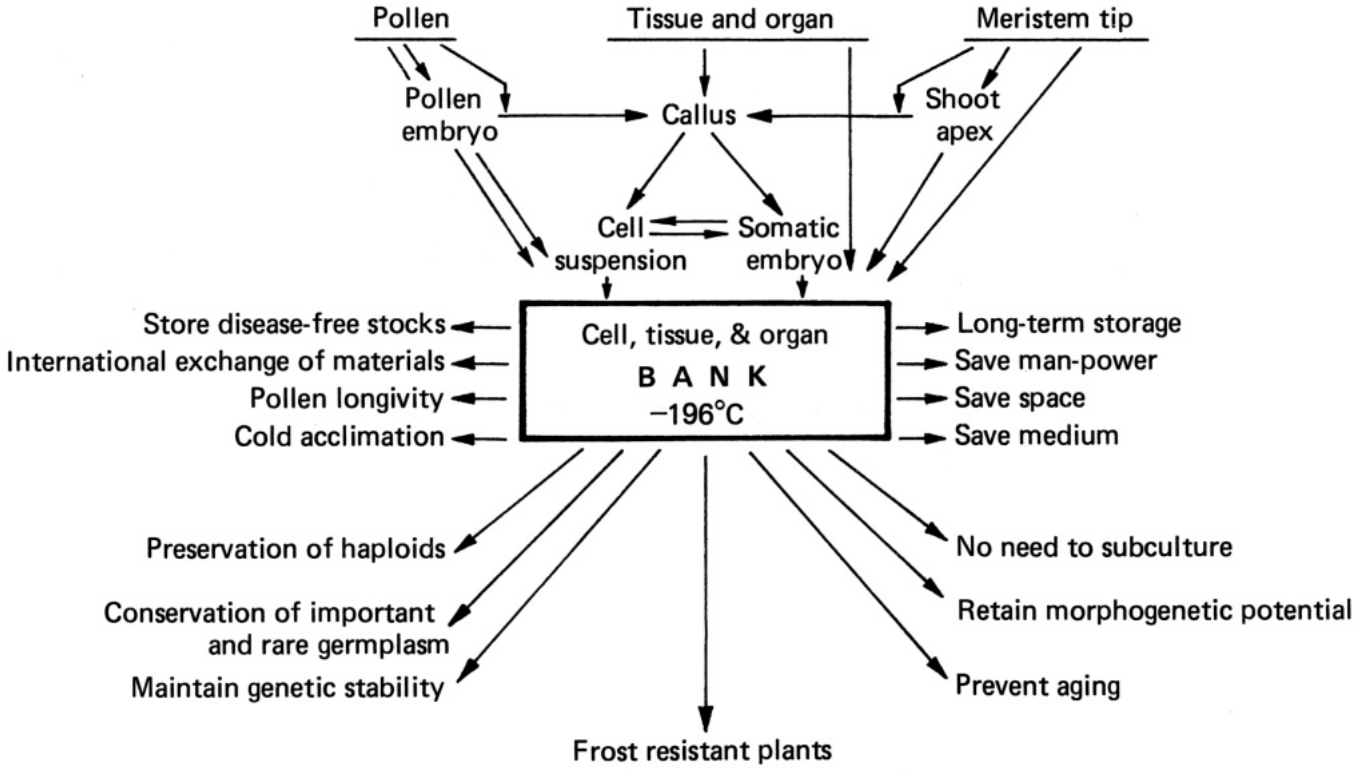


Fig. 7. Effects on TTC value of varying glucose—DMSO ratios. Total sample concentration of glucose + DMSO = 1.9 M. Com— position of the mixtures (percent of each component in the solution, w/v) and molar ratios of the cryoprotectants are indicated on the abscissa. Source: Finkle and Ulrich (1979).

A third component, polyethylene glycol (P), contributes an additional benefit (Table 1). But P, when added alone, is neither cryoprotective nor toxic (Ulrich et a1 1979). The addition of P may diminish the harmful effects of the other two compounds on the freeze—stressed cell. This combination of three compounds was used in subsequent freezing experi— ments.

Protoplasts and suspension cultures, callus cultures, growing tips and meristem cultures, and anther cultures have been studied. Many of these tissues have particular advantages in terms of genetic preservation or laboratory feasibility (Fig. 8). The liquid nitrogen preservation of anthers and microspores (Bajaj 1980, Coulibaly and Demarly 1978) and pollen (Barnabas and Rajki 1978) of cereal plants offer prom— ise. But we will discuss primarily suspension and callus culture preservation by low temperature freezing, with em—



FREEZE PRESERVATION AND ESTABLISHMENT OF GERMPLASM BANKS

Fig. 8. Diagrammatic representation showing the freeze storage of plant cells, tissues, and organs in liquid nitrogen at −196°C. Prospects for the establishment of germplasm banks. Source: Bajaj (1977a).

phasis on rice and other cereals.

Table 1. Viability values (A_{530}) after cryoprotective treatment of sugarcane cell suspensions.

Treatment temperature	Viability values (A_{530})		
	No cryoprotective compound	GD[a]	PGD[b]
0°C	3.10	1.78	1.95
	2.01	0.64	0.98
	3.39	–	–
	3.67	1.86	2.32
—34°C	0.02—0.1	0.40	0.69
		0.27	0.32
		0.47	0.76
		0.52	1.13

[a] 8% glucose, 10% DMSO. [b] 10% polyethylene glycol, 8% G, 10% D. (—) = no treatment.

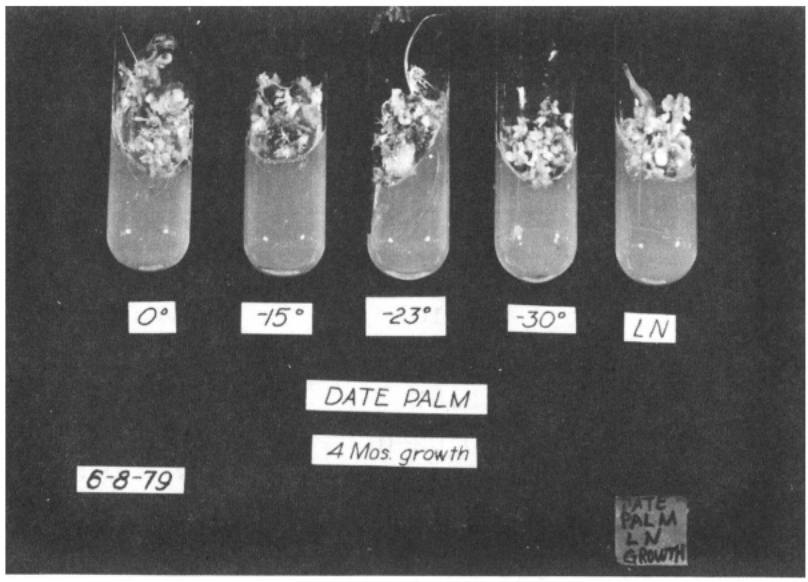
Table 2. Plant species in which callus cultures have survived freezing.

Frozen to -196°C	Frozen to other temperatures	
Alfalfa (3 lines)		
Apple	Elm	-30°C
Asparagus	Grape	-15°C
Carrot[a]	Grapefruit	-15°C
Palm (2 lines)	Phaseolus	-15°C
Rice (8 lines)	Tomato	-30°C
Soybean		
Strawberry[a]		
Sugarcane		
Wheat		

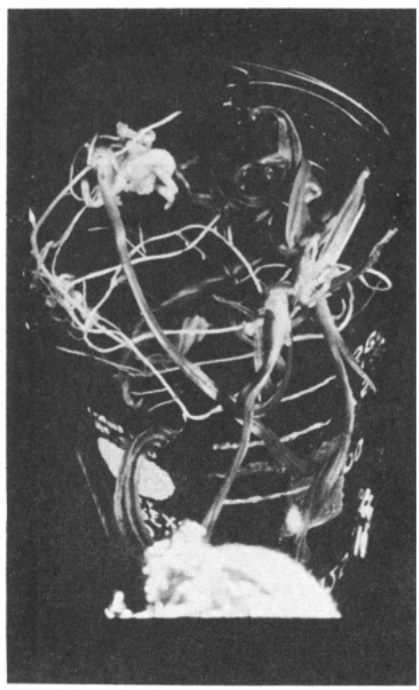
[a] Carrot suspension culture, strawberry meristem.

Freezing capabilities

Only a few dozen plant species so far have been demonstrated to be amenable to frozen storage in liquid nitrogen (Withers 1980a). However, these include examples of almost a third of the two dozen principal crop species of the world (Stapley and Gayner 1969). Using the method described here, liquid nitrogen freezing at —196°C was attempted with about 20 plant species, 10 of which survived. Others have survived intermediate freezing temperatures (Table 2). Callus cultures



A



B

Fig. 9. Comparison of 4 months growth of unfrozen date p callus (0°C) and callus rapidly thawed after slow freezing to -15°C, -23°C, -30°C, and -196°C. Source: Tisserat et a (1981).

of taro and redwood did not survive even —15°C.

The capability of cryogenic experimenters to freeze plant tissues at —196°C with recovery indicates a favorable prognosis of cryogenic storage of other species. Through increased understanding of and improvements in present methodology, it seems likely that many temperaturesensitive species will become amenable to ultralow temperature storage.

Many deep—frozen tissues have been regenerated into whole plants (Withers 1980a). We have grown alfalfa (Ulrich et al 1981a) and date palm (Tisserat et al 1981) callus cultures into plants after storage for several months at —196°C (Fig. 9). These plants now have progressed beyond the plantlet stage and are growing in soil. Tissue survival after freezing of tropical and desert—zone plants such as sugarcane (Ulrich et al 1979) and date palm holds promise for the possibility of cryogenic storage of many other agriculturally important warm climate plants.

Table 3. Relative position (R_p values) of isozymes from leaves of Phoenix dactylifera L. cultivars or specimens.

Enzyme	R_p values						
	Deglet Noor[a]	Khadrawy[a]	Dayri L7, BC_3[a)]	Medjool Field[a]	Medjool -C[b)]	Medjool +C[c)]	Medjool LN[d)]
			50				
			53				
Alcohol dehydrogenase	56	56	56	56	56	56	56
Esterase	77	77	77	77	77	77	77
	86	86	86				
Peroxidase	50	50	50	50	50	50	50
Phosphoglucose isomerase	7	7		7	7	7	7
	12	12		12	12	12	12
	20	20	20	20	20	20	20
	67	67	67	67	67	67	67
Phosphoglucose mutase	70	70	70	70	70	70	70
	73		73				

[a)] Mature leaf samples from verified cultivars grown in the field. [b)] Specimen leaf sample from untreated Medjool cultures; C = cryoprotectant. [c)] Specimen leaf sample from cryoprotectant—treated Medjool cultures. [d)] Specimen leaf sample from cryoprotectant—treated Medjool cultures frozen to —196 C.

Testing for genetic change

Isozyme patterns of frozen date palm tissue were examined to detect whether changes in genetic makeup, such as mutations that affect protein biosynthesis, may have occurred (Table 3). Extracts from the leaves of variety Medjool plantlets regenerated from callus frozen to —196°Cwere subjected to gel electrophoresis. The pattern of isozyme proteins in the extracts from regenerated plantlets showed no difference from unfrozen plants (Ulrich et a1 1981b). This indicates that no genetic modifications affecting the biosynthesis of these proteins appeared during freezing to —196°Cand emphasizes the potential value of lowtemperature freezing for the stable storage of reliable genotypes.

FREEZING RICE LINES

Recent studies on freezing compared a number of lines of rice (Oryza sativa) (Schaeffer and Sharpe 1981). The closely related lines are mutants selected from cells of Line BL-2 that survived culturing in a medium containing S-aminoethyl—L—cysteine, a competitive analogue of lysine. The comparisons measured differences in the responses of these close genotype to cryogenic treatments.

Methods

Callus taken 1—2weeks after the last transfer onto an agar medium was treated in a small petri dish in a sterile hood. After a batch treatment with PGD, callus tissue was subdivide and placed in glass tubes containing 1 ml of 10:8:10 PGD (1. M total combined concentration). The tubes were capped and frozen at a temperature drop rate of 1°C per minute. Samples were thawed at preselected temperatures. When -30°C was attained, designated tubes were plunged into liquid nitrogen for 4 minutes. Frozen samples were rapidly thawed by swirling in a 40°C bath just to the melting of ice. At this point samples containing PGD were washed with a simplified nutrient medium (Finkle and Ulrich 1979). The wash solution was maintained either at room temperature (22°C) or at ice temperature (0°C). The treated callus samples were transferred as duplicate standard portions onto nutrient agar plastes and incubated in an illuminated chamber at 28°C.

Results

The growth responses of closely related lines of rice callus varied with three different aspects of the cryogenic treatments (Table 4). Eight rice lines were examined, only six of which are shown.

PGD addition and removal. Rice lines showed large differ—

Table 4. Growth of rice lines after freezing treatment.

Line	Age (wk)	Fresh weight				
		Control (0°)[a] (mg)	+PGD (0°)[b] (%)	+PGD (—23°) (%)	+PGD (—30°) (%)	+PGD (—196°) (%)
BL—2	10	664	84.8	45.2	35.1	15.2
A—4	4	571	108.8	92.5	59.0	60.1
A—5	8	938	98.7			39.1
A—7	9	804	44.9			25.7
A—13	10	1249	87.4	46.8	15.1	15.6
A—15	8	1121	74.0	23.3	8.6	7.1

[a] All lines had a 100% fresh weight. [b] PGD (polyethylene glycol [10% w/v] —glucose [8%] —DMSO [10%] was added at 0°C and washing out was at 22°C.

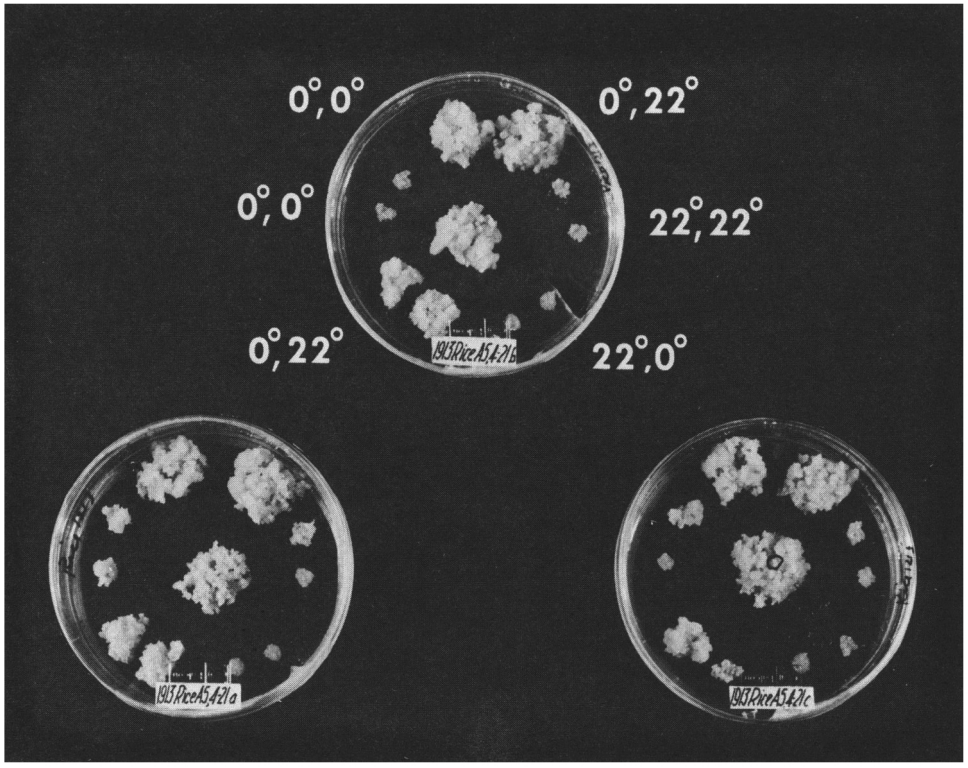


Fig. 10.Rice A — 5 calli after 7 weeks of growth on agar medium. Tempera— tures of addition and removal of PGD are labeled at six positions around the top petri dish only. Sample at the center of each petri dish is an untreated, unfrozen control. PGD in the treated but anfrozen calli (top position on dish) was added at 0°C (11 o'clock position) or 22OC (1 o'clock position). The remaining 4 samples, in duplicate, were frozen to

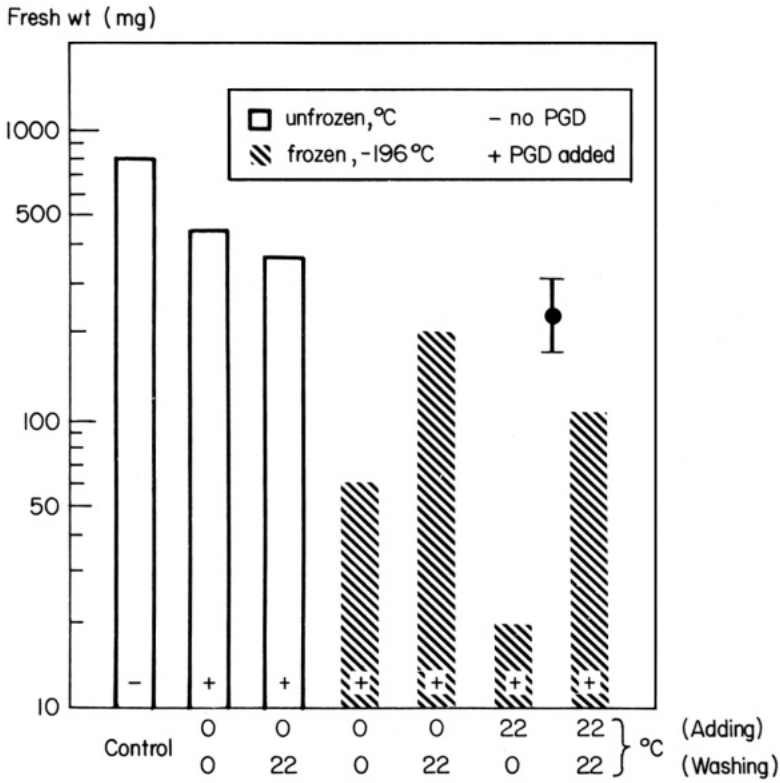


Fig. 11. Histogram of weight grain of unfrozen and frozen (-196°C) rice A-7 calli after 9 weeks of culture on agar. Final fresh weight (mg) is graphed on a logarithmic scale. Treatments (the same as in Fig. 11) are shown at the base of each bar. The top line of the abscissa designates the adding temperature and the bottom line the washing temperature. Analysis of variance error bar shows a 95% confidence interval.

ences in response to treatment with ice—cold PGD followed by washing at room temperature. Lines A-4 and A-5 (and A-11) not shown) were insensitive to PGD. Growth of BL—2,A-7, A-13, and A-15 was diminished by the transient addition of the cryoprotective mixture. Poor growth after exposure to PGD probably was caused by irreversible damage of a portion of the cells exposed to the cryoprotectants, as has occurred with sugarcane.

