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CELL AND TISSUE CULTURE IN FIELD CROP IMPROVEMENT

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CELL AND TISSUE CULTURE IN FIELD CROP IMPROVEMENT

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PREFACE

The use of cell and tissue culture for the identification and selection of useful variants at a cellular level has become one of the most powerful tools in modern plant breeding. Already some varieties and lines of major food crops which have been bred using these techniques are being released to farmers. This book is the Proceedings of the international seminar "*Cell and Tissue Culture in Field Crop Improvement*", sponsored by the Food and Fertilizer Technology Center for the ASPAC Region and co-sponsored by the National Institute of Agrobiological Resources, and the Food and Agriculture Research and Development Association of Japan. I should like to express my gratitude to the staff of the Institute, who were so generous with their cooperation and support during the work of preparing the seminar, which was held in Tsukuba.

The breeding of improved varieties is one of the most effective ways to help the small-scale farmer achieve higher and more stable yields. Not only do improved varieties make more efficient use of soil nutrients, they are often more resistant to disease, and give effective disease control without any increase in inputs such as chemical pesticides. Cell and tissue culture is likely to prove of great benefit, therefore, to farmers who cannot afford heavy investment into farm inputs, and who must make intensive use of a small area of land. The papers in this Proceedings bring together recent research by tissue culture specialists from the Asian and Pacific region and the United States. They cover such important topics as the *in vitro* selection of resistant cells which can be regenerated into resistant plants, the use of haploidy, embryo culture, and the production of hybrids by protoplast fusion.

Since, as one seminar participant pointed out during the discussion, tissue culture proceeds mainly by trial and error, the exchange of information is very useful in promoting further discoveries and avoiding duplicated research. In view of the rapid progress of research in this field, and the important practical implications of this research, there seems no doubt that cell and tissue culture will play a major role in the future development of crop production in the Asian and Pacific region. We hope that this book will make a useful contribution to the work of those who are already engaged in cell and tissue culture, and will also be of value to those who are now preparing to incorporate these techniques for the first time into their national plant breeding programs.

C. H. Huang

Cheng-Hwa Huang
Director, FFTC

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Section I.

Recent Advances in Tissue Culture Techniques

HIGH FREQUENCY AND SYNCHRONOUS SOMATIC EMBRYOGENESIS: A USEFUL SYSTEM FOR CROP IMPROVEMENT

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ABSTRACT

A high frequency and synchronous embryogenesis system from single cells was established in carrot suspension cultures. We attempted to transform single cells by the introduction of foreign DNA, using microinjection techniques, into the carrot system. A DNA fragment from the Ti plasmid, on which the ocs gene was located, could be introduced to selected single cells by microinjection. About 50% of microinjected single cells could differentiate into whole plants via embryogenesis. Transformation occurred at a frequency of 20% in regenerated plants, and expression of introduced foreign DNA was observed in 50% of transformants. Thus, overall frequency of expression of foreign DNA in regenerated plants from single cells was abou %. The results indicate that a high-frequency embryogenesis system from single cells and microinjection techniques are useful for obtaining transgenic cloned plants at a high frequency.

INTRODUCTION

During recent years, higher plants have been the subject of investigations to establish efficient methods of *in vitro* gene manipulation. Vectors have received relatively more attention than their hosts, and in many cases, protoplasts of cells of host plants have been the primary targets in attempts to transform plants. However, effective vectors and efficient methods of transformation remain elusive for most investigators. In addition, protoplasts have proved to be difficult to culture, and the frequency of regeneration from transformed protoplasts is still quite low. In the present study, we demonstrate that a high incidence of genetic transformation can be accomplished in carrot cells by using (1) a cell culture with a high frequency of embryogenesis, and (2) an improved microinjection technique.

A HIGH FREQUENCY AND SYNCHRONOUS EMBRYOGENESIS SYSTEM

Fujimura and Komamine[1] established a system in which embryogenesis occurred synchronously at high frequency from selected cell clusters in a carrot suspension culture derived from hypocotyls of seedlings of *Daucus carota* cv. Kurodagosun.

Before the induction of embryogenesis, cell clusters were selected by sieving and by density gradient centrifugation. Cells were sieved through a nylon screen, and cells of the size range 31-47 μM were collected. The collected cells were subsequently subjected to Ficoll density gradient centrifugation, and those sedimented in the heaviest fraction (18% Ficoll solution) were transferred to auxin-free medium for the induction of embryogenesis. Most of these differentiated to the globular stage of embryos six days after transfer. Thirteen days after transfer, more than 90% of cell clusters differentiated into torpedo shaped embryos and plantlets. The time course of embryo formation in this system indicated that embryogenesis occurred synchronously at a high frequency. This system provides a good source of material for artificial seeds, because a lot of embryos at different stages can be collected.

Using this system, we investigated the morphological, physiological and molecular mechanisms of embryogenesis from embryogenic cell clusters. The results are summarized as follows:

Three phases, Phase I, II and III, could be recognized in the early stage of embryogenesis. In Phase I, in which apparently undifferentiated cell division occurred at a slower rate, the active turnover of RNA and protein were observed. In Phase II, very active cell division occurred, and active DNA synthesis was observed in this phase, due to the reduction in repilcon size to one third of the control. During Phase II, synthesis of a new species of m-RNA and a new species of protein occurred, indicating that embryogenesis was regulated at a transcriptional level. High template activity, and changes in the relative composition of histone, were observed in the chromatin level. As a result of active cell division in Phase II, embryos reached the globular stage and developed to form heart- and torpedo-shaped embryos in Phase III.

However, the initial material of the system mentioned above was cell clusters, not single cells. These embryogenic clusters are called State I cells, and it would seem that differentiation into embryos was already determined in State I cells, because they differentiated into embryos at a high frequency in the auxin-free medium without any trigger. Therefore, the process of formation of State I cells from single cells is the most important process in which the determination of embryogenesis may occur. In order to investigate this process, which is called Phase 0, a system in which embryogenesis can be induced from single cells synchronously at a high frequency must be established.

Firstly, we attempted to collect single cells[2]. Cells were sieved through a nylon screen, and those in a size range of 10-16 μm were subjected to density gradient centrifugation in Percoll solution. Cells sedimented on 10% Percoll were collected. Ninety-eight percent of collected cells were composed of single cells. However, if these single cells were transferred directly to the auxin-free medium, no embryo formed, indicating that totipotency was not yet expressed in these single cells. We then examined the effect of various treatments of single cells for 0 to 8 days, and treated cells were transferred to the auxin-free medium containing zeatin, that is, the embryo inductive medium from cell clusters. The number of formed embryos was counted seven days after transfer.