Freezing and thawing. Freezing and thawing produced more cell death in all cases; the colder the frozen temperature, the more severe. But beyond -30°C, little additional cell damage was often seen. Metabolically, -196°C appears to be little different from -30°C. The major challenge is for cell to reach -30°C relatively unharmed.

There were large differences in response among the rice lines. Lines A-4, A-5, and A-7 (and A-1, not shown) demonstrated good survival in -196°C temperature (25-60% of control weights). Other lines demonstrated much less. These effects were found in repeated experiments.

Adding and washing temperatures. The temperatures at which PGD was added or washed out had profound effects on survival (Fig. 10). Adding PGD (compared to not adding PGD) had little effect on Line A-5 callus. The temperature used in washing out PGD (0°/0°vs 0°/22° treatments) also had little or no effect. Recovery after freezing to —196°C depended on the washing—out temperature. Adding PGD at ice temperature followed by washing out at room temperature was the only combination in which active growth of Line A—5 callus occurred after freezing to —196°C. The 0°C addition, 22°C washing temperature regime made the difference between survival and nonsurvival of calli frozen to —196°C.

Adding and removing PGD itself was detrimental to the A—7 line (Table 4, Fig. 11). Here again, the highest survival of callus occurred in the 0°C addition, 22°C washing regime. The reverse combination, 22°C addition, 0°C washing, resulted in no survival. In the usual practice of cryogenic freezing (Sakai and Yoshida 1967, Nag and Street 1975, Sala et al 1979), cold temperature is used both for adding and washing out the cryoprotectant (0°C/0°C treatment). In our rice experiments, this temperature combination gave poor survival rates compared with 0°C/22°C treatments.

The explanation for this large effect of a small change in washing temperature may lie in whether the lipids of the membranes are in a fluid state above the melting temperature or in a semirigid state below this critical temperature. The transition state of membrane lipids is in the range of 10—12°C for many plant cells (Lyons et al 1979). This range is between the two washing temperatures we used. The relative degree of membrane rigidity could be a key factor in surviva explaining the different results we observed from the os-

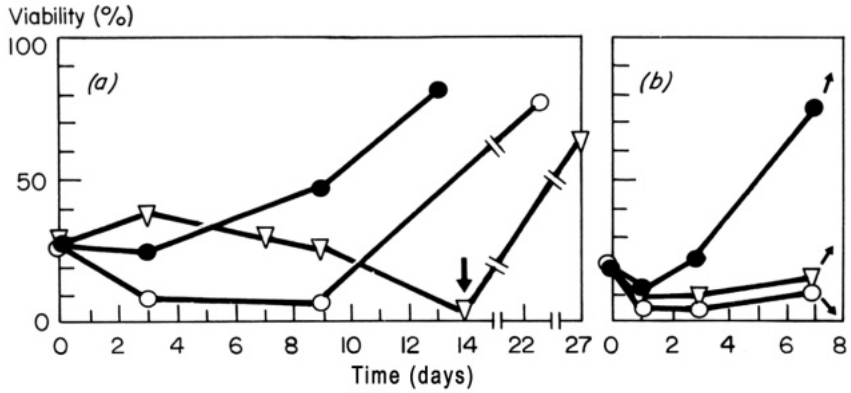


Fig. 12. Recovery growth in cells of Z. mays cryoprotected with 10% proline in culture medium, frozen stepwise, and thawed rapidly: a) Increase in viability (FDA staining) of cells returned to culture by layering over semisolid medium (o), in liquid medium (o), or in droplet culture (o). Arrow indicates the addition of fresh medium to droplets. b) Increase in viability of cells returned to culture by layering over semisolid medium and left in contact with original suspending medium (o), cells layered over semisolid medium but with the original suspending medium drained away (o), cells washed in water before layering over semisolid medium (o). Arrows indicate eventual recovery or death. Source: Withers (1980c).

motically disruptive washing out of cryoprotectants from frozen cells (Figs. 11 and 12). The relevance of the addition/washing temperature phenomenon is that the use of a higher washing temperature can be one critical factor enhancing survival of plant cells frozen for storage.

Suspension cultures

Sala et al (1979) succeeded in reviving a large fraction of rice cells suspended in 5% DMSO and slowly frozen to —196°C. Viability of the cells was estimated by TTC reduction and by growth. The multiplication of cells in the cultures was rapid after a 2—8day lag period, probably representing a relatively low rate of cell kill from the freezing.

FREEZING OTHER CEREAL SPECIES

Maize (Zea mays) and sorghum (Sorghum bicolor) have been reported to survive freezing to -196°C. In recent experiments we observed growth of wheat (triticum vulgare) callus after

liquid nitrogen storage.

Withers et al (1980a,c) described modifications of the growth medium and the cryoprotective compounds that were beneficial to freezing survival of Zea and Sorghum cultures. Proline in 10% solution added to suspension cultures a few days before slow—rate freezing brought about an improvement in recovery from freezing in liquid nitrogen. And, as with rice cultures, recovery was improved through post thawing manipulation. Large differences in recovery of Zea cells were promoted by manipulations of the recovery medium (Fig. 12a) that included the degree to which the culture medium was removed from recultured cells (Fig. 13b). The medium in which the cells were grown may have contained a factor in relation to the amount of medium present that was beneficial to cell recovery. Washing the cells seemed to be inadvisable.

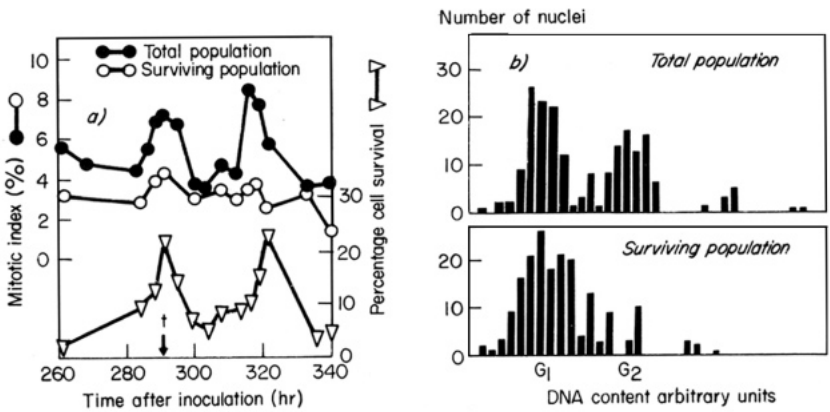


Fig. 13. Examination of the relationship between cell cycle stage and capacity to survive freezing and thawing (synchr—onized cell suspension of Acer pseudoplatanus). Cells were treated with 10% v/v glycerol and 5% v/v DMSO, frozen to -100°C at a rate of 1°C/min, stored in liquid nitrogen, and thawed at +40°C in a water bath. Viability was estimated by fluorescein diacetate staining. Dead cells were identified by staining with Evan's Blue. Subsequent counter staining with Feulgen reagent permitted cytological examination of surviving cells. a) Oscillations in mitotic index and cell survival over time, including fourth and fifth synchronous divisions. b) Nuclear DNA profiles of cells sampled at time 't' as indicated in a. The total population shows a clear separation of premitotic (G_2) nuclei and postmitotic (G_1) nuclei. Source Withers (1980b).

CONCLUSIONS

The main impact of the findings on cereal cultures is that rice, maize, and wheat tissues have been frozen to -196°C with survival of living, growing cells. The original, un—frozen callus tissues of rice and maize were not capable of differentiation, so no differentiation of frozen tissues was attempted. But when regeneratable types of cultured plant tissues have been found to survive the freezing step, re—generation has often followed, as was demonstrated in alfalfa and date palm. A total of 36 species of plants have survived freezing to -196°C, the temperature of liquid nitrogen (Withers 1980a). Other species that can survive this temper—ature will be found. The -196°C temperature is a storage condition under which living materials can be maintained with little change indefinitely (Ashwood—Smith and Friedman 1979, Whittingham et al 1977).

But a key question about survival of cryogenic freezing by tissue is: What degree of success can be attained with those species for which freezing has been reported to be not successful or has not been tried? A crude indication of the possibilities for survival might be drawn from our experi—ence with plant tissue survival of liquid nitrogen freezing. About half of the approximately 20 species tested have survived (Table 1). Two of the surviving species (sugarcane and date palm) are of tropical or desert origin.

Improved techniques can be expected to contribute further to survival. As an example, some mutant lines of rice appeared far more sensitive to freezing than other mutant lines, even though they are closely related genetical—ly. We have indicated where small changes in treatment made large improvements in the survival rate of the more freeze—sensitive lines. Other species of plants that are freeze sensitive might respond similarly; their sensitive factor could be overcome by some small shift in procedure. A period of growth on a medium containing proline improved cell survival (Withers 1980c). Similar small manipulations might improve survival in species that so far have not sur—vived cryogenic freezing. Further incremental improvements in tissue manipulations will continue to improve survival.

But such empirical improvements may not solve the larger problem of achieving the near—universal cryogenic storage of innumerable species that will be required to meet many of the world's needs. Much more attention will need to be paid to understanding the effects of structural and metabolic differences among plants and the influence of the growth stage that make some plant tissues more freeze sensitive than others. The importance of cell growth stage was underscored by observations made on synchronously grow—ing cultures of Acer pseudoplatanus (Withers 1980b). Cell survival of freezing depended on the stage of the cells in the mitotic cycle (Fig. 13). The small post—mitotic cells

in the G_1 phase of the cell cycle displayed a significant survival advantage.

Another area in which understanding is needed is the effect of cryoprotective agents themselves -- how they prevent injury from the freezing step, how they cause a degree of chemical and osmotic injury, and how, when used in synergistic mixtures and under appropriate conditions of treatment, they can further improve cell survival. Zavala et a1 (1981) have begun a study of how P,G, and D, added alone and in combination, affect the ultrastructure of sugarcane cells before and after freezing. Electron microscope studies hold promise as a concrete approach to visualizing experimental changes in organelle, membrane, and intercellular structures. Studies of this type could make a major contribution to understanding how cells react to temperature stress, how cryoprotectants benefit the freezing cell, and how the cryoprotective compounds interact.

Still another area of needed study is the all—but—neglected investigation of healing mechanisms and the use of postthawing aids to repair. There are indications that repair processes that lead to cell recovery are at work. Cella et a1 (1978) observed that attempts to prepare protoplasts (cells without walls) from just—thawed frozen rice cell suspensions gave poor yields. But within 4 days under growth conditions, the thawed cells produced protoplasts equivalent in yield to unfrozen control cells, presumably through a strengthening repair of the plasma membranes. Using a postthaw washing solution at room temperature on these thawed rice cells might have prevented the membrane structural damage that had to be repaired during the early postthaw steps in the cells' struggle for survival. A possible method for avoiding damage eliminates the post—thaw washing often used to remove cryoprotective solutions from frozen cells (Withers 1980a).

Electron microscope studies on frozen Zea mays (Withers 1980b) and other plant cells (Withers 1978, Zavala et al 1981) have located ultrastructural sites of cell damage. Details in the appearance of those cells capable of healing are beginning to be identified. This type of information eventually will contribute important insights into the mechanism of cell repair.

Improved understanding of freeze injury and its counteraction by chemical agents, of culture practices that will bring cells to the optimal growth stage to withstand freezing,and of means to promote healing of damaged cells should improve our ability to determine the optimal conditions for cryogenic freezing of great numbers of untried lines of cereals and other plant species. With continuing improvement of methods, we can begin to think realistically about the possibility of frozen tissue banks for long—range germplasm storage. Such banks would permit safe storage of large numbers of acquisitions of identified

cultivars in compact, economical facilities in many sites. Successful cryogenic methods also would allow the secure storage of rare plant clones or mutants for breeding or experimental purposes and the introduction of certified clonal material across national borders with a minimum of quarantine impediments to international exchange and research cooperation.

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PROTOPLAST CULTURE AND FUSION

O. Schieder

Development of viable seeds in most interspecific or intergeneric crosses has been prevented because of specific inhibition or elimination of key steps in pollination, pollen tube growth, fertilization, and embryo or endosperm development. But now, techniques for isolation, culture, and plant regeneration from protoplasts as well as methods that allow the fusion of protoplasts from genetically different lines or species can be used as new tools in studies on the genetic manipulation of important crop plants (Keller and Melchers 1973, Kao and Michayluk 1974, Vasil and Vasil 1980a). Major aspects of protoplast fusion are:

- production of fertile amphidiploid somatic hybrids of sexually incompatible species.
- production of heterozygous lines within a plant species which normally would be propagated only vegetatively (potato).
- Transfer of only part of the nuclear genetic information from one species to another using chromosome elimination.
- Transfer of cytoplasmic genetic information from one line or species to another.

REGENERATION OF PROTOPLASTS

To use protoplasts in genetic manipulation, they must be cultured to form colonies and subsequent plants. Protoplast regeneration has been studied recently on several plant species from different genera. Protoplasts can be regenerated to plants from members of the genera Nicotiana, Solanum, Datura, Petunia, Hyoscyamus, and Atropa in the Solanaceae family (Vasil and Vasil 1980b). Plants also have been raised from protoplasts of such species as Daucus carota (Grambow et al 1972), Asparagus officinalis (Bui—Dang—Haand Mackenzie 1973), Brassica napus (Kartha et al 1974, Thomas et al 1976), Ranunculus sceleratus (Dorion et al 1975), and Citrus sinensis (Vardi 1978). Of these, the most important is Solanum tuberosum, from which dihaploid (Binding et al 1978) and tetraploid protoplasts (Shepard and Totten 1977) can be regenerated to plants.

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Table 1. Somatic hybrids produced by the use of albino mutants.

Species fused	Regeneration	References
Nicotiana tabacum + *Nicotiana tabacum*	Plants	Melchers and Labib (1974) Gleba et al (1975)
Nicotiana tabacum + *Nicotiana sylvestris*	Plants	Melchers (1977a)
Nicotiana tabacum + *Nicotiana rustica*	Plants	Douglas et al (1981a)
Nicotiana tabacum + *Nicotiana glauca*	Plants	Evans et al (1980)
Nicotiana tabacum + *Nicotiana knightiana*	Plants	Maliga et al (1978)
Nicotiana tabacum + *Nicotiana rustica*	Plants	Nagao (1978), Iwai et a1 (1980)
Nicotiana tabacum + *Nicotiana glutinosa*	Plants	Nagao (1979)
Nicotiana tabacum + *Nicotiana alata*	Plants	Nagao (1979)
Petunia hybrida + *Petunia parodii*	Plants	Cocking et al (1977)
Petunia inflata + *Petunia parodii*	Plants	Power et al (1979), Cocking(1978)
Petunia parviflora + *Petunia parodii*	Plants	Power et al (1980)
Datura innoxia + *Datura innoxia*	Plants	Schieder (1978a)
Datura innoxia + *Datura stramonium*	Plants	Schieder (1978a)
Datura innoxia + *Datura discolor*	Plants	Schieder (1978a)
Datura innoxia + *Datura sanguinea*	Shoots	Schieder (1980a)
Datura innoxia + *Datura candida*	Plants	Schieder (1980a)
Datura innoxia + *Atropa belladona*	Shoots	Krumbiegel and Schieder (1979)
Lycopersicon esculentum + *Solanum tuberosum*	Plants	Melchers et al (1978)
Daucus carota + *Daucus capillifolius*	Plants	Dudits et al (1977)
Daucus carota + *Aegopodium podagraria*	Plantlets	Dudits et al (1979)
Daucus carota + *Petrosilium hortense*	Plantlets	Dudits et al (1980)

Binding and Nehls (1980) and Binding et al (1981) extended protoplast regeneration experiments to several species of different families. They succeeded in getting plants from protoplasts of some Compositae members and from two species of Cichoriaceae. Remarkable progress also has been made on two legumes and one gramineae species.

With embryogenic suspension cultures derived from immature embryos of Pennisetum americanum, two types of protoplasts were obtained -- large, vacuolated protoplasts that never underwent division and small, nonvacuolated protoplasts derived from embryogenic cell masses. Under appropriate conditions, the larger vacuolated protoplasts divide and produce plantlets via somatic embryogenesis (Vasil and Vasil 1980b). Embryoids obtained from the mesophyll protoplasts of Medicago sativa were regenerated to plants (Kao and Michayluk 1980, Dos Santos et al 1980). Cell suspensionderived protoplasts of Trifolium repens produced callus capable of differentiating into complete plants (Gresshoff 1980).

By 1980, the number of plant species whose protoplasts can be regenerated to plants was still limited. However, the regenerating legume and gramineae protoplasts reported so far should stimulate more studies. Rice seems to be a good candidate. Protoplast regeneration to callus has been reported (Academia Sinica, Beijing Institute of Botany, Somatic Hybridization Research Group and Cytobiochemistry Research Group 1975; Deka and Sen 1976) and several authors report plant regeneration from callus derived from rice seed (Niizeki and Oono 1968, Nakano and Maeda 1979).

SELECTION OF SOMATIC HYBRIDS

Induced fusions of protoplasts from two genetically different lines or species necessarily result in a variety of homokaryotic and heterokaryotic fusion products. Selection of the few true somatic hybrid colonies from a mixed population of regenerating protoplasts is a key step in successful somatic hybridization experiments. Two principle selection procedures for isolating somatic hybrids have been established (Table 1).

In the first, selection of somatic hybrids depends on the appearance of a different phenotype or growth pattern from the parent cell colonies or plantlets developed synchronously (complementation selection). In the second, there is direct mechanical selection of the fusion products after a short period of culture, either individually in microdroplets or in a nurse culture. Both methods attempt to isolate somatic hybrids as early as possible.

Table 2. Somatic hybrids selected by the use of two nonallelic auxotrophic mutants.

Species fused	Regeneration	References
Sph. donnellii + *Sphaerocarpos donnellii*	Plants	Schieder (1974)
Physcomitrella patens + *Physcomitrella patens*	Plants	Grimsley et al (1977a,b)
Nicotiana tabacum + *Nicotiana tabacum*	Plants	Glimelius et al (1978) Wallin et al (1979)

Table 3. Somatic hybrids selected by the use of resistant mutants.

Species fused	Regeneration	References
Petunia hybrida + *Petunia parodii*	Plants	Power et al (1976)
Nicotiana knightiana + *Nicotiana sylvestris*	Plants	Maliga et al (1977)
Nicotiana tabacum (tumor) + *Nicotiana tabacum*	Shoots	Wullems et al (1979)
Nicotiana sylvestris + *Nicotiana sylvestris*	Callus	White and Vasil (1979)
Daucus carota + *Daucus carota*	Callus	Harms et al (1981)

USE OF MUTANTS

Fusion of protoplasts from two nonallelic chlorophyll—deficient mutants leads to the formation of somatic hybrids expressing a wild phenotype. The first experiment to select somatic hybrids with the aid of albino mutants was performed on haploid tobacco (Melchers and Labib 1974), followed by reports of intraspecific and interspecific somatic hybrids in the genera Nicotiana, Petunia, and Datura (Table 1).

It is not always necessary to fuse two nonallelic mutants to select somatic hybrids. Cocking et al (1977) fused protoplasts of an albino mutant of Petunia hybrida, which can be regenerated to shoots in a defined medium, with wild—type protoplasts of Petunia parodii, which develop only into cell aggregates of at least 50 under the chosen culture condition. Selection of somatic hybrids was based on ability to develop and on green color.

A similar selection system was used for interspecific somatic hybrids of several other Petunia species and in the genera Nicotiana and Datura (Table 1). Dudits et al (1979, 1980) used the same procedure for experiments on intergeneric somatic hybridization of carrot and Aegopodium podagraria, and carrot and Petrosilium hortense. Somatic hybrids could be obtained in some combinations even where protoplasts of the wild—type species showed no divisions at all under the chosen culture conditions. This was the case in experiments on hybridization of Datura innoxia and four other Datura species.

Protoplasts of albino mutants also can be fused with green wild—type protoplasts which regenerate in culture. Selection of somatic hybrids was based on green color and different phenotype the regenerants developed synchronously from the protoplasts of the wild—type partner. For example, in intergeneric fusion experiments on Datura innoxia and Atropa belladonna, protoplasts from albino mutants of D. innoxia were fused with green wild—type protoplasts of A. belladonna because no albino mutant of A. belladonna was available. Division and subsequent regeneration of plantlets was possible in protoplast culture from both parents separately. Selection of somatic hybrid calli was based on green color, coded by the genome of Atropa belladonna, and the appearance of hairs, coded by the genome of Datura innoxia (Krumbiegel and Schieder 1979). In all other combinations, the somatic hybrids were selected after the development of shoots.

Although the use of auxotropic mutants for the selection of somatic hybrids is efficient because only the hybrid lines can survive, it has been successfully used in only three intraspecific combinations (Table 2). Obtaining such mutants in higher plants is difficult and limited to a few plant species.

The situation for mutants resistant to such chemicals

Table 4. Somatic hybrids obtained after fusion of wild—type protoplasts.

Species fused	Regeneration	References
Nicotiana glauca + Nicotiana langsdorffii	Plants	Carlson et al (1972) Smith et al (1976) Chupeau et al (1978)
Petunia hybrida + Petunia parodii	Plants	Power et al (1977)
Petunia hybrida + Parthenocissus tricusp.	Callus	Power et al (1975)
Petunia hybrida + Vicia faba	Callus	Binding and Nehls (1978)
Petunia hybrida + Atropa belladonna	Shoots	Gosch and Reinert (1976)
Solanum tuberosum + Solanum chacoense	Plants	Butenko and Kuchko (1980)
Datura innoxia + Datura quercifolia	Shoots	Schieder (1980b)

Table 5. Somatic hybrids obtained after visual isolation of fusion bodies.

Species fused	Regeneration	References
Glycine max + Nicotiana glauca	Callus	Kao (1977)
Arabidopsis thaliana + Brassica campestris	Plantlets	Gleba and Hoffmann (1978)
Nicotiana knightiana + Nicotiana sylvestris	Plants	Menczel et al (1978)
Nicotiana tabacum + Nicotiana knightiana	Plants	Menczel et al (1981)

as antibiotics or amino acid analogs is much better. Successful somatic hybridization using two different resistant mutants, leading to a double-resistant somatic hybrid (Table 3), has been reported only in Nicotiana sylvestris (White and Vasil 1979) and Daucus carota (Harms et al 1981) In many cases, such mutant cell lines have lost their morphological potential. Therefore, nearly no chance exists to get somatic hybrid plants. This was the case in hybridization experiments on Nicotiana sylvestris (White and Vasil 1979), where only somatic hybrid cell lines could be recovered.

Resistant factors also can be used in combination with wild-type protoplasts to select somatic hybrids. As has been mentioned, the protoplast of Petunia parodii do not form cell aggregates of more than 50 cells in a defined medium. This limited development of Petunia parodii protoplasts was not suppressed by the addition of 1 mg actinomycin D/liter. Protoplasts of Petunia hybrida, which normally can be regenerated into plants, did not divide in the presence of this antibiotic. After fusion of Petunia parodii and Petunia hybrida protoplasts on the selection medium, several actinomycin D-resistant calli could be selected which regenerated into plants (Power et a1 1976).

USE OF WILD-TYPE PROTOPLASTS

The first somatic hybridization with wild-type protoplasts reported was from protoplasts of Nicotiana glauca and Nicotiana langsdorffii (Carlson et al 1972). Selection of somatic hybrids was based on the ability of hybrid colonies to develop on hormoneless media. Parental colonies grow only on hormone-supplemented medium. Smith et al (1976) confirmed this result. However, the somatic hybrids showed higher chromosome numbers.

Similar selection procedures (Table 4) have been undertaken in interspecific hybridization experiments on Petunia (Power et al 1977) and on Solanum (Butenko and Kuchko 1980). Such systems, in which the different growth patterns of the parents and the hybrids in defined media are used for the selection of somatic hybrids, are useful only when sexual hybrids are available. Knowledge of the growth pattern of the hybrids in defined media is necessary for the selection. Obviously, the use of such a system is not practical for producing somatic hybrids between sexually incompatible species.

Some somatic hybrid cell lines and plantlets also have been isolated by their normal morphology. Binding and Nehls (1978) selected somatic hybrid colonies of Vicia faba and Petunia hybrida which showed an intermediate phenotype. However, a different phenotype of the hybrids already in the callus stage is not expressed in all combinations. In

several somatic hybridization experiments where the hybrids were selected by the use of mutants, a vigorous growth pattern of the somatic hybrids already in the callus stage could be observed (Schieder 1978a, 1980a; Douglas et a1 1981a). This phenomenon has been proposed for selecting somatic hybrids (Schieder and Vasil 1980) and has been demonstrated successfully after fusion of the wild—type protoplasts of Datura innoxia and Datura quercifolia. Among the 6,000 that developed calli, 5 showing a larger size were selected and regenerated to shoots. One of these proved to be a somatic hybrid (Schieder unpubl.).

ISOLATION BY VISUAL MEANS

Kao (1977) introduced a successful method of mechanically isolating heterokaryocytes and cultivating them individual—ly (Table 5). Fusion of colorless protoplasts of Glycine max derived from a cell culture with the green mesophyll protoplasts of Nicotiana glauca facilitated the isolation with micropipets of heterokaryotic fusion products. Trans—fer of the heterokaryotic fusion bodies into Cuprak dishes containing many small individual cells allowed the observa—tion of individual cells. With these manipulations, 20 hy—brid cell lines were obtained. Similar results were obtained by isolating heterokaryons of Arabidopsis thaliana and Brassica campestris with micropipets (Gleba and Hoffmann 1978).

An alternative to single cultivationof heterokaryons is a nurse culture in suspensions of phenotypically dif—ferent cells. Such a system is independent of the specific and complex media necessary for single protoplast culture (Kao 1977, Gleba 1978). Menczel et a1 (1978) fused protoplasts of a kanamycin—resistant cell line of Nicotiana knightiana, which lacks regeneration capacity and chlorophyll synthesis, with green wild—type protoplasts of Nicotiana sylvestris. Interspecific fusion products were transferred to protoplast cultures of the chlorophyll—deficient cell line of Nic—otiana knightiana. All green and shoot—producing calli could be selected as somatic hybrids.

CONFIRMATION OF SOMATIC HYBRID NATURE

Several methods confirmed the somatic hybrid nature of the selected regenerants. When sexual hybrids were available, comparison was undertaken (Power et a1 1976, Carlson et a1 1972). The intermediate phenotypes of organs such as flowers, leaves, trichomes, roots, or tubers often confirmed the hybrid nature (Smith et a1 1976, Schieder 1978b, Power et a1 1980, Dudits et a1 1977, Melchers et a1 1978). Additional evidence fop most of the selected somatic hybrids was chromosome numbers that were higher than those of the par—

ental lines or species.

In general, diploid protoplasts have been used to produce somatic hybrids in higher plants, resulting predominantly in tetraploid somatic hybrids. Double—diploid or amphidiploid chromosome sets in intraspecific and interspecific somatic hybrid cell lines or plants have been documented in Nicotiana (Melchers and Labib 1974, Melchers 1977b, Gleba et al 1975, Chupeau et al 1978, Carlson et a1 1972), Petunia (Power et al 1977, Cocking et al 1977), Daucus (Dudits et al 1977), Datura (Schieder 1978a, 1980a), and Solanum (Butenko and Kuchko 1980), together with somatic hybrid lines that showed either higher ploidy levels or aneuploid chromosome numbers. In the intergenic combinations of Nicotiana glauca + Glycine max, Arabidopsis + Brassica campestris, Datura innoxia + Atropa belladonna, and Petunia hybrida + Vicia faba, the somatic hybrid nature of selected lines also could be confirmed by the different size of the chromosomes of both parents (Kao 1977, Gleba and Hoffmann 1978, Krumbiegel and Schieder 1979, Binding and Nehls 1978).

The different isoenzyme pattern after gel electro—phoresis of the parental species often was used to confirm the somatic hybrid nature of selected regenerants. The enzymes used for the determination were alcohol dehydro—genases and aspartate aminotransferases (Wetter 1977), esterases and glucose—6—phosphate dehydrogenases (Maliga et al 1977), peroxidases (Dudits et a1 1977), superoxid dismutases (Douglas et al 1981b), and amylases (Lonnendonker and Schieder 1980). The somatic hybrids between Solanum tuberosum and Lycopersicon esculentum were confirmed by the analysis of fraction—1 protein (Melchers et al 1978).

In a few cases, the hybrid nature of the selected somatic hybrids was demonstrated genetically. The intras—pecific somatic hybrids of tobacco produced after fusion of haploid protoplasts of two genetically different albino mutants (Melchers and Labib 1974), after self—crossing, showed offspring in green and mutated seedlings comparable to those of the diploid sexual hybrids (Melchers 1977b).

The hybrid nature of the tetraploid intraspecific hybrids of two albino mutants of Datura innoxia was demon—strated by anther culture (Schieder 1978b). Power et al (1978) compared flower color segregation after self crosses of sexual tetraploid hybrids of Petunia hybrida and Petunia parodii with two somatic hybrids. Minor differences were detected. In the progeny of the amphidiploid somatic hy—brids of Datura innoxia and Datura stramonium or Datura discolor, small portions of albino seedlings were detected (Schieder 1980b). One albino mutant of Datura innoxia was used for the experiments. The appearance of albino seedlings could be the result of the formation of a very small percentage of quadrivalents in meiosis I. However, the seed—derived green seedlings were uniform in three sexual

generations. These somatic hybrids are the only two examples so far that are self—fertile and that can be propagated as somatic hybrids through seeds.

CHROMOSOME ELIMINATION

In intergeneric fusion products, the chromosomes of both parents often can be distinguished by their size. In such cases, chromosome elimination, if it occurs, can be studied. The somatic hybrid cell lines of N. glauca and Glycine max produced by Kao (1977), in which the chromosomes of both parents can easily be distinguished, were unstable. The number of chromosomes of N. plauca decreased during culture. Similar observations were made on the somatic hybrid cell lines of Vicia faba and Petunia hybrida (Binding and Nehls 1978), where a stable line that contained the whole, chro— mosome set of V. faba and only 4 chromosomes of P. hybrida could be obtained (Binding pers. comm.). The chromosome numbers of the somatic hybrid cell lines of Arabidopsis thaliana and Brassica campestris, as well as of Datura innoxia and Atropa belladonna, appeared stable over a long period of culture (Gleba and Hoffmann 1978, Krumbiegel and Schieder 1979). However, with the appearance of shoots, a loss of chromosomes could be observed, either from B. campestris or from A. belladona (Gleba and Hoffmann 1979, Krumbiegel and Schieder 1981). This could not be confirmed for all regenerated shoots of the somatic hybrids of A. thaliana and B. campestris. A nuclear coded albino mutant of D. innoxia was used for the selection of the D. innoxia + A. belladona somatic hybrid cell lines. The appearance of shoots with albino sectors and of totally albino shoots indicated the loss of the chromosomes from A. belladonna (Krumbiegel and Schieder 1981). However, one green line possessing only 6 chromosomes and a second possessing only 4 chromosomes of A. belladonna, together with the whole chromosome set of D. innoxia, could be obtained. These were totally stable for more than 10 months. Such results indicate that some chromosomes can be transferred from one species to the other via intergeneric protoplast fusion.