Auxin treatment was found to be essential for induction of embryogenesis from single cells. No embryo was formed in the auxin-free medium, but if single cells were treated with auxin, embryos formed after transfer to the auxin-free medium. The optimum conditions of pretreatment for induction of embryogenesis from single cells can be summarized as follows:

Auxin (2,4-D at 5×10^{-6}M) was essential. Cell density (5×10^{5} cells/*ml*) was also an important factor. Cytokinin (zeatin 10^{-6}M), mannitol (10^{-3}M) and a high concentration of oxygen (40%) were promotive. However, even under optimum conditions, the frequency of embryogenesis from single cells remained only 30%. We then examined what type of single cells could differentiate into embryos under these conditions, by serial observation of the process of embryogenesis from single cells.

We found three types of single cells. Small, round, cytoplasm-rich cells could differentiate into embryos at a rate of more than 90%. From ovary shaped cells, only 10% of embryos were formed. Elongated cells did not differentiate at all. We therefore collected the small, round cells, called State 0 cells, by picking them up manually under an inverted microscope.

Fig. 1 shows the whole procedure of induction of a high frequency embryogenesis system from single cells. Preculture in the medium containing 2,4-D, zeatin, and a high concentration of $CaCl_2$, was effective in increasing the relative number of State 0 cells. Cells in the suspension culture were sieved, and fractionated by a density gradient centrifugation. Fractionated cells were cultured in a medium containing 2,4-D, zeatin and mannitol for seven days, and then transferred to an auxin-free medium containing zeatin. The frequency of embryogenesis from single cells was 85-90% when this system was used.

Using this system, we investigated which events occur in Phase 0, that is the process of development from single cells to State I cell clusters.

It was revealed that unequal cell division occurs first, together with the polarized localization of DNA synthesis. RNA synthesis, especially m-RNA synthesis, and free calcium also appeared during Phase 0. These biochemical polarities may play an important role in molecular mechanisms of embryogenesis, because they did not occur under non-embryogenic conditions.

The system established here is useful, not only for the investigation of the molecular mechanisms of somatic embryogenesis but also because it may provide a useful system for crop improvement. If, for example, foreign DNA can be introduced into single cells by microinjection,

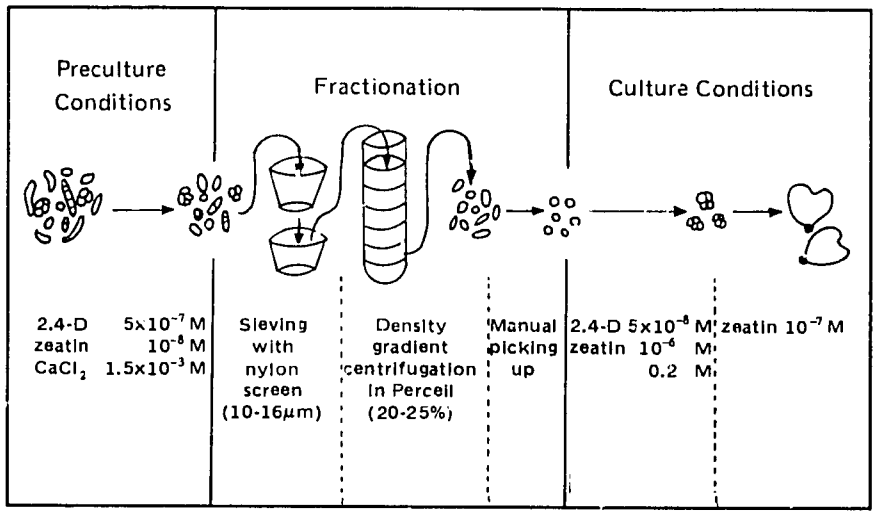

Fig. 1. Establishment of a high-frequency, synchronous embryogenesis system in carrot suspension cultures

a high frequency of genetically manipulated single cells can differentiate into a transformed whole plant. Thus, we can produce transgenic cloned plants at a high frequency.

MICROINJECTION

Microinjection was performed by using an inverted microscope with a hydraulic micromanipulator[1]. A single cell was held with a holding pipette, with a coating of soft Gellan Gum as a cohesive agent on the bottom of a Petri dish. Nuclei of single cells could be microinjected with a Lucifer Yellow CH. Seventy percent of injected cells could divide. Microinjected cells could differentiate into embryos at a frequency of 50%. We then tried to introduce foreign DNA into single cells by microinjection, and examined replication and expression of the introduced foreign DNA during embryogenesis.

The DNA fragments used in this experiment were Hind III No. 1 fragment from Ti plasmid PTiAch5, which was digested by Sma I. The fragments were cloned using vector YIP5. The ocs gene, which encodes lysopine dehydrogenase responsible for octopine biosynthesis, is located on this fragment.

DNA replicated during embryogenesis was detected by Southern hybridization, using a DNA chemiprobe kit made by Orgenics Ltd. Cytosine residues of the probe were sulfonated and hybridized with the DNA from regenerated plants via embryogenesis. The monoclonal antibody against sulfonated DNA was conjugated with the hybridized probe. The alkaline phosphatase, labelled secondary antibody, was conjugated with the primmary monoclonal antibody, and the activity of alkaline phosphatase was detected.

We could detect alkaline phosphatase activity in the sample of DNA from regenerated plants differentiated from microinjected single cells. These results show that the injected foreign DNA was replicated in regenerated plants via embryogenesis.

We then tried to detect lysopine dehydrogenase activity, by analyzing the reaction products of crude enzymes from transformants or non-transformants incubated with arginine, the precursor of octopine, using high voltage electrophoresis.

In the sample of transformants, formed octopine was clearly detected on electrophoretograms. The results were confirmed by using labelled arginine, indicating that the introduced foreign DNA was expressed in regenerated plants via embryogenesis.

The frequency of transformation was 20% in regenerated plants, and expression of foreign DNA was observed in 50% of transformants. Since microinjected single cells differentiated into embryos at a frequency of 50%, the overall frequency of expression of introduced foreign DNA was about 5% in plants regenerated from single cells.

The high-frequency embryogenesis system and microinjection techniques reported here are useful, not only for the investigation of molecular

mechanisms of somatic embryogenesis, but also for crop improvement. If we can introduce useful foreign DNA, such as cytoplasmic male sterile genetic factors, and if this can be expressed during differentiation, we could obtain useful transgenic cloned plants at a high frequency.

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DISCUSSION

Q. (A.N. Rao)
Can the system you describe be used for plants other than carrots?

A. As you know, it is relatively easy to induce embryogenesis in carrots, which is why we began with this plant. We are now trying to apply the system to other plants, such as rice. We have not succeeded as yet, but it should be possible.

Comment: (A.N. Rao)
In our work with tree tissue we didn't separate out the cells according to size and shape as you have done, but we did try to select smaller cells, and tried them with different concentrations and applied hormones. However, it didn't work. Small globular bodies formed, but these didn't differentiate. Perhaps the timing is important, as in your experiments.

A. If you want to apply the system to other plants, it has to be modified, and different hormones and media must be used.

Q. (F.J. Zapata)
After the whole plant was regenerated, did you find any variation? In theory, there should not be any variation with somatic regeneration.