The phenomenon of chromosome elimination in inter— generic fusion products seems to be useful, not only for the transfer of some chromosomes, but also for the transfer of some genetic information, perhaps via somatic recombina— tion. Dudits et a1 (1979) fused protoplasts of a nuclear coded albino mutant of Daucus carota with green wild—type protoplasts of Aegopodium podagraria which never underwent divisions. Selected green plantlets possessed only the chromosome set of Daucus carota. Several chemical studies have shown that some genetic information from A. podagraria was present in the selected plants. Similar results were obtained after fusion of protoplats from an albino mutant

Table 6. Somatic hybrids that contain only part of the nuclear genetic information from the second parent.

Species fused	Regenerat ion	References
Petunia hybrida + Parthenocissus tricusp.	Callus	Power et al (1975)
Daucus carota + Aegopodium podagraria	Plantlets	Dudits et al (1979)
Daucus carota + Petrosilium hortense	Plantlets	Dudits et al (1980)
Arabidopsis thaliana + Brassica campestris	Plantlets	Gleba and Hoffmann (1979)
Vicia faba + Petunia hybrida	Callus	Binding and Nehls (1978)
Datura innoxia + Atropa belladonna	Plantlets	Krumbiegel and Schieder (1981)

Table 7. Somatic hybridization which resulted in cybrids.

Species fused	References
Nicotiana tabacum "albino" + Nicotiana tabacum "susu"	Gleba et al (1975)
Nicotiana tabacum "CMS" + Nicotiana tabacum "fertile"	Belliard et al (1978)
Nicotiana tabacum "albino" + Nicotiana tabacum "CMS'	Gleba (1979)
Nicotiana sylvestris "fertile" + Nicotiana tabacum "CMS"	Zelcer et al (1978) Aviv and Galun (1980)
Nicotiana plumbaginifolia "light sensit." + Nicotiana tabacum "light resistant"	Sidorov et al (1981)
Nicotiana tabacum "strept. res." + Nicotiana sylvestris "strept. sen."	Medgyesy et al (1980)
Petunia hybrida "CMS" + Petunia axillaris "fertile"	Ishar and Power (1979)

of Daucus carota with X—rayirradiated wild—type protoplasts of Petrosilium hortense (Dudits et al 1980). These results (Table 6) require further confirmation.

TRANSFER OF CYTOPLASMIC GENETIC INFORMATION

In contrast to sexual crossings, in somatic hybridization experiments the cytoplasms of both parent plants are mixed. The cytoplasms segregate during development, leading to cells containing the cytoplasm of only one parent. This fact, in combination with the possible elimination of one of the nuclei, can result in hybrids containing the cytoplasm of one and the nuclear genetic information of the other parent. Successful transfer of the cytoplasmic markers albinism (Gleba et al 1975), male sterility (Gleba 1979, Zelcer et a1 1978, Belliard et al 1978, Izhar and Power 1979), sensitivity to light (Sidorov et al 198l), and streptomycin resistance (Medgyesy et a1 1980) have been reported in several intraspecific and interspecific fusion experiments (Table 7). Often the protoplasts from one parent were inactivated by X—irradiation (Zelcer et a1 1978, Sidorov et a1 1981). This procedure may be of general applicability in male sterility—based seed breeding.

CONCLUSION

These results of somatic hybridization are limited predominantly to model plants from the genera Nicotiana, Petunia, Daucus, Solanum and Datura. Somatic hybridization experiments have not yet been done with important crop plants because of the limited number of species from which protoplasts can be regenerated into plants. However, the results demonstrate that it is possible to recover fertile and stable amphidiploid somatic hybrids after protoplast fusion, as was shown with the somatic hybrids Datura innoxia + Datura stramonium and Datura innoxia + Datura discolor.

Intraspecific somatic hybridization can be used for plant improvement in instances where vegetative propagation is the only method. Wenzel et al (1979) proposed a breeding scheme for potato that combines classical breeding methods with parthenogenetic and androgenetic reduction of the chromosomes and somatic hybridization. The breeding scheme is based on the addition by protoplast fusion of different genomes containing various resistant genetic information.

The results obtained in intergeneric fusion experiments leading to asymetric hybrids after partial or total elimination of the chromosomes of one fusion partner open

the possibility of incorporating via protoplast fusion only parts of the nuclear genetic information from one species into another. However, for further successful somatic hybridization in the direction of plant improvement, it is essential that the number of species from which protoplast regeneration into plants is possibly be increased. This is especially necessary for important crop plants such as cereals and legumes.

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GENETIC ENGINEERING OF PLANTS

W. R. Scowcroft

The plant breeder skilled in the conventional approach to genetic improvement of crop species may justifiably scoff at the term genetic engineering of plants and retort: "You are merely reinventing the wheel! We have been genetically engineering plants for the good of mankind for years." Indeed, he is correct. However, perhaps the dramatic rise of molecular and cellular biology may help that wheel turn more efficiently and meet the ever increasing demand for food and fiber resources a little faster.

It seems that specific and directed gene transformation is imminent, if it is not already upon us. This prediction stems from:

- The phenomenal increase in DNA—slicing technology for the isolation and characterization of specific genes and the construction of functional molecular vectors to transfer these genes from one species to another (Setlow and Hollaender 1979, 1980; Malik 1981).
- The increasing sophistication of plant cell culture, not only to provide recipient host cells for molecular donors but also to facilitate the holistic evaluation of cellular—wrought changes.
- The unequivocal demonstration of vector—mediated genetic transformation in eukaryotes, such as in mammalian cells (Wigler et al 1979a) and in yeast (Ilgen et al 1979).
- The knowledge that crown gall induction by Agrobacterium tumefaciens in plants is a consequence of DNA transfer in vivo (Hooykas et al 1979).

My purpose here is to look at some, but by no means all, of the strategies being developed to generate a reliable technology for genetic transformation in plants.

At the outset, it is important to distinguish between shotgun—treatment of plant or plant cells with DNA and precise and controlled gene transfer. Many reviews (Kleinhoffs and Behki 1977, Scowcroft 1977) have evaluated claims and counterclaims for the success of early general approaches. Most of the earlier transformation events presumed to have taken place in plants are equivocal largely because it was not possible to identify or recover the DNA sequence responsible for the transformation—like event.

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THE CONCEPT OF GENETIC TRANSFORMATION

Following, the insertion of DNA into cell, transformation results if there is a permanent or long—term change in the genetic constitution of the recipient cell. The stable retention of DNA in a cell or plant depends largely on the trans—forming DNA's ability to multiply in the host and to be in—tegrated into the genome of the recipient. For this to occur, the transforming DNA must be associated with sequences that facilitate self—replication or genome integration or both. For this to occur, a vector is required.

In bacteria, plasmids, and bacteriophages have provided the essential vectors for transformation. For yeast and ani—mal cell transformation, vectors that perform a dual func—tion have been developed. They will multiply in bacteria to facilitate the construction, amplification, and identifica—tion of the requisite vector and they will multiply in the relevant eukaryotic cell for which transformation is required These vectors are called shuttle vectors.

An example of an E. coli—yeast shuttle vector is one made by joining the 2 μM plasmid of yeast with a bacterial plasmid and inserting the yeast leucine gene. It contains a bacterial gene for tetracycline resistance, which expresses in E. coli, and the leucine gene from yeast, which expresses in both bacteria and yeast (Ilgen et al 1979).

Mammalian cell shuttle vectors are constructed from the part of the Simian virus 40 (SV40) containing the origin of replication, a bacterial plasmid containing a selectable gene such as ampicillin resistance, and a gene, such as thy—midine kinase from herpes simplex virus, to permit selection in the recipient cell (Wigler et al 1979b).

PLANT VECTORS

Stable and reproducible transformation in plants will require specifically constructed vectors. Primarily, they should con—tain a plant replicon, such as a DNA sequence at which DNA replication can be initiated in the transfected plant cell. They also should contain a sequence that permits the in—tegration of the vector into the host genome. There are sev—eral possible sources of potential replicons.

DNA viruses

Double—strand DNA viruses, the caulimoviruses of which cauli—flower mosaic virus (CaMV) is the best known, could serve as a source for a plant vector (Kado 1979). But difficulties have been experienced in the potential of CaMVas a cloning vehicle. It has a limited host range and the yield of vir—ions from infected plants is low. Moreover, when cloned entire, CaMV seems to lose infectivity when it is excised by

restriction endonucleases and religated.

Organellar genome replicons

Plant organellar genomes (chloroplasts and mitochondria) are potential sources of replicators. Langridge (1981) has utilized yeast transformation to recover plant chloroplast replicators. These vectors contain a bacterial replicator from the ColE1 plasmid, the bacterial transposon TN7 which carries trimethoprim resistance, the kanamycin resistance gene from plasmid pML21, the his-3 gene of yeast coding for imidazole glycerophosphate dehydratase, and the replicator from tobacco chloroplast DNA.

By using yeast as a host for the selection of plant replicators, it may be possible to isolate replicons from the nuclear genome of plants. Attempts are being made to recover such replicators from ribosomal DNA. This source may have the added advantage of sufficient sequence homology to facilitate integration.

Agrobacterium tumefaciens and the Ti-plasmid

It is now well established that the crown gall disease of plants is the result of the transfer of part of the DNA (the T-DNA) of the Ti plasmid of A. tumefaciens into plant cells (Hooykas et al 1979, Chilton et al 1980). The T-DNA found in sterile crown gall tissue appears to be localized in the nucleus covalently linked to plant DNA (Chilton et al 1980). The T-DNA contains genes that are transcribed, one of which codes for the crown gall specific enzyme lysopine dehydrogenase. Foreign DNA can be inserted into the T-DNA of the Ti plasmid and transferred into the nucleus of a recipient plant (Hernalsteens et al 1980).

However, until recently the potential value of the Ti plasmid as a vector was constrained by the inability to transfer T-DNA coded properties through a sexual cycle. Otten et al (1981) used a genetically altered Ti plasmid to demonstrate that T-DNA can be inherited with Mendelian segregation ratios and that the lysopine dehydrogenase activity was transmitted to both pollen and eggs. The genetically altered Ti plasmid was produced by transposon mutagenesis which inactivated the tumor functions of the plasmid. Tumors formed by A. tumefaciens containing this plasmid could be produced to proliferate shoots which developed fertile flowers.

Thus, the T-region of the A. tumefaciens Ti plasmid is a suitable genetic element for use in the construction of plant vectors. It also has the advantage of utilizing a natural delivery system which would restrict its use to dicotyledonous species. I am aware of no evidence to suggest that T-DNA will infect monocots.

SELECTABLE GENE FUNCTIONS

Bacteria, yeast, and mammalian systems

The availability of DNA sequences coding for functions that can be efficiently selected is mandatory for any genetic transformation system. In bacteria, this requirement has been satisfied largely by drug—resistant genes. In yeast, genes coding for amino acids, particularly the his—3 and leu—2 genes, have provided the selectable genes. For mammalian cells, genes coding for thymidine kinase (tk) from herpes simplex or for adenine phosphoribosyltransferase (APRT) have been preeminent in transformation studies.

A necessary complement for both yeast and mammalian cells is the availability of strains or cell lines which were deficient for the gene functions for which marker rescue was imposed.

Plant genes

There is a relative paucity of gene—controlled functions for plants which might be incorporated into transforming vector to ensure an efficient selection system. While it is true that mutant plant cell lines are being isolated with increasing veracity (Maliga 1980), many of these are resistant to antimetabolites. Such mutants offer little or no value in demonstrating the acquisition of a genetic characteristic by transformation. The mutants required are those deficient in some essential or conditionally lethal biochemical function. Considerable difficulty has been experienced in isolating nonleaky amino acid auxotrophs in plants.

Alternative and available deficient mutants which may be of value are those deficient for nitrate reductase (NR) (Muller and Grafe 1978) or, for example, alcohol dehydrogenase (Adh). However, isolation of the DNA sequence coding for nitrate reductase is proving intractable. That for Adh may be somewhat easier.

Maize Adh

Colleagues in the Division of Plant Industry (Sachs et a1 1981) are advanced in cloning the Adh gene of maize. Adh is induced in maize by anaerobic conditions. This feature enabled the purification of polyA RNA partially enriched for Adh mRNA for the construction of anaerobic cDNA clones. Anaerobic specific cDNA clones were isolated by the hybridization of these labeled cDNA clones with aerobic and anerobic RNA profiles. Clones that specifically code for a polypeptide of 40,000 daltons, the molecular weight of the ADH polypeptide, were identified by hybrid release translation. Further definitive characterization was achieved using the presumptive Adh cDNA probe to detect electrophoretically

different hybrid release translation products in mutant stocks carrying an allele known to produce an electrophoretically different polypeptide. These cDNA clones will be used to recover genomic Adh clones from a library of maize DNA.

It is essential to have a recipient cell line for transformation with the Adh gene when it becomes available. We have adopted two approaches to this (Horst Lorz, Division of Plant Industry, CSIRO, pers. comm.).

In the first approach, we have attempted to culture Adh—null mutants by immature embryo culture. In contrast to Black Mexican Sweet (Green and Phillips 1975) and an F1 of BMS and Adh—null, the Adh—null line failed to survive under prolonged culture conditions. Lorz has found that, under standard culture conditions, Adh activity of a good maize cell suspension line Z4 is rather high. However, under strictly anaerobic conditions (argon) Adh activity can be elevated about ten fold. If it can be established that the very poor culturability of Adh—null lines is strictly a function of the Adh deficiency, this can be used as a selection system following transformation.

The second approach will attempt to isolate Adh—deficient lines from the Z4 line for which protoplast isolation and culture are well defined. The selection is based on allyl alcohol where Adh^+ cells convert allyl alcohol to the toxic compound acrylaldehyde. Adh— mutants will not carry out this conversion and so remain resistant

Other selectable genes

In developing a generalized transformation system for plants, it may not be necessary to isolate and insert plant genes such as alcohol dehydrogenase or nitrate reductase into cloning vehicles. It is possible that genes which have been successfully utilized in prokaryote and yeast transformation can be used in plants. For example, we have established that the ubiquitous enzyme dihydrofolate reductase analogs trimethoprim and methotrexate inhibit both cell growth and protoplast culture of tobacco.

Similarly, the 2—deoxystreptamine antibiotic G418, which inhibits cell—free protein synthesis in plants (Jimenez and Davies 1980), also inhibits cell growth and protoplast culture at relatively low concentrations. Prokaryote genes coding for resistance to these drugs are available and my colleague John Langridge is currently incorporating these genes into *E. coli*—plant shuttle vectors.

In this context, it is worth recording that the prokaryotic gene coding for methotrexate resistance was used to construct a recombinant eukaryote expression vector based on SV40 which could transform mouse fibroblasts to methotrexate resistance (O'Hare et al 1981). Transformed cells produced a prokaryote didhydrofolate reductase RNA tran—

script which presumably was translated into a functional product. Admittedly, this expression vector was sophisti—cated in that it contained the promoter and cap site of SV40 and the donor and accepted splice site and polyadenyla—tion site of the rabbit β—globin gene. It must be concluded that these accessory functions are necessary for the correct transcription and translation of a prokaryotic gene in a eukaryotic cell environment. The important point is that prokaryotic genes may also be useful in developing trans—formation systems in plants.

It is also possible that a class of plant specific genes could be isolated for use in vector construction. Many her—bicides interfere directly with plant cellular metabolism. For example, amitrole is known to interfere with histidine biosynthesis. Glyphosate (N—(phosphonomethyl) glycine) ap—pears to inhibit a target enzyme in the shikimic acid path—way. If such herbicide—resistant genes could be isolated and inserted into a plant expression vector, they might provide for generalized transformation.

OPTIMIZING TRANSFORMATION

Many other facets are involved in the development of genetic transformation in plants. It will be essential to optimize DNA uptake and minimize donor DNA polymerization. Encap—sulating vector DNA in liposomes, which readily fuse with protoplasts, has been advocated as a possible way to min—imize vector DNA degredation (Lurquin 1979).

Poly—L—ornithinealso tends to reduce the severity of nuclease activity (Hughes et al 1979). With mammalian cells, for which there is an efficient selection procedure, the frequency of cells expressing donor gene functions can be greatly increased by direct microinjection of vector DNA into the nucleus of the recipient cell. High frequency (1 in 5 cells injected) transformation requires co—injection with SV40 DNA (Capecchi 1980).

Although I do not wish to trivialize experiments that seek to optimize DNA uptake and minimize DNA degradation, I feel that these are secondary features. The primary objec—tive is to achieve reproducible transformation. From the experience gained with other eukaryote systems, once this is achieved (albeit initially at a low frequency), the ancillary aspects will be rapidly optimized because there is a basis for comparative experimentation.

COTRANSFORMATION

The establishment of a reproducible, generalized transforma—tion can then be used to simultaneously cotransfer any DNA sequence without the need to actually insert that sequence

into a vector molecule. It has been found that a high proportion of mammalian cells transformed for the selectable marker thymidine kinase simultaneously integrate other exogenous DNA sequences at a high frequency. Such cotransformed cloned sequences include ϕX174, rabbit ß—globin (Wigler et al 1979b), and human growth hormone (Robins et al 1981), which at the time of DNA treatment were physically unlinked to the tk^+ vector.

Thus, provided there is a way to recognize exogenous transforming vector nonlinked sequences, cotransformation is a real possibility. The benefits of cotransformation could be boundless in plants and ultimately in plant improvement.

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TRANSFER OF TUMOR CHARACTERISTICS BY PROTOPLAST FUSION

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Crown gall is a plant tumor caused by a large plasmid (Ti plasmid) present in Agrobacterium tumefaciens. It has been shown (Braun and Wood 1976) that a neoplastic disease is induced when a portion of Ti plasmid (T—DNA) enters plant cells. The transformed plant cells, in addition to producing tumors, acquire several new characters which can be stably inherited. These new characters include inde— pendent growth of the phytohormone and the ability to synthesize and catabolize the amino acid derivatives octo— pine, nopaline, and agropine.

The genes responsible for these characters are located on the DNA fragments of Ti plasmid (Genetello et a1 1977). For example, the lysopine dehydrogenase (LpDH) responsible for octopine synthesis is determined by one of several genes on T—DNA. The nopaline dehydrogenase (NpDH) respon— sible for nopaline synthesis also is located on T—DNA fragments. Therefore, the occurrence of octopine or no— paline in plant cells may be an indication of the transfer of Ti plasmid from bacteria to plant cells. Octopine or nopaline could be used as a marker for screening trans— formed or hybrid cells.

This natural process of continuous tumorgenesis is thought to be an example of natural plant genetic engineer— ing. Since Ti plasmid can infect most dicotyledonous plants (such as tobacco, sunflower, kalanchoe, and birch), it can be used as a vector for transferring some useful foreign genes into plant cells.

In the past decade, studies on plant protoplasts have developed rapidly. Whole plants have been regenerated from protoplasts of more than 40 species. Plant protoplasts provide useful systems for gene transfer and a potential way to express prokaryotic genes in eukaryotic genomes. Now it is possible to transfer T—DNA or hybrid molecules carrying Ti plasmid into plant cells by the transformation of plant protoplasts with the plasmid or by fusion between tumor cells and normal cells. This may lay a sound founda— tion for the practical use of genetic engineering in plants. In addition, it is important to reveal the genetic mechanism

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of tumor cell transformation and gene expression and regulation in higher plants.

Some critical questions remain to be answered:

- What is the fate of T—DNA in transformed cells?
- What is the stability of the T—DNA in transformed cells?
- Can prokaryotic genes be transferred into any plant genome and can it be expressed in it?

CHARACTERISTICS OF PLANT TUMORS

Tumor cells have many specific properties in morphology, biochemistry, and genetics. They can continuously proliferate and autonomously grow in vitro without the addition of phytohormones. Opine metabolism is another property. Tumors induced by different plasmids differ in morphology and in metabolic function (synthesis and breakdown). Three main metabolites have been found so far:octopine, nopaline, and agropine. The opine produced by a tumor cell is not dependent on the host plant, but on the type of bacterial plasmid introduced. The tumor induced by octopine—type plasmid can synthesize octopine, whereas the nopaline plasmid—induced tumor synthesizes nopaline. These products are degraded and utilized by the inducing bacterium as sources of nitrogen and carbon.

Ti plasmid has been considered to be a group of conjugative genes, probably because it contains transferring genes which could promote conjugation and transfer of plasmids between the recipient and the donor (Schell et al 1979, Kerr et al 1977, Genetello et al 1977). These genes are depressed under normal conditions, but octopine—type plasmid conjugation often can be specifically induced by octopine. The mechanism of conjugation and transfer possibly is correlated with Ti plasmid transfer from bacteria to plant cells (Schell et al 1979).

Insertion of T—DNA into the plant genome is the causative agent for tumorgenesis and the synthesis of the unusual metabolites. Recently it has been demonstrated that T—DNA can be inserted into the nuclear DNA of a host plant (Zambryski et al 1980, Thomashow et al 1980). The nuclei isolated from octopine—type tumor cells contain T-DNA-transcribed RNA (Drummond et al 1977). Only a part of the T—DNA is responsible for LpDH activity and hormone independence. But how the T—DNA is inserted and what the insertion mechanism is are unanswered questions.

TRANSFER OF GENETIC INFORMATION

Genetic information is transferred by protoplasts in two ways. In the first, protoplasts are used to take up foreign

DNA fragments. In the second, tumor cells carrying exogenous macromolecules are fused with normal protoplasts. Wounding is the prerequisite to A. tumefaciens infection of a plant. In infecting, only Ti plasmid released by bacteria enters the plant cells; bacteria are only absorbed by the plant cell wall. Therefore, protoplasts removed from the cell wall by enzyme digestion are becoming a useful system for bacterial infection.

Marton et al (1979) used 3-day-old protoplasts of a streptomycinresistant mutant of tobacco (SR1) incubated with various types of A. tumefaciens. He selected trans—formed tumor cells based on their capability for autonomous growth and for streptomycin resistance. As a result, the selected cell line contained tumor—specific LpDH or NpDH activity. The transformation frequency depended on the bacterial strain used and the ratio of recipient and donor densities. Two clones lost their hormone independence after they had been subcultured for two generations on a medium without hormone. He suggested that this might have resulted from the complete loss of T—DNA genes in the transformed cells.

Davey et al (1980) obtained several transformed cell lines of Petunia by the transformation of Petunia meso-phyll protoplasts with Ti plasmid in the presence of poly-L—ornithine. The transformation frequency was about 1×10^{-5}. Electrophoresis analysis revealed the presence of LpDH activity and octopine in the transformed cells. He thought this was due to the introduction of Ti plasmid or some DNA fragments of it into plant cells and suggested that intact bacterial cells might be unnecessary for transforma-tion. Since only transformed cell clones and not differ—entiated plants were obtained, it is not clear how long these tumorous properties can be stablely maintained.

The cell wall has been an important factor for bacteri-al infection. Recently, Hasezawa et al (1981) reported that higher transformation frequencies ($2.3 - 7.6 \times 10^{-4}$) were obtained by the transformation of Vinca rosea protoplasts with A. tumefaciens spheroplasts. The transformants were detected by the capacity of octopine synthesis. He suggested that these improved results might be because the Ti plasmid introduced into plant protoplasts was intact. The cell clones grew on an MS medium without hormones and possessed tumor—specific octopine and its synthetase. However, this was analyzed only on a cell clone level. Whether the transformed clone was stable and able to regenerate or whether it became an obstacle to differentiation has not been reported.

TRANSFER OF GENETIC INFORMATION BY FUSION

Since it has been demonstrated that tumor cells containing

Ti plasmid DNA or T—DNA segments can induce tumor formation (Thomashow et al 1980), retransfer of T—DNA from tumor cells to normal cells is possible. In this way, isolated Ti plasmid can be prevented from degrading, as in the case when cells or tissues are used as recipients.

By fusing protoplasts of a tobacco tumor line B6S3 and of a streptomycin resistant mutant SR1, then selecting transformants on K_3 medium without hormones, Wullems et a1 (1980) obtained some cell lines that were both streptomycin resistant and hormone independent. The transfer of tumor characters undoubtedly is based on the transfer of the T-DNA of Ti plasmid.

In the same year, we made a protoplast fusion of tobacco tumor B6S3 with N. tabacum Xanthi (Li et al 1981a.). Fusion frequency was about 12%. Regenerated calli were differentiated into plantlets on a medium without phytohormones. The discretion of the hybrid was based on the presence of LpDH. Microscale electrophoresis analysis of various plant leaves (Otten and Shilperoort 1978) revealed that 21 plants derived from calli contained the LpDH responsible for the synthesis of octopine. The shape of the plants and their leaves was similar to that of N. tabacum Xanthi, indicating that their LpDH originated from the tobacco tumor. Their chromosome sets were aneuploid.

These results show that after intraspecific fusion of tobacco protoplasts, a gene(s) coding for LpDH may be transferred from tumor cells to normal cells, ruling out the possibility of cellular chimera and artificial contamination.

INTERGENERIC FUSION

Can these characters of tobacco tumor be transferred to somatic cells of distant and sexually incompatible plant recipients? Plant protoplast fusion has been done between sexually incompatible parents with obvious opposite characters, for example tobacco B6S3 and petunia W43 (Li et al 1981b).

The fusion frequency of the two species was 12—15%. After culture had been developed in fused cells, some hybrid clones were selected on a medium lacking hormones. Thirty green hybrid plants and four albino shoots were differentiated in turn from the seven green clones selected. These plants were more or less similar to the petunia in such morphological traits as leaf shape, leafstalk, and stem. But some plants had thickly growing shoots, compact leaflets, and no or few roots. Microscale electrophoresis analysis showed that the leaves of 21 plants contained LpDH. Repeated analysis revealed that LpDH activity was still obvious in six of the plants. In the petunia W43

control, no LpDH activity was detected, suggesting that the gene(s) controlling LpDH had been transferred into petunia cells via fusion and were being expressed (Table 1).

Table 1. Properties of tobacco tumor B6S3, Petunia hybrida W43, and their hybrid[a].

Properties	T-B6S3	P-W43	Hybrid of tobacco tumor B6S3 × P. hybrida W43
Phytohormone independence	+	-	+
Green callus tissue	-	+	+
Regenerated plant	-	+	+
LpDH activity	+	-	+

[a] + = existing, - = not existing.

Because the plants originated from a small amount of leaf tissue, the possibility of chimeric origin was ruled out. It was also shown that the inclusion of tumor cells could not inhibit the morphogenesis of petunia shoots. The chromosome numbers of somatic cells was greatly changed and cells with 23, 26, 31, and 40 or more chromosomes appeared. This surpasses the chromosome number of the parental petunia (2n = 14).

These results imply that, even in the case of distant species, the genetic information of Ti plasmid can be transferred from tumor cells to normal plant cells, resulting in somatic hybrids.

In addition, several plants contained weak activity of LpDH and this activity was absent in four other plants. This phenomenon may be due to chromosome elimination in mitosis of the somatic hybrids or to relatively less of the T-DNA fragment being transferred. Seed progeny have not yet been produced. Therefore, the stability of LpDH remains to be investigated.

CONCLUSION

Recently rapid progress has been made in the molecular genetics of Ti plasmids. The possibility of using Ti plasmids as vectors of desirable genes appears near. A similar status exists for plant protoplast culture, fusion, and transformation. Whole regenerated plants derived from

protoplasts of more than 40 dicotyledonous species are now available. For cereals and legumes, however, it is still a hard task, although some breakthroughs have been made.

Nevertheless, protoplasts are a good experimental system for genetic manipulation. The genes of Ti plasmid have been expressed in plant genomes by the transformation of protoplasts with A. tumefaciens spheroplasts or isolated DNA. Through the protoplast fusion of crown gall tumor and a normal plant, it has been shown (especially in somatic hybrids) that the genetic information of Ti plasmid can be expressed in intraspecific cell hybrids and even in an intergeneric hybrid of somatic cells.

As is well known, tobacco and petunia are taxonomically distant and sexually incompatible and hybrids have not yet been obtained. But when protoplasts of tobacco tumor cells and cells of wild petunia were fused, hybrid plants were likely to form. This is probably associated with the conjugation caused by the Tra gene (Schell 1979). If this is the case, an inducing factor of the Tra gene that may be present in petunia protoplasts may help overcome the incompatibility of these two species.

To what extent the genetic information of a transferred tumor or T—DNA can be stably maintained in the host plant, how to prevent backmutation, and whether or not it can be expressed in seed progeny are important problems remaining.

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BEYOND HAPLOIDS

Peter S. Carlson

Researchers in plant genetic manipulations are, to varying degrees, intellectual artists captivated by the logic and consequences of their experimental manipulations. The purpose of plant breeders is not to produce bubbles of ever—more—intricatebeauty, but to speed the production of varieties to meet pressing human needs. This requires harnessing intellectual artistry to the realities of production agriculture. Although plant breeders have produced a number of remarkable feats of genetic engineering in developing current crop varieties, comprehension of the parameters and mechanisms underlying genetic alteration is still sketchy.

Therein lies the challenge for new genetic manipulations: to develop additional genetic techniques, to rationalize and explain the why of desired breeding manipulations, and to create standard procedures for their production and recovery.

A number of recent publications (Proceedings of symposium on plant tissue culture 1978, Fourth international congress of plant tissue and cell culture 1978, Fifth international protoplast symposium 1979) chronicle the increased interest in and efforts toward applying plant cell and molecular genetics to plant breeding. The reports by Chinese scientists and others of varietal development in rice, wheat, and other crop species via anther culture—derived haploids are a major first step in verifying the utility of these new techniques (Academia Sinica Institute of Genetics and IRRI, this volume).

Genetic modifications produced with embryo culture, cellular mutant selection, and gene transfer techniques will certainly play a role in future varietal development. This expectation requires that plant breeders take a major responsibility in defining the directions and goals of particular research efforts and that they participate in the evaluation of modified genotypes. Because gene effects vary with environment, any type of gene modification has meaning only for the environment under which the particular effect was measured. Selection of a group of genotypes because of a superior phenotype in one environment is likely to mean less superiority and maybe even inferiority when the same array of genotypes is placed in another environ—

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ment. Genetic modifications recovered in vitro often may not perform in vivo. Plant breeders will make the decision whether or not to use a particular genotype in a crop improvement program.

Currently, most plant cell and molecular geneticists are more comfortable with the concepts of microbial genetics than with the concepts of plant breeding. This is to be expected, because the techniques, assumptions, and logic of the field are largely derived from microbiology. I believe that future applications of plant cell and molecular genetics for plant improvement will be enhanced if and when we begin to deal with the traditional difficulties of plant breeding. Quantitative inheritance, heterosis, genotype-environment interaction, and heritability are phenomena we must come to understand and with which we must work. These phenomena have been described and, to a certain extent, manipulated statistically.

The problem, of course, is that microbial genetic methodologies were not designed to deal with polygenes, population means, and nonreproducible environments. This is not to say that defined single gene modifications or transfers based on microbial paradigms will not be important. I am confident they will be. Salt tolerance, disease resistance, herbicide tolerance, and many other relatively simple traits based on direct selection schemes can be used by breeders. The important points are that our scientific challenges go beyond those traditionally faced by microbial geneticists and that these challenges present unique scientific opportunities.

I will not recount the numerous important results of plant cell and molecular genetic research here. Nor will I enumerate the experimental and technical difficulties encountered in this research. These items are well covered in other publications (Chaleff 1980). I also will not include related topics which I have discussed elsewhere, such as attempts to analyze hetorosis, single gene overdominance, and polygenic inheritance and accomplishing introgression in vitro (Carlson 1980). Instead, I will present some speculations and preliminary data from my own work and from that of others to illustrate in a rudimentary way how we may begin to approach the complex genetic opportunities presented by the need for plant improvement.

Wright, in his Evolution and the genetics of populations (1977) made this observation:

> "I have recognized from the first that a high degree of precision is out of the question in dealing with evolutionary phenomena in nature, or even ordinary laboratory experiments, because of the complexity of the interactions among the factors and consequent excess of the number of parameters required for full description over that determinable from observation or even from

well—controlledexperiments."

It seems that a similar statement is applicable in any attempt to apply the reductionistic methods of analytical cell and molecular genetics to plant breeding. We must establish correlations and, in many instances, be satisfied even if these correlations do not provide direct cause—and—effect information about gene action.

VARIABILITY OF PLANTS REGENERATED FROM IN VITRO CULTURE

Experiments with sugarcane, potato, and tomato (Heinz et a1 1977, Shepard et a1 1980, D. Evans pers. comm.) have dem—onstrated that plant cells cultured in vitro and subsequent—ly regenerated into entire plants show a range of phenotypic variability. This is unexpected, given that genetically identical cells presumably comprise the starting material. In some instances, these variations have been shown to be due to chromosomal and other mutational changes which oc—curred during in vitro culture. Several of these variations have been transmitted sexually.