A. Unfortunately, we haven't studied this.

Comment: (F.J. Zapata)
If there is no somaclonal variation, this will be a splendid technique for plant breeding, since it will be a means of creating identical plants (clones).

PRODUCTION OF DISEASE RESISTANT PLANTS USING SOMACLONAL VARIATIONS

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ABSTRACT

Traditionally, the breeding of disease resistant crops has been achieved by crossing cultivars with resistant wild varieties. Cell and tissue culture has now made possible the more rapid breeding of resistant plants, by exposing calli derived from plant materials to a series of stresses, including pathogens, or the toxins produced by pathogens. This paper describes the selection of tobacco plants resistant to Alternaria and Cercospora by cell culture.

INTRODUCTION

So far, the breeding of disease resistant crops has been achieved by crossing cultivars with wild plants which have disease resistant traits. Recently, biotechnology, which includes cell breeding, cell fusion and gene manipulation, has been developed as a means of expanding the gene source, thus enabling us to produce new disease resistant plants.

In this paper, I should like to describe the cell breeding of disease resistance, using somaclonal variations. The success of regeneration from a protoplast[1] has contributed greatly to cell breeding techniques. In 1973, using this technique, Carlson[2] succeeded in producing a tobacco resistant to wildfire disease, selecting tobacco mesophyll protoplasts by the use of chemical stress. After this achievement, further disease resistant plants were produced by selecting calli derived from a single cell using various stress materials, the pathogens themselves, pathotoxins, chemical substances and culture filtrates (Table 1).

In our laboratory, the production of *Alternaria* and *Cercospora* disease resistant tobacco has recently been accomplished. *Alternaria* and *Cercospora* secrete during spore germination an AT toxin (host specific toxin)[19] and a cercosporin (non-specific toxin)[20], respectively. Calli originating from a mesophyll protoplast of tobacco were selected by AT toxin and paraquat (similar to cercosporin)[20]. Some of these plants resistant to AT toxin and paraquat were also resistant to *Alternaria*[5] and *Cercospora*[21] disease. The properties of these plants are discussed below.

Table 1. Disease resistant plants obtained by screening through conventional *in vitro* procedures

Pathogen	Plant	Ref. No.
Helmintnosporium maydis	*Zea mays*	3
Helminthosporium sacchari	*Saccharum officinarum*	4
Alternaria alternata	*Nicotiana tabacum*	5
Helminthosporium victoriae	*Avena sativa*	6
Fusarium oxysporum	*Dianthus caryophyllus*	7
Fusarium oxysporum	*Medicago sativa*	8
Fusarium oxysporum	*Lycopersicon esculentum*	9
Fusarium oxysporum	*Solanum tuberosum*	10
Pseudomonus syringae	*Nicotiana tabacum*	5
Phoma lingam	*Brassica naps*	11
Helminthosporium oryzae	*Oryzae sativa*	12
Rhizoctonia solani	*Solanum tuberosum*	13
Sclerospora sacchari	*Saccharum officinarum*	4
Ustilago scitaminea	*Saccharum officinarum*	14
Fiji disease	*Saccharum officinarum*	15
Phytophthora infestans	*Solanum tuberosum*	16
Tobacco mosaic virus	*Lycopersicon esculetum*	17
Verticillium albo-atrum	*Medicago sativa*	18

MATERIALS AND METHODS

Protoplast Isolation and Culture

Fully expanded leaves of tobacco plants (*Nicotiana tabacum* L. cv. Samsun), which had been grown in a greenhouse in natural daylight at 25°C, were used for protoplast isolation. The leaf surface was initially sterilized with 70% ethanol for 15s, and then with 0.2% mercuric chloride for 15s. After rinsing the leaf three times with sterile water, the lower epidermis was removed and pieces of the leaf were floated (treated side downward) on a proteplast isolation solution containing 9.1% mannitol, 1% cellulase and 0.05% Macerozyme. The pH of the enzyme solution was adjusted to 5.8 with 0.2 N KOH, prior to sterilization through a Millex-GS filter. All subsequent operations were carried out aseptically in a laminar flow cabinet. Leaf materials were kept in the enzyme solution at 30°C for 3 hrs without agitation until a mesophyll protoplast suspension formed. This suspension was filtrated through four layers of cotton cheesecloth. The filtrate was then placed in a centrifuge and spun at 100 xg for 2 min. After removal of the supernatant, the pellet was washed four times with sterilized 0.5 M mannitol solution containing 0.1 mM CaCl, and suspended in a small amount of the same solution to give the desired protoplast concentration (approximately 5 x 10^6 cells/*ml*).

Protoplasts were cultured at a concentration of about 1 x 10^5 cells/*ml* in plastic Petri dishes 9 cm in diameter, containing 5 *ml* of Nagata and Takebe's medium[22] supplemented with 3 mg/*ml* naphtyl acetic acid (NAA) and 1 mg/*ml* bentylaminoprine (BAP), and 0.8% agar. These dishes were sealed with Parafilm M and incubated, first in the dark for seven days and then in diffuse light of 700 lux at 26°C for 5 weeks.

Plant Regeneration from Calli

Cell colonies (0.3-0.8 mm) that appeared on the Nagata and Takebe medium after 4-6 weeks of incubation were transferred to Linsmaier and Skoog[23] basal medium containing 2 mg/*l* NAA, 0.2 mg/*l* BAP and 1-1.5% agar (LS-1 medium) for callus differentiation. The transferred calli were maintained at 26°C under continuous illumination of 800 lux. After two weeks of incubation, each callus was cut into pieces of c. 2.5 mm. The LS-2 agar medium (Linsmaier and Skoog basal medium supplemented with 0.1 mg NAA and 5 mg BAP/*l*) was used for plant regeneration. Calli were transferred to this medium and incubated in a growth chamber adjusted to 26°C and 50% relative humidity under a 16 hr/day of 13,000 lux. Three to six weeks later, the shoots which had developed were transferred to LS-3 medium (Linsmaier and Skoog basal medium containing 0.1 mg NAA and 0.5 mg BAP/*l*) for further shoot development and root initiation.

Pathogen and Toxin Preparation

Alternaria alternata pathotype tobacco was supplied by Dr. Nishimura of Nagoya University, Japan. This fungus was maintained on PSA (potato sucrose agar) medium at 28°C, and was subcultured at two-week intervals.

To prepare the toxin, we cultured the fungus in Richard's medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 50 g sucrose and 1 g yeast extract/*l*) and kept the culture stationary for three weeks at 28°C.

Mycelial mats from 5 *l* of the culture fluid were removed by passing the fluid through Whatman No. 2 filter paper. The toxic filtrate was absorbed by 0.5% activated charcoal for 30 min. The charcoal was then washed twice with distilled water, and collected on Whatman No. 2 filter paper by filtration. The toxin was extracted from the charcoal by 70% acetone, which was removed from the toxin extract by evaporation at 40°C. The remaining water solution was adjusted to pH 4.0 with 1 N HCl. This acidified solution was treated with ethylacetate to remove any admixtures in the water (Fig. 1).