The important observation concerning these variant regenerated plants is that a portion of them exhibit a marked increase in vigor even the original parental genotype. This is unexpected because ordinarily the vast majority of mutations are deleterious. It is easy enough to demonstrate that passage through in vitro culture results in genetic abberations. But why should these mutations result in a high proportion of superior genotypes?

A possible explanation comes from a series of experi—ments designed to study the effects of random nonlethal mutations in heterozygous conditions (Wallace 1963, Mukai 1954). The findings, also unexpected, demonstrated the in—teresting phenomenon that random mutations, when heter—ozygous, may have a favorable effect on viability and fit—ness in an otherwise homozygous genetic background. General—ly, the relative viabilities of random mutations of heter—ozygotes were significantly superior to nonmutant homozygotes (significant at the 0.01 level). As expected, individuals homozygous for the random mutations showed a marked decline in viability.

These experiments were performed on caged populations of Drosophila using X—rays as a mutagen. However, even though animal material and a known dosage of a mutagen were used in this work, the conclusions may explain the observa—tions on plant cells cultured in vitro. In some instances, random nonlethal mutations can result in heterosis when they are heterozygous with a nonmutant allele.

If single gene overdominance is even partially the explanation for the superior genotypes recovered from in vitro culture, then several points become important.

- The superior genotypes recovered are heterozygous

for mutations which, when homozygous, will be deleterious; that is, they will carry a heterotic load. This load is acceptable if the crop is normally propagated vegetatively (sugarcane and potato) but is unacceptable if the crop is planted as an inbred (tomato). The variation may be important if hybrid seed is produced.

- The variation should appear and be recovered in diploid or higher level ploidy materials. Haploids would not be expected to randomly yield superior unselected genotypes through culture.
- The moderate use of additional mutagenic agents may increase the frequency of desirable variation recovered from in vitro culture of plant cells.

Another event which may be caused by genetic alterations induced from in vitro anther and pollen culture is the production of albino haploid plants. This particular problem in cereal species appears to be related to medium composition, time in culture, and genotype. The observation that haploids produced from female gametophyte tissue result in fewer albinos suggests that it also is related to the differentiated state of the starting tissue. Certainly, albino production is frustrating for experimenters trying to produce large numbers of haploid lines for varietal screening as homozygous diploids. The frequency of such albinos may be reduced by better nutritional conditions in vitro, by a more rapid passage of the tissue through the in vitro stage, or by the addition of antimutagenic agents.

However, once standardized haploid production procedures are adopted, the frequency of albinos may provide an estimate of genetic variation occurring in vitro. Mutation breeders, particularly those working with cereals, have used the frequency of albino production in M2 seed lots to estimate the efficiency of mutagenic treatment (Nilan 1974). For example, in barley, lesions at more than 200 loci can give rise to albino phenotypes. The frequency of albino haploids may be a basis for estimating the expected frequency of other, less frequent and less obvious, mutant phenotypes. It also may allow an estimation of what proportion of the genetic variability found in haploids is due to mutagenesis occurring in vitro and what proportion is due to segregation in the heterozygous parental plant material.

RECOVERY OF INBREDS WITH HIGH COMBINING ABILITY

The production and selection of inbred lines via haploid techniques provide a proven method for varietal production of inbred crop species. However, it appears that many inbred crop species will soon be cultivated as hybrids. For example, the development of cytoplasmic male sterility now permits

the rapid production of high—yielding hybrid rice varieties. Increasing numbers of hectares in China and throughout the world are being planted to hybrid rather than to inbred varieties. No techniques exist now for rapid assessment of the combining ability of inbred genotypes, even though this character will become increasingly important. We have been attempting to develop such a technique.

Griffing and Langridge (1963) have examined heterosis in F1 hybrids in 38 inbred races of Arabodopsis thaliana grown in environmentally controlled in vitro conditions. In recording fresh weight 14 days after germination, they dis—covered an intriguing phenomenon. Growth was determined at 3°C intervals from 16° to 31°C and, as expected, the races differed considerably in optimal growth temperature. In crosses between 1 race and 4 others, the increase in fresh weight over the midparent value of the F1 was 25% at tem—peratures from 16° to 25°C. However, the F1 value increased to 97% over the midparent at 28°C and to 368% at 31°C. As expected, these values all decreased in the F2 populations.

These results also were found when a number of inbreds and their F1 hybrids were examined. Heterosis was accentu—ated at high temperatures. These findings were interpreted on the basis of the multiple loci at which the alleles encode for polypeptides of different thermolability and on optimal temperatures. Heterozygotes, with two alleles and different polypeptide products, presumably have more versatility than do homozygotes. This versatility is espe—cially manifested at high temperatures, when a homozygous genotype is more likely to suffer from thermolability than is the hybrid. Irregardless of the validity of this inter—pretation, these findings demonstrate that inbreds are more sensitive to temperature than are heterotic F1 hybrids. Hybrids are better able to withstand a stress situation.

We confirmed these conclusions with tobacco inbreds and their F1 hybrids. Seedlings were grown in vitro with, known environmental conditions (P.S. Carlson, Michigan State University, unpubl. data). A number of inbreds and their F1 hybrids were examined under normal (23°C) and high (31°C) temperature. In addition, we investigated several other stress conditions to accentuate heterosis, including media stress (low NO_3^- , or $PO_4^{\equiv}$, or $SO_4^{\equiv}$), low light stress, high osmotic pressure stress, salt (NaC1) stress, and herbicide—induced stresses. As in the case with Arabidopsis, the most striking data came from temperature stress.

We used 10 different F1 hybrids and their inbred parental lines. Four hybrids were heterotic under field conditions (from 12% to 23% over the midparent), six were not. In the 4 heterotic hybrids, a marked accentuation of heterosis occured in seedlings under high temperature condi—tions (from 97% to 215%). In the six nonheterotic hybrids, there was no statistically significant increase in the Fl's over their inbred parents at high temperatures.

We then had 8 inbreds (parent of the four heterotic Fl's) with good combining ability and 12 inbreds (parent of the nonheterotic Fl's) with apparently poor combining ability, at least in those hybrids. We have attempted to differentiate between these genotypes and to determine a general rapid test for combining ability. Again, our focus was on examining tolerance and growth in high temperature. Specifically, treating germinated seedlings to a 24-hour temperature shock of 40°C, followed by growth at 31°C, proved most effective. Seedlings of all 8 inbreds with good combining ability survived and continued growth. Only 3 of the 12 inbreds with poor combining ability grew after this temperature treatment.

The test was repeated with anther-derived haploids from the 20 inbred genotypes. Although the haploids were not as vigorous as the diploid seedlings, the results were identical.

These observations indicate that survival and growth under such stress conditions as high temperature may provide a preliminary screen for selection of inbred lines with high combining ability. We have not attempted to use this system to select haploid genotypes with high combining ability from heterozygous tobacco diploids. Hence, these experiments must be considered to constitute a model system without proven applicability.

PHENOGENETICS OF ASEXUAL REPRODUCTION IN VITRO

From studies on chromosomal changes occurring in vitro, genetic variability induced in culture appears as a continuum of genotypes. The extent and magnitude of this genetic variability depend on the starting genotypes, the length of time in culture, the composition of the culture medium, and other, as yet undefined factors. Such a continuum is not found when the phenotype of cells in culture is examined.

Epigenetic variation occurring in vitro results in discontinuous growth habits. A more refined analysis of molecular phenotypes via twodimensional gel electrophoresis to separate polypeptides points to the same conclusion. Cell cultures exist as subpopulations with a finite number of discrete molecular phenotypes, not as a continuous range of phenotypes (Carlson 1979; P.S. Carlson, Michigan State University, unpubl. data). In long-term cultures, the number of discrete phenotypes can be reduced and stabilized by selection and growth conditions.

This phenomenon is important because it indicates that the molecular characteristics of any cell line are unique and not repeatable in other cell lines, even though they are derived from the same initial genotype and cultured under identical conditions. Consequently, the types of genetic

variants which can be recovered via selection in vitro may differ among cell lines. If a gene product is not being expressed by a cell line, variants in that gene product will not appear. Likewise, epigenetic variants may appear as putitive genetic changes by an altered gene expression pattern in vitro.

These events may account for the frequent recovery of nongenetic phenotypes in response to selection pressures. It would not be expected to contribute as significantly to the selection of phenotypes at the whole—plant level after in vitro culture.

In many aspects, the production of discontinuous phe—notypes in vitro is analogous to the presence of multiple selective peaks described by Wright (1977). Selection from an initial gene pool and epigenetic configuration tend to move the cellular phenotype to an epigenetic selective peak which is better adapted for growth in vitro. This selection occurs simultaneously in a number of different cells and their asexual clones, resulting in the production of a number of discrete peaks.

In long—term cultures, further selective pressures result in populations moving by epigenetic changes to other selective peaks which may or may not be more adapted to cul—ture conditions. Selection by growth rates in vitro then determines which of these discrete subclones will survive, for example, which ones have reached a higher peak. We know that epigenetic events occur frequently in vitro at rates of 10^{-3} to 10^{-5} per cell generation and that epigenetic events produce stochastic distributions of cell populations.

This phenomenon in plant cells displays some of the characteristics found in asexual reproduction of Tetrahymena pyriformis (Allen and Gibson 1973). In this ciliate, there is a regular and frequent (approximately 10^{-3} locus per cell generation) diversification of phenotype within a clone which occurs in both heterozygotes and homozygotes.

The basic finding is that in heterozygous clones, after they manifest the dominant or codominant phenotype for several cell generations after mating, a number of distinct and stable subclones appear with the expression of only one of the two alleles. This process has been termed allelic assortment. The subclones have a stochastic distribution and are not genetic in origin, because the subclones breed as heterozygotes. Additionaly, allelic assortment occurs in—dependently at each genetic locus, resulting in a tremendous array of different phenotypes.

Although the mechanisms underlying allelic assortment are not understood, the process has been used to recover normally recessive genetic phenotypes from genetically heterozygous genotypes. The result of allelic assortment is a phenotypically discontinuous population of subclones which are genetically identical in their genetic behavior in breeding.

The result of these discontinuous phenotypes in plant cell cultures is a bit like Müllerian mimicry. The cell population contains a number of subclones, all of which have come to resemble one another in their growth characteristics but which differ markedly in their polypeptide composition (often by more than 20% of their total polypeptides). All subclones should be expressing basic metabolic pathways, because they are growing in a medium where these are essential. Hence, cell selection screens designed to recover variants in these pathways should be successful in every subclone of a population. However, selection screens designed to recover variants in pathways not essential for growth in culture either may not be successful or may yield confusing or irreproducible results with in a population or between different cell lines.

Genetic variants of most interest to plant breeders often are not those that involve basic housekeeping functions, but those that determine other physiological and developmental characters. Polypeptides controlling these characters probably are most commonly those fluctuating in response to epigenetic variation, the most difficult in which to recover genetic variants,

Additionally, polypeptides determining characters of interest are expressed within a fluctuating background of changing epigenetic expression. Consequently, it may be impossible to recognize the effects of genotype environment interaction, heritability, dominance, and epistasis in cell culture systems where the quantitative and qualitative expression of polypeptides is unpredictable. For the time being, we should select variants for complex agronomic characters at the whole—plant or organ level. Repeatable and sequential epigenesis occurring in organisms provides a better matrix for mutant selection of agronomic characters than does in vitro cell culture. The challenge of controlling epigenetic variation remains unmet, despite its great importance. We have only the beginnings of a taxonomic catalog of the possible epigenetic configurations which a single cell can assume.

TYPES OF GENETIC VARIABILITY AND THEIR USE

Plant cell and molecular geneticists have developed or will soon develop genetic manipulations which permit a wide array of modifications. These genetic manipulations will be important in altering a number of agronomic characteristics of crop species. However, different individual manipulations may have specific utility in approaching defined agronomic goals. The use of mutant induction and selection will provide adequate solutions to some problems, whereas the introduction of a small number of genes via genetic transformation may provide solutions to others. We do not as yet have the

necessary experience to predict the optimal use for each type of manipulation. Work on microbial systems, particularly on the production of and resistance to antibiotics, provides an initial comparison of the effectiveness of different types of genetic alterations. This comparison is instructive as a model for work with plant materials.

The production of the antibiotic penicillin by members of the Peniccillium crysogenum group of fungi is a well-studied, although not completely understood, process. A number of genetic manipulations have been used in attempts to increase penicillin titer, including mutant induction, heterokaryon production, and manipulation via the parasexual cycle. The most productive approach has been mutant induction and selection. There is little evidence that any technique other than mutant selection has been used successfully with P. chrysogenum.

Variation in penicillin production among wild isolates growing in submerged culture ranges from 0 to 80 units/ml. However, one isolate that produced 100-200 units/ml was recovered. This one strain, NRRL 1951 B25, derived from a single haploid nucleus provides the basis for the vast majority of all industrial strains derived by mutation and selection. During the 1950s, mutations which raised the yield of that original narrow genetic base to 250, then to 400, then to 1000, and finally to 2,500 units/ml were selected. This was accomplished by selecting for a final phenotype without any clear knowledge of the intermediary biochemical steps. In these highly selected strains, degeneration and loss of viability was common but could be controlled by environmental manipulations.

A difficult and somewhat unpredictable problem was that of scaling up production from laboratory testing conditions to large industrial fermenters in which environmental conditions varied. From studies of parasexual crosses between different strains of P. chrysogenum followed by mitotic recombination and haploidization analysis, it is clear that genes determining penicillin yield are limited in number and are located throughout the genome and that alleles for increased production are recessive. The expression of alleles is modified by the genetic background and by the culture environment (Burnett 1975). The highly selected genotypes require a specific environment for their expression.

Acquisition of antibiotic resistance by bacteria is another case in which genetic mechanisms have been studied. Although mutations to resistance are often recovered in laboratory experiments and might be expected to provide a common mechanism operating in natural populations, this is not the case. Antibiotic resistance in natural populations seems to have arisen in almost every case, not by de novo mutation and selection, but by mobilization of genes from other organisms via plasmids, viruses, and transposons (Reanney 1976). Mobilization of genetic information from

other organisms appears to provide pre-existing mechanisms to overcome specific growth limitations. This genetic material has already been selected and refined by environmental forces. Genetic change is accomplished by acquisition rather than by mutation.

Most systems in which mutations that alter the specificity of an enzyme or other protein have been selected show that these structural changes have secondary effects. Proteins with altered specificity are less thermostable and are more rapidly denatured in widely varying natural conditions. Fitness of an organism or a natural population is better served by utilizing genetic transfer systems to confer resistance. Natural resistance to antibiotics exists in species of bacteria, fungi, and higher organisms (Koch 1981).

An interesting case is the acquisition of trimethoprim resistance in the enterobacteria. Trimethoprim is an inhibitor of dihydrofolate reductase in all organisms. However, the bacterial enzyme is about 10,000 times more sensitive to the antibiotic than is the mammalian enzyme (Burchell 1975). Bacterial mutants that have up to 6-fold higher levels of dihydrofolate reductase have been selected in the laboratory (Sheldon and Brenner 1976).

This mechanism has not been observed in clinical resistance, perhaps because deregulation of this vitamin biosynthetic pathway interferes with other bacterial functions and limits population growth rates. Clinically resistant bacteria carry a qualitatively different dihydrofolate reductase, which is plasmid meditated. The new dihydrofolate reductase which supplements the normal chromosomal encoded enzyme is 10,000 less sensitive to trimethoprim, does not increase the biosynthetic rate of the pathway, and is not readily denatured (De Datta and Hedges 1972). The source of this plasmid-carried gene is not known, but it seems certain that it did not arise in bacteria via mutation over the few years of use of this drug. It is not an example of structural gene evolution (Koch 1981).

What are the lessons from these two microbial examples for efforts in plant genetics? They both relate to genotype-environment interactions. If the environmental component is minor or can be controlled, then mutant selection for a desired objective may prove adequate. This is particularly true if variability for the character does not exist in natural populations or if the character is genetically complex.

However, if there is a major environmental component in determining the character, then gene transfer techniques may be more successful. Gene transfer techniques are also appropriate if the character is genetically simple and biochemically defined. Experience with plant materials will provide the best lessons.

CONCLUSIONS

Plant cell and molecular geneticists must be candid about where and how their manipulations can assist plant breeders. They must realize that, for plant breeders to adopt a new technique, the technique must provide a unique or improved solution to a problem, be relatively simple, and provide guaranteed results. Additionally, they must begin to deal with the statistical descriptions of the world which underlie the science of plant breeding and realize that the goal is no longer the laboratory demonstration of a manipulation in a model system but its application in a farmer's field with a crop species.

Plant breeders can remain interested skeptics but should provide information, discussion, and direction. Most importantly, they must be ready to actually use the new techniques, once they are developed and shown to be effective.

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POSTER ABSTRACTS

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1. Chen Ying: Anther Cold Pretreatment and Preculture Effects on Initiation and Development of Isolated Rice Pollen.
2. Liang Chen—ye: Cytoplasmic Origin of Pollen Plants Obtained from Anthers.
3. Ling Ding—hou: Cytogenetic Study of Homologous Asyndetic Triploids Derived from Rice Anther Culture.
4. Sun Li—hua: Induction of Diploid Plants from Haploid Rice Tissues.
5. Tian Wen—zhong: Increasing Differentiation Frequency of Green Plantlets in Rice Anther Float Culture.
6. Zhao Cheng—zhang: Characteristics of Rice Plants Regenerated In Vitro and their progeny.
7. He Ding—gan: Response of Wheat Anthers at Different Stages to In Vitro Culture.
8. Lu Wen—liang: Cell Division in Differentiation of Wheat Pollen.
9. Zhuang Jia—jun: Increasing Differentiation Frequencies in Wheat Pollen Callus.
10. Wei Zhi—ming: Wheat Pollen Culture and Regeneration of Plantlets.
11. Zheng Wan—Zhen: Effects of Plant Hormones on Maize Anther Culture.
12. Xu Zhi—hong: Conditioning Factors in Barley Anther Culture.
13. Chen Zhi—zheng: Induction of Rape Embryoids from Stage Float Culture of Anthers.
14. Chen Zheng—hua: Induction of Sugarcane Haploid Plants.
15. Kuo Chung—shen: The Culture of Unfertilized Rice Ovaries.
16. Zhu Zhong—chun: Induction of Haploid Plantlets from Unpollinated Wheat Ovaries.
17. Shao Qi—quan: In Vitro Fertilization of Maize Ovaries and Ovules.
18. Zhu Zhong—chun: Production of Haploid Plantlets from Unpollinated Ovaries.
19. Wang Ching—chu: Endosperm Culture of Wheat/Rye.
20. Hu Zhong: Culture of Young Haploid Rice Panicle Fragments.
21. Chen Shao—lin: Isolation of BUdR—Resistant Mutant in Soybean Cells.
22. Wang Ching—chu: Effect of Actinomycin D on Induction of Pollen Callus.
23. Cai Qi—Gui: Regeneration of Plants from Datura Callus Protoplasts.
24. Sun Yong—ru: Regeneration of Somatic Hybrid Plants.

25. Wang Pei-tian: Interspecific Hybrid Plants by Protoplast Fusion.
26. F.T. Wang: Effects of Surface Charge on Protoplast Fusion.
27. Kuo Chung-shen: Morphology and Cytology of Embryoids Derived from Maize Callus.
28. Liu Ying-jin : Assay of Rice Plant Tissue for Cold Injury.

ANTHER COLD PRETREATMENT AND PRECULTURE EFFECTS ON INITIATION AND DEVELOPMENT OF ISOLATED RICE POLLEN

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Initiation and development of isolated rice pollen were strongly influenced by cold pretreatment of anthers. Isolated pollen grains remained at the uninucleate stage, even when cultured in a conditioning medium after being separated from fresh anthers.

Some pollen isolated from anthers precultured for 1 day divided several times, but all degenerated. Pollen isolated from anthers precultured for 2—3 days sometimes developed into multicellular pollen, but those isolated from anthers precultured for 5 days gave only multicellular masses and calli.

When cold pretreatment was increased to 10—30 days, initiation frequency and androgenesis level were enhanced. Some calli and green plantlets were obtained.

The best results were obtained from a combination of 10—15 days cold pretreatment and 3—4 days preculture. A large amount of pollen developed into multicellular masses or calli, part of which differentiated into green plantlets

Pollen were examined cytologically in the anther after pretreatment or preculture. Most pollen grains isolated from 5—day precultured, 20— to 30—day pretreated anthers divided once. Some divided several times.

CYTOPLASMIC ORIGIN OF POLLEN PLANTS OBTAINED FROM ANTHERS

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The cytoplasmic origin of the pollen plant derived from rice anther culture was studied using the male sterile gene as a marker.

Pollen plants induced by anther culture from the sterile lines (ms) were still male sterile. Three kinds of pollen plants (male sterile, male fertile, and male semi-fertile) were obtained from a three-line hybrid in which the male-sterile parent was an abortive type of wild rice. When the male-sterile or fertile pollen plants derived from anther culture of a three line hybrid were topcrossed and backcrossed with the typical maintaining line of the abrotive type of wild rice as male parent, the offspring were all male sterile. But when the male sterile or fertile pollen plants were crossed with the restoring line as male parent, the offspring were all male fertile.

These results confirm that male sterile pollen plants derived from the hybrid had the same genetic characteristics as the male sterile line of wild rice. Sterility and fertility restoration in the male sterile line of the abortive type of wild rice was determined by the interaction of the nucleus and cytoplasm.

CYTOGENETIC STUDY OF HOMOLOGOUS ASYNDETIC TRIPLOIDS DERIVED FROM RICE ANTHER CULTURE

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Two homologous asyndetic triploid plants were discovered among 23 pollen plants of the first generation derived from an intercultivar hybrid of rice. Cytogenetic study of meiosis of these triploids found no chromosome pairing in diplotene, diakinesis, and metaphase I. Thirty—six univalents were observed. The distribution of lagging chromosomes was continuous in anaphase I.

Other abnormal phenomena in meiosis were: 1) abnormal spindle appearances were divergent spindle in V shape, curved spindle in C shape, and polypole spindle; 2) syncytium made from 2 to more than 10 PMCs; 3) tetrad division; and 4) abnormal microspores (from monads to octads) in the tetrad stage. These two triploids may be considered asyndetic because evidence of nonpairing chromosomes had not been obtained before in diplotene.

The origin of the asyndetic gene of these two asyndetic triploids might be a result of 1) recessive mutation, 2) dominant mutation, or 3) asyndetic gene segregated from hybrid F_1 pollen. We consider recessive mutation the best explanation.

INDUCTION OF DIPLOID PLANTS FROM HAPLOID RICE TISSUES

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Tissues of haploid plants derived from rice pollen (internode, node, young spikelet, leaf sheath and blade, and axillary bud) were cultured. The doubling ratios of diploid plants (2n=24) induced were 50% for stem and node and 10% for young spikelet. No diploid plants we're obtained from the axillary bud and no seedlings developed from leaf sheath and blade culture.

The basic medium was MS. Two mg 2,4—D/liter and 2 mg kinetin/liter were added to the dedifferentiation medium; 2 mg kinetin/liter, 0.25 mg NAA/liter, and 0.25 mg IAA/liter were added to the differentiation medium, and 0.1% active carbon was added to the rooting medium.

INCREASING DIFFERENTIATION FREQUENCY OF GREEN PLANTLETS IN RICE ANTHER FLOAT CULTURE

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Induction of calli from rice anthers can be greatly increased by float culture (a mean maximum number of calli per anther up to 8), but the differentiation frequency is low.

Four induction and four differentiation media were tested. A japonica rice cultivar was used. Anthers with microspores at the late uninucleate stage were pretreated at 8—10°C for 10 days before inoculation, then floated on a liquid medium.

The effect of the induction medium on the differentiation ability of calli was higher than the effect of the differentiation medium. Callus differentiation of green plantlets as high as 50% was obtained from potato medium sterilized by filtration. Calli floating on the liquid medium had a higher differentiation ability than those that sank to the bottom of the medium. The earlier calli were transferred to the differentiation medium, the higher the differentiation.

CHARACTERISTICS OF RICE PLANTS REGENERATED IN VITRO AND THEIR PROGENY

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Variations in quantitative characters of growth period, plant height, panicle number, 1,000-grain weight, and grain number per panicle and qualitative characters of, awn presence and panicle shattering were observed in 4,282 D_1 rice plants regenerated from somatic cells and dehusked seeds of young rice panicles of 23 varieties and 1,087 pedigrees.

Genetic alterations in most D_2 pedigree lines were stable and heritable. Some promising lines in D_2 populations preserved most of the traits of the original varieties and had new desirable single or integrated characters.

Quantitative characters in the D_2 and D_3 were compared with those of the original varieties. Significant differences were found in the inducibility of some character variations. An increase in panicle number and a shortening of plant height occurred, probably induced by the reactivation of genes that had been inhibited during the tissue culture process.

RESPONSE OF WHEAT ANTHERS AT DIFFERENT STAGES TO IN VITRO CULTURE

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Wheat pollen was divided into ten developmental stages: 1) pollen mother cell, 2) meiosis, 3) tetrad, 4) early uninucleate, no germ pore, 5) early uninucleate, germ pore, 6) mid-uninucleate, 7) late uninucleate, 8) premitosis, 9) binucleate, and 10) trinucleate.

Two anthers each from the first and second florets at the middle part of the spikelet were inoculated on potato-II medium. One anther from the same floret was fixed and stained with aceto-carmine to ascertain the developmental stage. Experiments with five different materials showed that mid- and late-uninucleate stages were most suitable for anther culture. In some materials, anthers of any stage could produce calli and plantlets.

Root tips from plantlets growing on differentiation medium were stained with modified carbo1 fuchsin to examine their ploidy. Preliminary results showed that both haploid and diploid plantlets can be obtained from anthers at tetrad, early uninucleate, mid-uninucleate, late uninucleate, premitosis, and binucleate stages.

CELL DIVISION IN DIFFERENTIATION OF WHEAT POLLEN

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Observation of cytological characteristics of various types of pollen grains at the initial stage of culture showed: 1) mitosis frequently arising during days 1—5 culture was mainly first division in differentiation instead of first division in dedifferentiation; 2) mitosis after 6 days of culture was a dedifferentiation division; 3) amitosis after 6 days of culture was a dedifferentiation division. Amitosis made up about 97% of the divided cells and mitosis made up about 3%. The dedifferentiation division was po— larity—free. The appearance of amitosis might be related to the loss of polarity.

INCREASING DIFFERENTIATION FREQUENCIES IN WHEAT POLLEN CALLUS

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The differentiation frequency of green plantlets on 190-2 medium was 38.3% higher than on modified MS medium (major inorganic salts reduced by half). Medium 190-2 also was better for plantlet differentiation than N_6 media containing 3% and 8% sucrose. Differentiation medium 190-2 contains: KNO_3(1,000 mg/liter), $(NH_4)_2SO_4$ (200 mg/liter), $Ca(NO_3)_2 \cdot 4H_2O$ (100 mg/liter), KH_2PO_4 (300 mg/liter), $MgSO_4 \cdot 7H_2O$ (200 mg/liter), KC1 (40 mg/liter), $FeSO_4 \cdot 7H_2O$ (27.8 mg/liter), $Na_2EDTA \cdot 2H_2O$ (37.3 mg/liter), $MnSO_4$ $4H_2O$ (8 mg/liter), $ZnSO_4 \cdot 7H_2O$ (3 mg/liter), H_3BO_3 (3 mg/liter), KI (0.5 mg/liter), glycine (2 mg/liter), thiamine-HC1 (1 mg/liter), pyridoxine-HC1 (0.5 mg/liter), nicotinic acid (0.5 mg/liter), meso-inositol (100 mg/liter), sucrose (3,000 mg/liter), agar (7,000 mg/liter), NAA (0.5 mg/liter), and kinetin (0.5 mg/liter).

When 3% sucrose was added, a green plantlet differentiation rate of 42.5% was obtained. With 6% sucrose, differentiation decreased to 27.5%; with 9% sucrose to 20%.

Calli cultured on a liquid medium containing 0.04% colchicine at 8°C for 72 hours before transfer to agar medium differentiated 35% more green plantlets than calli cultured on a medium without colchicine. Treatments with 0.01% and 0.02% colchicine also were efficient. Spontaneous chromosome doubling was higher in colchicine—treated callus than in untreated callus.

When kinetin concentration was increased from 0.5 mg/liter to 1 mg/liter and 2 mg/liter, green plantlet dif—

ferentiation did not increase but albino plantlet differ—entiation did.

Calli from anthers cultured at normal temperature for 35 days differentiated 56% and 52.8% plantlets. Calli from anthers cultured 107 days differentiated 16.6% and 7.5% plantlets. Subcultures had slight influence. Preservation at low temperature maintained differentiating ability for a long time and enhanced spontaneous chromosome doubling. The effects varied with genotype.

WHEAT POLLEN CULTURE AND REGENERATION OF PLANTLETS

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Excised wheat flower buds were cold treated at 4—5°C for 8 days, then anthers were floated on a liquid medium. C medium (pH 5.8) for small calli induction was supplemented with 800 mg glutamine, 100 mg serine, 5 g myoinositol, 2—3 mg 2,4-D, and 50 g sucrose/liter. When only the anthers were inoculated at the middle or late uninucleate stage, shed pollen developed into small calli. Small calli were transferred to fresh agar medium.

At 2—3 mm diameter, calli were transferred to MS medium (pH 5.8) supplemented with 0.3 mg IAA, 1 mg KT, 30 g sucrose/liter. Some gave rootless green or albino shoots. Rootless green shoots transferred to 112 MS plus 0.5 mg IAA, 0.1 mg KT, and 20 g sucrose/liter for rooting differentiated into intact plantlets.

EFFECTS OF PLANT HORMONES ON MAIZE ANTHER CULTURE

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Plant hormones as auxin (2,4-D, NAA), antiauxin (2,3,5-TIBA), and cytokinin (kinetin, 6-BA) were added to the basal medium N_6 or Yupei in maize anther culture. The response of anthers varied with type of hormone.

The maize anther was not sensitive to auxin (2,4-D, NAA).

Response to antiauxin was unstable. In the presence of 0.5 mg 2,3,5-TIBA/liter, the induction frequency of embryoid and callus of hybrid Chinghuang 13 on Yupei medium usually was enhanced. No effect was found on N_6 medium.

The maize anther was rather sensitive to cytokinin. Kinetin (1 mg/liter) either alone or in combination with 2,4-D or 2,3,5-TIBA, appeared to be most effective.

Although the addition of appropriate hormones enhanced the yield of pollen embryoids, exogenous hormones were unnecessary to start pollen cell division. Embryoids and calli were produced even on unsupplemented basal media (N_6 or Yupei). However, induction frequencies usually were lower than in treatments supplemented with exogenous hormones.

Hormones also played an important role in embryoid formation. Raising the level of 2,4-D from 2 mg/liter to 4 mg/liter apparently inhibited embryoid formation. Induction of embryoids was enhanced as the level of auxin was lowered and the level of cytokinin was raised.

The chromosome number and the ploidy level of H_1 pollen plants and embryoid cells usually were affected by the type of hormone supplement in the induction medium. In the presence of a combination of hormones (2 mg 2,4-D/liter + 1 mg KT/liter + 2 mg 6-BA/liter), the number of chromosomes, especially in diploid cells (2n=20), increased and the ploidy level of pollen plants was raised. In the presence of 2,3,5-TIBA, the number of chromosomes was reduced, the number of haploid cells (n=10) was increased, and the proportion of pollen plants with a predominance of haploid cells was enhanced to 85%.

CONDITIONING FACTORS IN BARLEY ANTHER CULTURE

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Productivity of cultured barley (Hordeum vulgare cv. Sabarlis) anthers was enhanced by using media previously conditioned by anthers of the same genotype. Batch anthers produced 7—82 times more calli in conditioned media than in the control media. Anthers averaged more than 50 calli and virtually all anthers were responsive.

Anthers containing pollen at the early bicellular stage gave the most effective conditioning. The optimal length of contact between anthers and medium was 7 days.

Adding exogenous hormones was not important to the formation of multicellular structures when anthers were cultured in a conditioned medium or at high inoculation density although exogenous hormones stimulated callus growth. The potato medium was better than N_6 medium for sustaining size and vigor of pollen—derived calli growth.

Glutamine at 160 or 800 mg/liter was ineffective or inhibitory. On the other hand, m—inositol at 1,000 mg/liter partly replaced the effect of the conditioned medium. Inositol interacted synergistically with callus formation. The enhancing influence of the conditioned medium was destroyed by heat, suggesting that callus formation is thermolabile. The synergism of callus formation with growth supplement also was destroyed by heat.

The need for high inoculation density could be replaced by using conditioned media or by the culture of relatively few anthers in drops of medium (0.2 ml).

INDUCTION OF RAPE EMBRYOIDS FROM STAGE FLOAT CULTURE OF ANTHERS

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Two cultivars of Brassica napus L., Tower and Marnoo, were used. Histological and cytological evidence for development from microspore to embryoid showed that microspores at different embryogenesis stages required different culture conditions. Development from microspores to embryoids visible to the eye was in three stages: early cell division (ECD), multicellular mass development (MMD), and embryoid formation (EF).