The crude toxin was partially purified by eluting it with 1 N NH_4OH from a Dowex 50 x 8 column. Ammonia was removed by evaporation, and the volume of the crude toxin was then reduced to 50 *ml* (a 100-fold concentration of the content of the original solution). The toxin samples were filtrated before use through a Millex-GS filter, in order to sterilize them.

Selection of Toxin and Paraquat Resistant Calli

Calli were cut into pieces *c.* 2.5 cm in diameter, and placed in Petri dishes (9 cm in diameter) containing 15 *ml* LS-1 medium, to which 0.7% *A. alternata* toxin (v/v) was added aseptically as the selective agent. At this concentration, the toxin produced modest growth inhibition. The dishes were sealed with Parafilm M, and kept under a dim light of 400 lux at 26°C for 3-6 weeks.

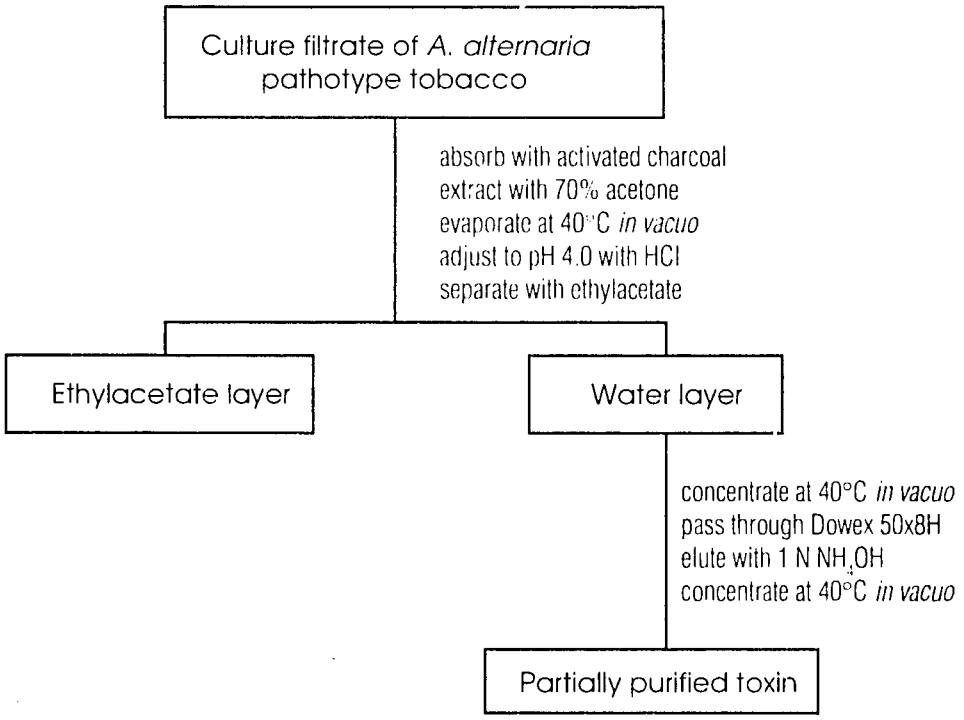

Fig. 1. Preparation of *Alternaria alternata* pathotype tobacco toxin

The resulting calli were then transferred to Petri dishes (9 cm in diameter) containing 15 *ml* of the LS-1 medium, which contained 125 μM paraquat (PQ). The dishes were sealed with Parafilm M and kept at 26°C for 2-3 weeks under a light of 13,000 lux for 16 hr a day. Calli which survived on the PQ medium were transferred to the LS-1 medium containing 250 μM PQ, and incubated for 2-3 weeks under the same conditions. Calli that survived on the second PA medium were cultured again in the LS-1 medium containing 250 μM for three weeks. As shown in Table 2, PQ concentration was increased gradually, and finally the paraquat resistant calli survived even in the presence of 2 mM PQ.

The PQ resistant calli were then transferred to LS-3 medium for shoot and root formation in the presence of 250 μM PQ.

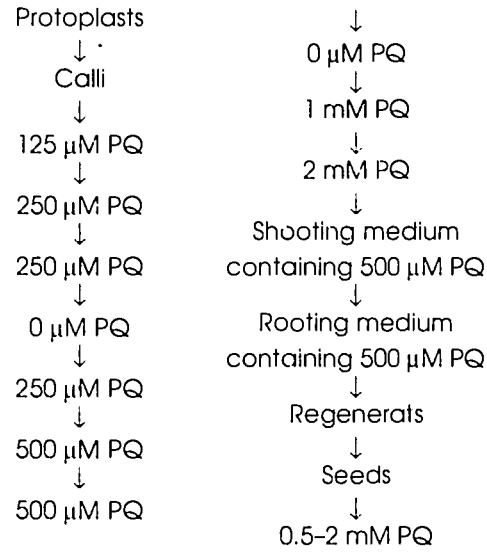

Fig. 2. Selection of paraquat (PQ) resistant tobacco plants

Testing Plant Resistance to Pathogens

A. Alternata Pathotype Tobacco

Leaves were detached and tested by spraying them uniformly with a spore suspension (10^5 spores/*ml*) and maintaining these at 28°C under 10,000 lux for 3-4 days. Susceptibility to the fungus was shown by the number of small necrotic flecks which appeared on the inoculated leaf (Fig. 3). Resistant leaves (R) were indicated by 0-10 necrotic flecks/cm^2, intermediate (I) by 11-50 necrotic flecks/cm^2, and susceptible (S), by more than 50 flecks/cm^2.

Table 2. Number of regenerated plants showing a different reaction to infection with *A. alternata* pathotype tobacco

No. selection cycles	No. calli isolated	No. regenerated plants[z]		
		R	I	S
1	36	0 (0.0)	74 (10.6)	621 (89.4)
2	5	5 (21.7)	7 (30.4)	11 (47.0)

[z] R: resistant, I: intermediate, S: susceptible

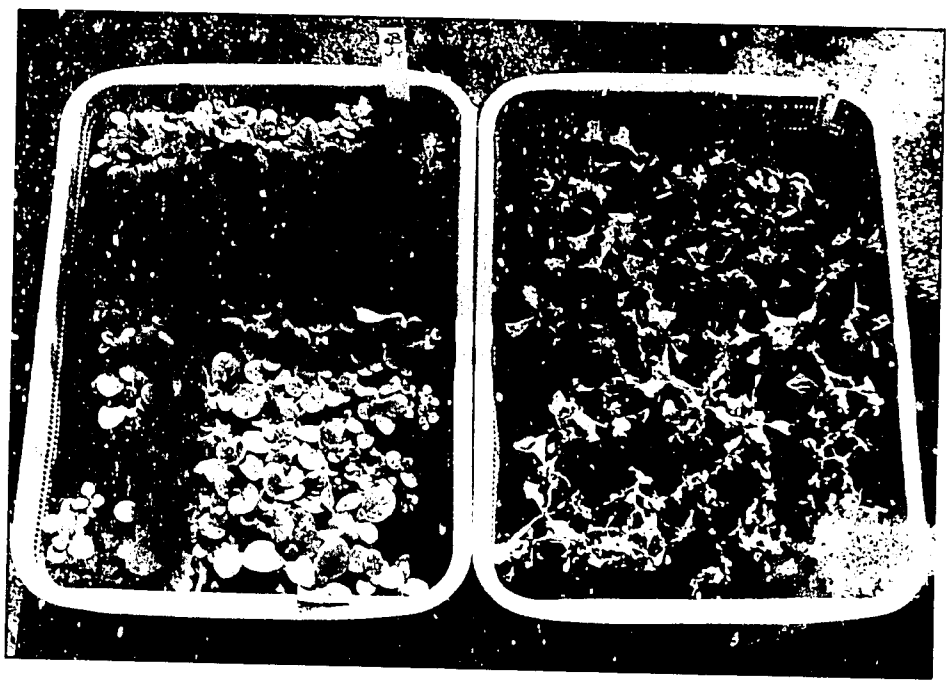

Fig. 3. Paraquat resistant tobacco plant. Left: resistant; Right: susceptible

C. Nicotiana

Paraquat resistant tobacco leaves were detached, injured by a razor blade, and inoculated with an agar block of cultured mycelial mat. The samples were incubated at 28°C under 10,000 lux for 3-4 days.

RESULTS

In Vitro Selection for Plants Resistant to Toxin and Paraquat

AT Toxin

From the large number of cell colonies formed on the culture plates, 700 were chosen at random, and transferred to a LS-1 medium containing 0.7% toxin. Results of our selection of calli resistant to this toxin through two cycles of selection are given in Table 2. Calli that survived after the second transfer to medium containing toxin were allowed to regenerate into plants. The number of plants regenerated ranged from four to 30 per callus.

Paraquat

About 3,000 calli were used to select plants resistant to paraquat. When calli 0.3-0.8 mm in diameter were transferred to the LS-1 medium containing 125 μM paraquat, 1-5% of calli survived.

The concentration of paraquat was increased gradually, and paraquat resistant calli were selected. Finally, calli that survived on the medium in the presence of 2 mM paraquat were allowed to regenerate into plants.

The regenerated plants were tested by spraying them with 500 μM paraquat solution, and paraquat resistant plants were selected (Fig. 3).

Disease Resistance of Regenerated Plants

Alternaria Disease

Results of the response of regenerated plants to the pathogen are given in Table 2. Plants regenerated from calli which were not treated with a pathogenic toxin were all as susceptible as the stock of cv. Samsun. Calli which survived one selection cycle produced large numbers of plants, but 80-90% of these were susceptible to the pathogen. No resistant plants were obtained from these calli.

The number of regenerated plants was greatly reduced after the passage of two cycles. Of the plants regenerated, five were resistant to *A. alternata*, while the percentage of plants susceptible to the pathogen was greatly reduced. This clearly shows that the use of two cycles of selection to select resistant calli is very effective in obtaining resistant plants from callus.

To determine whether plants regenerated from a single callus vary, all regenerated plants were classified according to their susceptibility to disease. The results are shown in Table 3, and show that variation in reaction to disease does in fact exist in different plants regenerated from a single toxin resistant callus. Plants resistant to *A. alternata* which had been produced from callus No. 48 had a normal morphology and showed vigorous growth. The offspring of these plants were given a progeny test. Approximately 9.3% of the progeny of 48R plants were resistant to *Alternaria* leaf spot. Our results clearly indicate that the trait is heritable, and can be transmitted to offspring through sexual reproduction (Table 4).

Table 3. Variation in response to *A. alternata* pathotype tobacco in plants regenerated from individual toxin-resistant calli

Isolation No. of original calli	No. regenerated plants	Disease reaction[z]		
		R	I	S
48	10	3	5	2
293	5	1	2	2
458	5	2	1	2
496	5	–	–	5
500	3	–	1	2

[z] R: resistant, I: intermediate, S: susceptible

Table 4. Segregation ratio of progenies of 48R plant showing symptoms of brownspot disease from *A. alternata* pathotype tobacco

Generation	No. plants tested	Reaction to pathogen		% resistance
		R	S	
R_1	461	43	418	9.3
R_2	187	130	57	74.8
Samsun	60	0	60	0.0

[z] R: resistant, S: susceptible

Cercospora Disease

Results of the response of regenerated plants to *Cercosporin* and *Cercospora* fungus are shown in Figs. 4 and 5. Paraquat resistant plants were also resistant to *Cercospora*, the action of which is similar to that of paraquat, and resulted in resistance to *Cercospora* disease.

DISCUSSION

All published results, and those obtained in our laboratory, indicate that the variability in various characters, including disease resistance, found among regenerates might be present beforehand in the cells from which the plants are derived. It is also possible that cell or callus cultures, or both, might cause genetic alterations to occur in the cells of callus or in regenerated plants.

In fact, variants do exist in plants regenerated from callus of single cell origin (Table 3). In such cases, genetic differences are probably generated during cell division *in vitro*. Nevertheless, toxin-selected calli do yield a high percentage of disease resistant plants.

Our results indicate that if the mode of action of stress materials is similar to that of pathotoxins, disease resistant plants can be selected by using these stress materials.

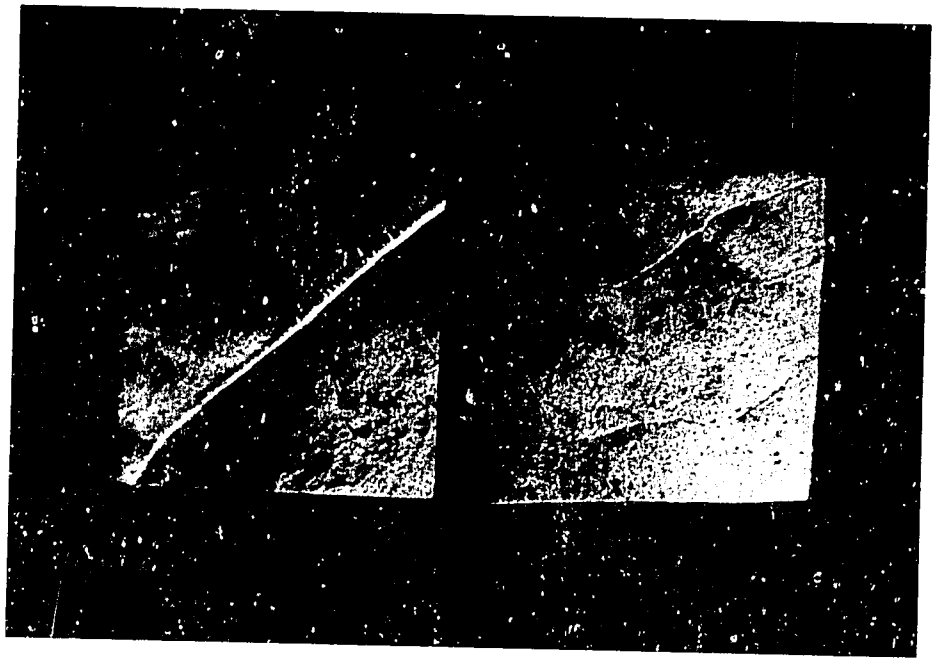

Fig. 4. Effect of *cercosporin* on paraquat resistant tobacco leaf and susceptible leaf. Left: resistant; Right: susceptible