Media used for developmental stages were: ECD, liquid basic medium containing 10% or 20% sucrose and free from hormones; MMD, liquid basic medium containing 10% sucrose and 0.1 mg 6—BA/liter, 0.1 mg 2,4—D/liter, and 0.1 mg NAA/liter; and EF, solid B_5 medium containing 2% sucrose and free from hormones.

Cold pretreatment of buds before inoculation significantly affected embryogenesis, with maximum embryo yields when materials were pretreated 5—6 days at 6—9°C.

Stage float culture, cold pretreatment, and elevated culture temperature were combined to induce a maximum relative embryo yield (no. of embryoids obtained/no. of anthers cultured) of more than 800%.

INDUCTION OF SUGARCANE HAPLOID PLANTS

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Current work began in 1974 and the first green plantlet from sugarcane anther culture was obtained in March 1979. Since then, 30 calli have been obtained from which hundreds of green plantlets have been differentiated. These plants grow normally in the field. In 1980, 80 calli were obtained from 8 lines. Hundreds of green plantlets have been dif— ferentiated from calli orginating from five lines.

Systematic microscopic observations on the development of microspores in culture were made to choose the best line: for culture, the optimum stage of anther development for inoculation, and the optimum medium. The suitable stage of pollen for inoculation was the diadtetradspore stage. Modified MS or N_6 medium was used as the basic medium for callus induction. Twenty percent sucrose appeared to be the optimum concentration. On a medium supplemented with 2 mg 2,4—D/liter and 2 mg kinetin/liter, microspores developed normally and the multicellular masses grew rapidly.

Modified MS medium (5% sucrose) supplemented with 2 mg 6—benzylaminopurine/liter and 2 mg napthalene ascetic acid/ liter was used for embryoid differentiation. The number of green plantlets was increased by the addition of lacto— protein hydrolysate and various amino acids.

Cytologenetic observation showed that the chromosome number of somatic cells of the original lines was 120; that of the root tips of the plantlets ranged from 50 to 90. These results demonstrate that the plantlets originated from pollen grains.

Examination of the pollen plants transplanted in 1979 showed that agronomic characters segregated intensively. One plant had many agronomic characters better than the donor and a brix of 24—25% (3% higher than that of donor plant). This plant has been cloned.

THE CULTURE OF UNFERTILIZED RICE OVARIES

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The inducing medium was N_6 supplemented with 2 mg 2,4-D/liter, 500 mg casien hydrolysate/liter, and 4% sucrose. The differentiating medium was N_6 supplemented with 2 mg kinetin/liter, 500 mg casein hydrolysate/liter, and 3% sucrose. Four cultivars and two crosses were used as experimental materials.

Differences in induction frequency of calli occurred among cultivars. Frequency of induction was higher in crosses than in cultivars. Twelve green plantlets and 3 albino plantlets were obtained and the chromosomes of 11 green plants were examined. Among them, 6 plants were haploid (n = 12) and 5 were diploid. The embryoids were located at the micropolar end. Some possessed a suspensor similar to zygote embryos.

Calli were from different origins. One originated from haploid tissue derived from the nucleus in the embryo sac, another originated from diploid tissue in the integument or ovary wall, and one originated from the unfertilized ovary.

INDUCTION OF HAPLOID PLANTLETS FROM UNPOLLINATED WHEAT OVARIES

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In preliminary experiments, all haploid plantlets regenerated from unpollinated ovaries were derived from differentiating callus. In subsequent experiments, healthy green haploid plantlets with 19 tillers and strong root systems were obtained through the direct development of ovaries instead of from callus transferred to differentiation media.

Tillers of these plantlets, separated and cultured individually, developed many new healthy plantlets.

IN VITRO FERTILIZATION OF MAIZE OVARIES AND OVULES

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Unpollinated ears 4-5 days after silk emergence were dehusked. Silks were cut to 1 cm length and ears were cut into blocks with about 10 ovaries each. Ovary blocks were pollinated with sterilized maize pollen and cultured in test tubes at 25-30°C. Seeds formed in 7 days and mature seeds were produced 30 days after pollination. Average seed set was 9.1%. Intact plants have been obtained from the 70 mature seeds obtained.

The upper third of corn ovaries was removed and the naked ovules pollinated. The same culturing procedures as for ovaries were followed. The average seed set was 0.42%. Seeds germinated 20-22 days after pollination and developed into plants.

PRODUCTION OF HAPLOID PLANTLETS FROM UNPOLLINATED OVARIES

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Haploid plantlets regenerated from unpollinated ovaries of Nicotiana tabacum, first reported in 1979, were derived directly from ovules. The chromosome number of the root tips was haploid, which gave rise to sterile seeds. Haploid plants were more dwarf and had weak growth vigor and small flowers compared with diploids. The style of haploid plants was longer than the filament.

Morphological traits and banding patterns in electro—phoresis differed. between embryo sac plants and pollen plants.

Haploid plants also were obtained from unpollinated ovary cultures of haploid plants derived from ovary culture. Plantlets derived directly from the ovules. The chromosome number of the root tips was haploid, which gave rise to sterile seeds.

Most ovules were at the megaspore mother cell stage before culture. After culture, the megaspore mother cell of most ovules underwent division and megaspore, free nuclei, cell masses, and further embryoid or multiembryoids were formed. The haploid plantlets obtained derived from the immature embryo sacs.

ENDOSPERM CULTURE OF WHEAT/RYE

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The endosperm of wheat/rye was inoculated on different culture media with different combinations of plant hormones 7-14 days after crossing. W medium containing 2 mg 2,4-D/liter, 0.5 mg kinetin/liter, and 8% sucrose was effective for the induction of endosperm callus. After one week in culture, a white callus appeared from some endosperm faces.

When the calli were transferred onto an auxin stepdown medium for plantlet regeneration, both shoots and roots were formed. Of the media tested for shoot differentiation, w medium supplemented with 0.2 mg NAA/liter and 2 mg kinetin/liter was best.

Four endosperm plantlets were obtained. The chromosome number in root-tip cells of one endosperm plantlet was unstable. Chromosome numbers varied from 7 to 30. No chromosomes numbered 49. There were a large number of aneuploid cells.

CULTURE OF YOUNG HAPLOID RICE PANICLE FRAGMENTS

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Fragments of young haploid panicles cultured on N_6 medium with 1 mg 2,4—D/liter are easy to induce into calli. About 30% of the calli were transferred onto a medium with additional NAA 0.5 + kinetin (2 mg/liter). Shoot buds initiated from tissue at the base of young spikelets and from the cut end of fragments, not from young glumes and ovaries. About 12 clusters of plantlets could be regenerated from fragments of 1 panicle.

Albino plantlets were less than 2%. About 98% of the regenerated plants were haploids.

Treating calli with colchicine at 200 mg/liter in 2% dimethyl sulphoxide (DMSO) water solution for 24 hours at 26°C produced 50% fertile diploids in regenerated plants without a decrease in callus differentiation frequency.

ISOLATION OF BUdR-RESISTANT MUTANT IN SOYBEAN CELLS

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Cells of soybean cell line SB—1 were irradiated with 1,000 g—rays and cultured on g—ray—irradiated B_5 medium for 20 days. After 10 days, culture on B_5 medium containing 20 µg/ml BUdR and µg uridine/ml, most cells were dying. Five cell colonies survived and could grow on BUdR—B_5 medium in subculture.

Colonies of BUdR—resistant mutant transferred on B_5 medium for 84 days showed as vigorous growth on BUdR—B_5 medium as did the control on B_5 medium. The BUdR—resistant characteristic was retained but irradiation of the cells alone was ineffective. Control cells on BUdR—B_5 medium all died.

We concluded that: 1) A stable BUdR—resistant mutant in soybean cell line SB—1 has been isolated. This mutant might be useful as a genetic marker in somatic hybridiza—tion. 2) Chemical changes in the culture medium might be induced by irradiation in addition to the direct effects of irradiation on the cultured cells. Combining the direct and indirect effects of irradiation may be useful in muta—tion induction.

EFFECT OF ACTINOMYCIN D ON INDUCTION OF POLLEN CALLUS

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The yield of pollen callus in some cereals can be increased by treatment with 10 μg actinomycin D/ml for 1—2 days during the early stage of anther culture. Induction frequency of pollen callus was 8.4% in coix, 28.8% in wheat, and 69.4% in rice (0.9, 1.1, and 4 times more than the controls, respectively). Cytological observation showed that devel—opment of pollen cells was slow, callus stayed in the uninucleate stage for a longer time, and division of cells was generally delayed. But once division occurred, the num—ber of pollen cells developing toward the sporophyte pathway increased. Apparently, in the early stage of culture the actinomycin D affected the DNA template of the pollen cells and inhibited its normal transcription.

REGENERATION OF PLANTS FROM DATURA CALLUS PROTOPLASTS

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Diploid calli derived from stem segments (Datura innoxia Mill) were subcultured on modified DPD medium. Calli were incubated in cellulase (3%), driselase (0.5%), hemicellulase (0.5%), macerozyme (1%), potassium dextran sulfate (0.5%), and 1 mM $CaCl_2 \cdot 2H_2O$ in Murashige-Skoog medium with 0.6 M mannitol at 28° C for 3 hours.

Protoplasts were cultured in the modified liquid culture medium for tobacco protoplast. The first division of the newly formed cells was completed after 3 days of culture. After 1-2 weeks, protoplasts developed into a small cluster. After 4-5 weeks, calli 1 mm in size were transferred to an auxophyton. When calli were 2-3 mm in size, they were transferred to modified B-MS differentiation medium in which normal shoots and shoots with cup-shaped leaves differentiated.

REGENERATION OF SOMATIC HYBRID PLANTS

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Protoplasts of N. glauca and P. hybrida were fused by PED solution and cultured in liquid medium. Three morphological types of calli were observed on MS medium + 0.2 mg IAA/liter and 1 mg 6—BA/liter. Loose callus was light green and grew fast, similar to N. glauca. Compact callus was dark green and grew slowly, similar to P. hybrida. A third callus was intermediate in growth rate, quality, and color with a flat plate shape.

Shoots differentiated from nine calli of the interme— diate type. Clump shoots without roots differentiated from one callus on rooting medium (MS medium + 0.2 mg NAA/liter).

Some young plants were morphologically different from both parents but mature plants were similar to the N. glauca parent.

Analysis of peroxidase isoenzymes and cytology showed that characteristics of regenerated plants were similar to both parents. The isoenzyme bands were in the same positions as those of both parents. Chromosome number of hybrid plants G—H was 2n=32 to 2n=38. Chromosomal bridge, breakage of chromosomal bridge, and chromosomal adhesion were observed at anaphase. These abnormal phenomena are often found in cells of hybrids with distant parents.

The regenerated plants were somatic hybrids of N. glauca/ P. hybrida. In fusion experiments, it is possible to use morphological calli traits to select hybrids.

INTERSPECIFIC HYBRID PLANTS BY PROTOPLAST FUSION

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CY4 (N. tabacum) and Yellow Flower (N. rustica) have been used in protoplast fusion studies started in 1979. CY4 has a long stem, long flower tube, long ovoid leaf without leafstalk, and large red corolla. Yellow Flower has a short stem, short flower tube, ovoid leaf with leafstalk, and small yellow-green corolla. A sexual cross between them is difficult.

The fusion procedure was modified by replacing the hanging drop method with sedimentation in petri dishes. A low rate of protoplast damage and a high frequency of protoplast fusion were obtained.

Some regenerated plants blossomed in June but most blossomed in August 1980. They divided into three types:

Type 1. Twenty-two plants with long stem, flower tube, ovoid leaf without leafstalk, and large red corolla similar to CY4 were developed from protoplasts of CY4.

Type 2. Nineteen plants exhibiting intermediate characteristics of long stem, large light-purple corolla, middle flower tube, and ovoid leaf without leafstalk were somatic hybrids between CY4 and Yellow Flower.

Type 3. Four plants regenerated from one piece of callus with characters identical to those of CY4 except for pink flowers were assumed to be somatic hybrids by partial fusion

No plants identical to N. rustica were observed, probably because protoplasts isolated from this species could not be grown by this method.

N. tabacum and N. rustica are diploids (2n=48). The chromosome number of plants with red and pink flowers was ca. 48. In plants with light-purple flowers, it was 57-91. Most had 60-80.

Zymogram patterns of peroxidase and cytochrome oxidase of plants with red flowers were same as CY4. Zymograms of plants with light- purple and pink flowers were complementary to those of the parents. This indicated that such plants were somatic hybrids.

EFFECTS OF SURFACE CHARGE ON PROTOPLAST FUSION

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The surface charge of protoplasts (carrot root, celery mesophyll, and tobacco mesophyll) was determined by cell electrophoresis and protoplast fusion frequencies in different ions was calculated. There was no positive correlation between the reduction of protoplast surface negative charge and protoplast fusion frequency. Electricity of protoplasts is one of many factors that influence the fusion percentage.

MORPHOLOGY AND CYTOLOGY OF EMBRYOIDS DERIVED FROM MAIZE CALLUS

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Most embryoids were produced from the surface layer cells of callus, with a few from the inside layers. The initial cell of the embryoid possessed denser protoplasm and a larger nucleus than other cells.

The developmental sequence of the initial cell was similar to that of the zygotic embryo. The first division gave rise to two daughter cells, basal and terminal. The basal cell either divided or did not divide and changed into the suspensor.

The terminal cell divided first longitudinally. Then four cells formed through transverse divisions. As the four cells further divided, the embryo proper was formed. The embryoids possessed one, two, or polycotyledonous embryoids. Production of embryoids from the callus was not synchronous and embryoids in different development stages could be found in the same callus.

ASSAY OF RICE PLANT TISSUE FOR COLD INJURY

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Aseptic plant tissue assays are being done to study the mechanisms of cold injury and tolerance of rice as well as for breeding by cell line selection. Preliminary results show that:

Cold—injury symptoms in test—tube seedlings of indica cultivars Bao—Shi No. 2, Ru—Ba Dwarf, and Kwe—shao No. 2 were the same as those that appeared in the field.

Cold treatment eventually disrupted the cellular integrity of rice plant tissue. Subsequent leakage of cell contents from callus tissue measured by DDS—11A type electrical conductivity meter indicate that this assay can be used to characterize rice lines or cultivars for cold tolerance.

Cold temperature causes a disproportionate decrease in respiration in cold—sensitive species. The reductive capacity of triphenyl tetrazolium chloride was assayed using 480 nm spectrophotometer. Results on callus tissue of three rice cultivars with different cold tolerances indicate that this assay can be used as a rapid indicator of ability to respire. Reductive capacity usually decreases during cold injury.

WORKSHOP RECOMMENDATIONS

The knowledge and understanding among researchers in the basic sciences of the goals and problems of scientists in the applied areas has been limited. That premise leads to these recommendations:

General:

- Realize the importance of interactive team work among plant breeders, physiologists, geneticists, pathologists, and scientists from other disciplines in the practical application of tissue culture.
- Arouse the interest of plant breeders in these innovative approaches and initiate breeding programs in which tissue culture techniques can be applied to the improvement of crops.

Somatic Cell Culture:

- Improve techniques of cell suspension culture for the selection of mutant cell lines tolerant of or resistant to such stresses as toxins, salt, and aluminum.

Haploids:

- Establish a more efficient system for haploid plant production through pollen or anther culture and ovary/ovule culture.
- Intensify research on other methods of haploid plant production, such as by chromosome elimination.
- Develop a reliable technique for doubling the chromosomes of haploid plants.

Incompatibility:

- Recognize the value of and emphasize research on embryo culture as a way to overcome abortion and other incompatibility problems due to poor affinity in wide crosses.
- Conduct extensive research on plant regeneration from protoplast culture for future studies on the production of somatic hybrids by protoplast fusion.
- Initiate research on in vitro fertilization.

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CELL AND TISSUE CULTURE TECHNIQUES FOR CEREAL CROP IMPROVEMENT

The workshop on "Cell and Tissue Culture Techniques For Cereal Crop Improvement" jointly organnised by the International Rice Research Institute (IRRI) and the Institute of Genetics of the Academia Sinica was held on October 19–23, 1981 in Beijing. Fifty scientists from Australia, Denmark, England, the Federal Republic of Germany, France, India, Japan, Peru, Philippines, USA and the People's Republic of China attended the meeting.

Sixty dissertations are compiled in this proceedings including 32 reports and 28 abstracts (i.e. the posters exhibited in the meeting).

The following topics and corresponding rapid progress, especially the investigations and prospects of the application of plant somatic genetics, have been reviewed and discussed. These include: the integration of cell and tissue culture with plant breeding genetics, biochemistry and phytopathology on plant improvement and somatic genetics; screening of resistant mutants and development of various variants by cell and tissue culture technique; overcoming of incompatibility in distant hybridization by young embryo culture; somatic hybridization, genetic manipulation and plant molecular genetics.