Fig. 5. Effect of *Cercospora* fungus on paraquat resistant and susceptible tobacco leaf. Left: resistant; Right: susceptible

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DISCUSSION

Q. (F.J. Zapata)
Have you studied the genetics of plant resistance to paraquat? Is it monogenetic or polygenetic?

A. No, not yet. Brownspot resistance is probably polygenetic. Paraquat resistance is probably monogenetic.

Q. (M. Vajrabhaya)
When you culture tissue to induce callus, you generally find two types of cell, one a compact type, long and tubular, and the other a loose type. Have you studied any material from the loose type?

A. Sometimes one can observe long tubular cells in Indica cultivars. However, after culturing Indica long cells, some of the meristematic region then differentiates into long cells.

Q. (J.D. Chung)
Do you think your method of inducing disease resistance can be used for other crop species?

A. Many crops are already being selected by this method, on the basis of their resistance to herbicides.

Comment: (D.A. Somers)
We have recently initiated a project with a new herbicide called sethoxydim. Initially we didn't

know the mechanism of action of this herbicide, but we have now learned that it works by inhibiting Acetyl-Coenzyme A Carboxylase (ACCase), which is a regulatory enzyme in the fatty acid synthesis pathway. This is a grass herbicide, used to control grass weeds in soybeans, which also kills corn tissue cultures. Specific sethoxydim-tolerant callus lines have an elevated level of ACCase, which is exactly the same pattern as is seen in Dr. Furusawa's experiments. We are now analyzing the enzyme in the mutants to determine whether there is a change in the enzyme protein or if gene amplification causes elevated expression of ACCase.

CELLULAR DIFFERENTIATION AND MORPHOGENESIS IN PLANT TISSUE CULTURE

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ABSTRACT

Studies of plant cell differentiation have been carried out in our laboratory for the past two decades. Rice scutellar epithelium exhibits various types of activity under different conditions. Epithelial cells cease activity for a short period during germination. They retain their haustorial nature for a long period in the root culture system, and divide to form callus masses when cultured on 2,4-D medium. Microtopography of the callus surface after it is transferred to a shoot-forming medium is important in the successful induction of shoot buds. Surface stabilization of the callus is essential for shoot-bud formation.

INTRODUCTION

Culturing of rice (*Oryza sativa*) seeds on a medium containing a high concentration of 2,4-D induces callus masses to form, mainly from the epithelial layer of the scutellum. It is well-known that the scutellar epithelium in rice seed is a mature and non-dividing tissue, which is however a haustorium for endosperm materials. Callus formation thus allowed us to study the scutellar tissue in two quite different ways.

The first was to identify the ultrastructural changes in the scutellar tissue, under conditions similar to those of the germinated seed. The excised root with attached scutellum, after the excision of endosperm and plumule, was cultured under aseptic conditions using two separate media. A sucrose concentration of 12.5% was usually supplied via the scutellum, while the root tip was immersed in the inorganic nutrients of Murashige and Skoog's medium[12]. The ultrastructure of the scutellar tissue was studied, using a transmission electron microscope, after various incubation periods. These results were compared to those from normally germinated seeds[13]. The ultrastructural changes in each were quite different. In the normally germinated seeds, the cellular constituents of the epithelial cells were almost consumed within two weeks of germination. In the root culture system, on the other hand, the scutellar cells remained healthy for a long period, for more than three months' culturing. The vacuoles were small in size, while lipid droplets and starch grains remained abundant. A highly developed endoplasmic reticulum was often present.

The second approach in our work was to study the emergence of callus from the epithelial cells, and the factors inducing its formation. Furthermore, our study extended to the mechanism by which callus gave rise to shoot buds, and the factors controlling differentiation. The results revealed that the callus cultures gradually exhibited a number of different morphological features on the callus surface, which varied temporally and spatially. Scanning electron microscopy at early stages of callus growth provided a detailed picture of these sequential events, which finally resulted in shoot-bud formation. The fragile nature of the external materials coating the superficial cells was most apparent in the callus which did not differentiate into shoots. In shoot-forming callus, on the other hand, a stabilized epicuticular layer seemed to develop gradually on the outer surface of the superficial cells. In the regenerated epidermis-like layer, the cells were slightly elongated and arranged with a single orientation. These observations

suggested that the changes in the topographical features of callus cultures take place in order to maximize plantlet formation.

This result reveals that the scutellar tissue, which is a mature and non-dividing tissue in normally germinated seeds, is able to show a range of differentiation patterns under different physiological conditions. A thorough understanding of the patterns of differentiation, and the factors controlling its development, may enable us to improve the frequency of shoot-bud differentiation.

We therefore describe, firstly, the fine structures of the scutellum, which had a different appearance in aseptic callus cultures and root cultures. Secondly, we offer a short overview of the experimental results concerned with the structures emerging on the surface of shoot-forming callus.

CALLUS CULTURE

As early as 1965, it was demonstrated in our laboratory that epithelial cells give rise to callus masses by sequential division in medium containing a high concentration of 2,4-D[9,10]. There is also evidence that cell division in rice epithelium can be induced by the application of ABA[5]. However, callus induction occurs at the outer layer of the central cylinder in excised seminal roots (Figs. 1 and 2), and at the cut end of excised coleoptiles in the 2,4-D medium (Figs. 3 and 4). Callus induced from the excised roots takes a spherical shape, with stratified cellular layers arising from specific sites on the central cylinder which are claimed to be the sites of lateral root primordia (Figs. 1 and 2). However, it is interesting that callus formation is absent when the extreme tip of the root is used (Fig. 2), in view of the hypothesis[14] that lateral roots develop away from the extreme tip of rice seminal roots. However, a high rate of callus formation occurred when scutellar epithelium from intact seeds was inoculated in the medium (Fig. 5). Electron dense materials accumulated rapidly in the vacuoles of the cells. Important events associated with the dedifferentiation and proliferation of scutellar cells thus seemed to result in a slower rate of digestion of the starchy endosperm.

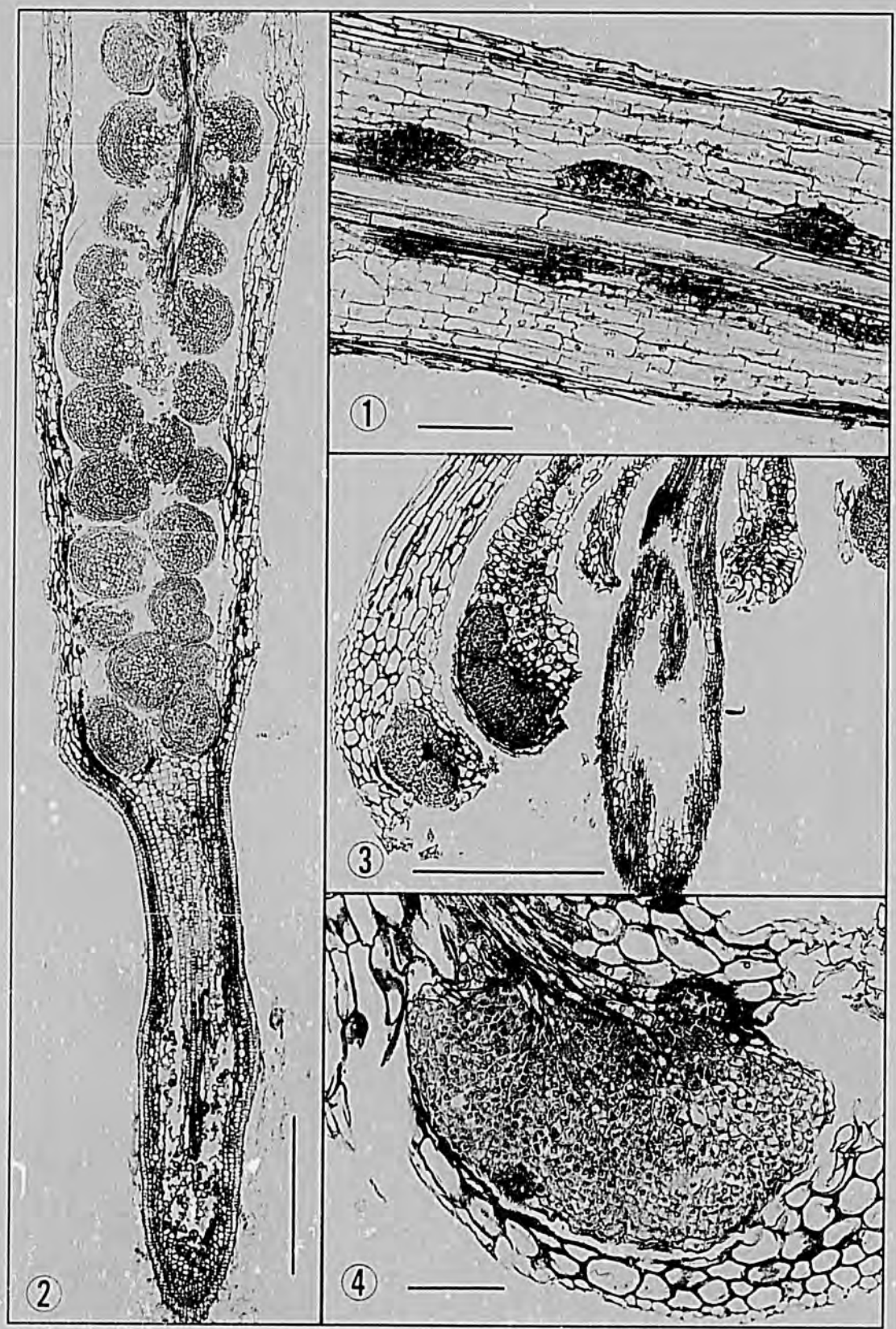
The ultrastructure of the epithelial cells in the callus induction process is sharply different from that found in germinated seeds and the root system. Consequently, it should be emphasized that the scutellar cells of rice seeds are particularly interesting for the cytological study of dedifferentiation and redifferentiation of plant cells.

Recently, several reports have suggested that plantlet formation occurs through somatic embryogenesis in cereal callus cultures. These papers have indicated that the scutellum structure develops from callus masses, and that such scutellum development is an important criterion in identifying somatic embryogenesis. However, the cytological nature of regenerated scutellum has hardly been demonstrated. Comprehensive studies on the scutellar ultrastructure must be carried out, in order to determine whether somatic embryos are actually present in cereal callus cultures. Furthermore, a comparison of early events in somatic and zygotic embryogenesis is an urgent need, to clarify the problem of somatic embryogenesis in rice callus cultures. From another aspect, it would be of great interest to determine the developmental stage at which the uppermost layer of scutellum starts to differentiate as an epithelium, because this would facilitate the induction of callus from the epithelium on an aseptic medium. Differentiation of the epithelium has been revealed 15 to 23 days after anthesis in barley, rye, oats and wheat[17], and has been clearly demonstrated in wheat 51 days after anthesis[16].

SEED GERMINATION

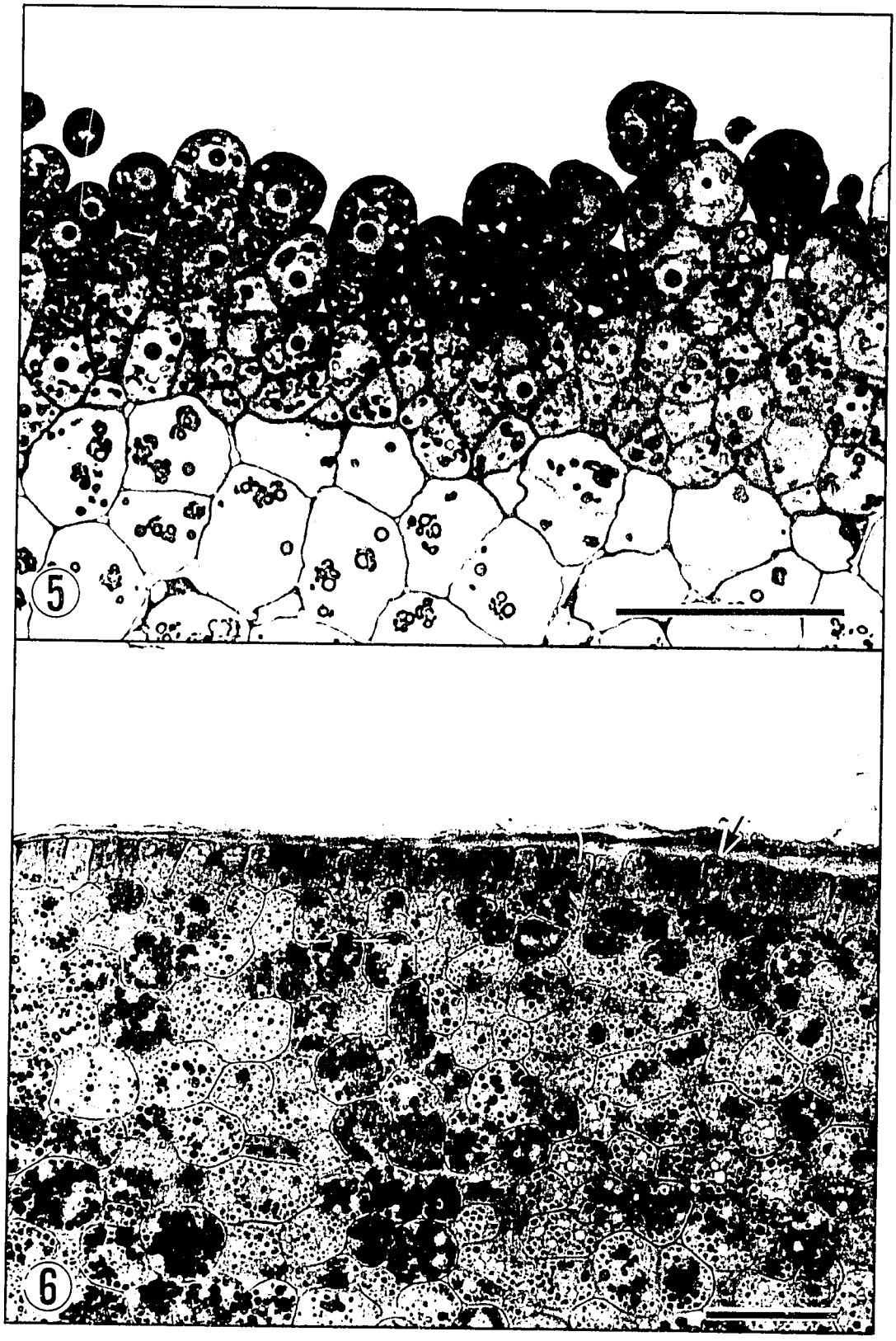
The epithelium and parenchyma of scutellum in mature dry seeds are shown in Fig. 6. In this state, the development of the mitochondrial cristae in the epithelial cells is very poor, suggesting that the cell is relatively inactive. Well developed mitochondria are never seen before germination. A high concentration of lipid droplets are present, making a complete cover to the epithelial cell wall. Electron dense materials are abundantly present in contact with the cell wall at the distal end of epithelial cells. We performed a thin film analysis, using an X-ray electron microscope, of these electron dense deposits in seeds four days after germination commenced. This analysis showed an accumulation of phosphorus and other elements in the deposits, which were confirmed as phosphate-containing particles.

As germination proceeds, the mitochondria become more conspicuous. After one week of germination, they are in contact with the epithelial cell wall. At this stage, lipid droplets have already migrated away from the cell wall to the inner part of the cytoplasm. Vacuoles are clearly present. Small vacuoles occasionally appear close to the sites where the materials in lipid droplets have



Figs. 1-4: Morphological aspects of excised rice roots and coleoptiles on callus induction medium

1. Lateral root initials emerged from central cylinder of a root after one day's culture (bar 100 µm)
2. Growth of small callus masses that are still enclosed in cortical tissue after 10 days' culture (bar 500 µm)
3. Callus growth at the cut end of a coleoptile after 10 days' culture (bar 500 µm)
4. Higher magnification of callus appearing in coleoptile tissue (bar 100 µm). Note the close connection between the callus masses and the vascular strand



(Top) Fig. 5 Vigorous division of scutellar epithelial cells in a rice seed cultured for 6 days on callus induction medium (bar 50 μm)

(Bottom) Fig. 6 Inactive state of both starchy endosperm and scutellum in a mature rice seed (bar 100 μm). An arrow indicates an unicellulated layer of the epithelium

dissolved. This suggests that small vacuoles may sometimes be the result of lipid digestion. Relatively large vacuoles are present in the proximal half of some epithelial cells. By the second week of germination, all the epithelial cells contain a large vacuole, leaving a narrow cytoplasmic layer along the cell wall. After this, the epithelial cells of rice seeds promptly degenerate, absorbing sugar materials converted from the starchy endosperm.

THE ROOT CULTURE SYSTEM

Rice grains were sterilized and germinated for three days. The endosperm and young bud were then excised from the scutellum, and the remaining seminal root with the scutellum was inserted into a small tube and passed through a pore in the distal end of the tube, leaving only the scutellum inside the tube. The tube pore was sealed with a mixture of melted gutta percha, lanolin and rosin. The tube was filled with one *ml* of sucrose, or a mixture of sucrose and phytohormones. The tube was held in cotton wool inside a 100 *ml* Erlenmeyer flask containing 10 *ml* of one-fourth strength MS inorganic medium. The cultures were incubated at around 27°C in darkness[12].

Growth of the seminal root was significantly affected by the sucrose concentration. After four weeks' culture, the fresh weight, the dry weight, the total root length and the diameter of the root base all increased with a higher sucrose concentration, up to an optimum of 12.5%. Auxins accelerated root growth for a period of more than four weeks.

After one week of cultur , there was no detectable difference in mitochondria and lipid droplets in the excised roots, compared to seeds germinated normally for one week. However, rough endoplasmic reticulum was well developed, and no extensive vacuolation was present after two weeks or even four weeks of culturing. This pattern is strikingly different from that found in normal germination.

Rough endoplasmic reticula generally developed near the distal portion of epithelial cells. This suggests a high level of protein synthesis in this region, which is directly supplied with sugar and phytohormones. The epithelium of seeds germinated for two weeks lost most of its internal constituents. However, even after four weeks of culturing, there was no marked degeneration of the cells.

Stacks of the rough endoplasmic reticulum sometimes surrounded a large lipid droplet and a malformed plastid, as in Fig. 7. This Figure shows an interesting structure of invagination, which extended from the cell wall to the vicinity of the stack. The cultured root was able to continue growth for more than three months (Table 1).

This clear difference in the structure and behavior of the epithelial cells demonstrates the suitability of the root culture method to study the factors controlling ultrastructural changes in epithelial cells.

Table 1. Effect of different incubation periods on the growth of excised, cultured rice seminal root with attached scutellum[14]

Incubation period (months)	Fresh weight (mg)	Primary root length (cm)
1	75	24
2	116	31
3	188	37

ANTHER CULTURE

Factors in the successful culture of rice anthers have been examined by several researchers. The frequency of callus induction from microspores depends greatly on the cultivars used for anther culture, as well as on the culture conditions. Cold pretreatment of panicles has been shown to have a favorable effect in rice anther culture. When young ears with microspores at the uninucleate stage are treated at 10°C for about 10 days, the frequency of callus formation certainly increases.

On the other hand, the seed ripening of cold-sensitive cultivars is greatly influenced by low temperature treatment, which results in an increase in the number of unripened seeds. Many abnormal microspores associated with tapetal hypertrophy are found, together with a decrease in the number of mature pollen grains, when panicles at the young microspore stage are kept under cool conditions[10, 15]. Varietal differences in callus formation can thus be expected to correlate with the susceptibility of cultivars to a cold environment. Fig. 8 shows that callus formation in anther culture tends to increase if cold sensitive cultivars are used[2]. It seems reasonable to suggest that a high frequency of abnormal pollen in cold sensitive cultivars leads to vigorous callus induction. We still do not have

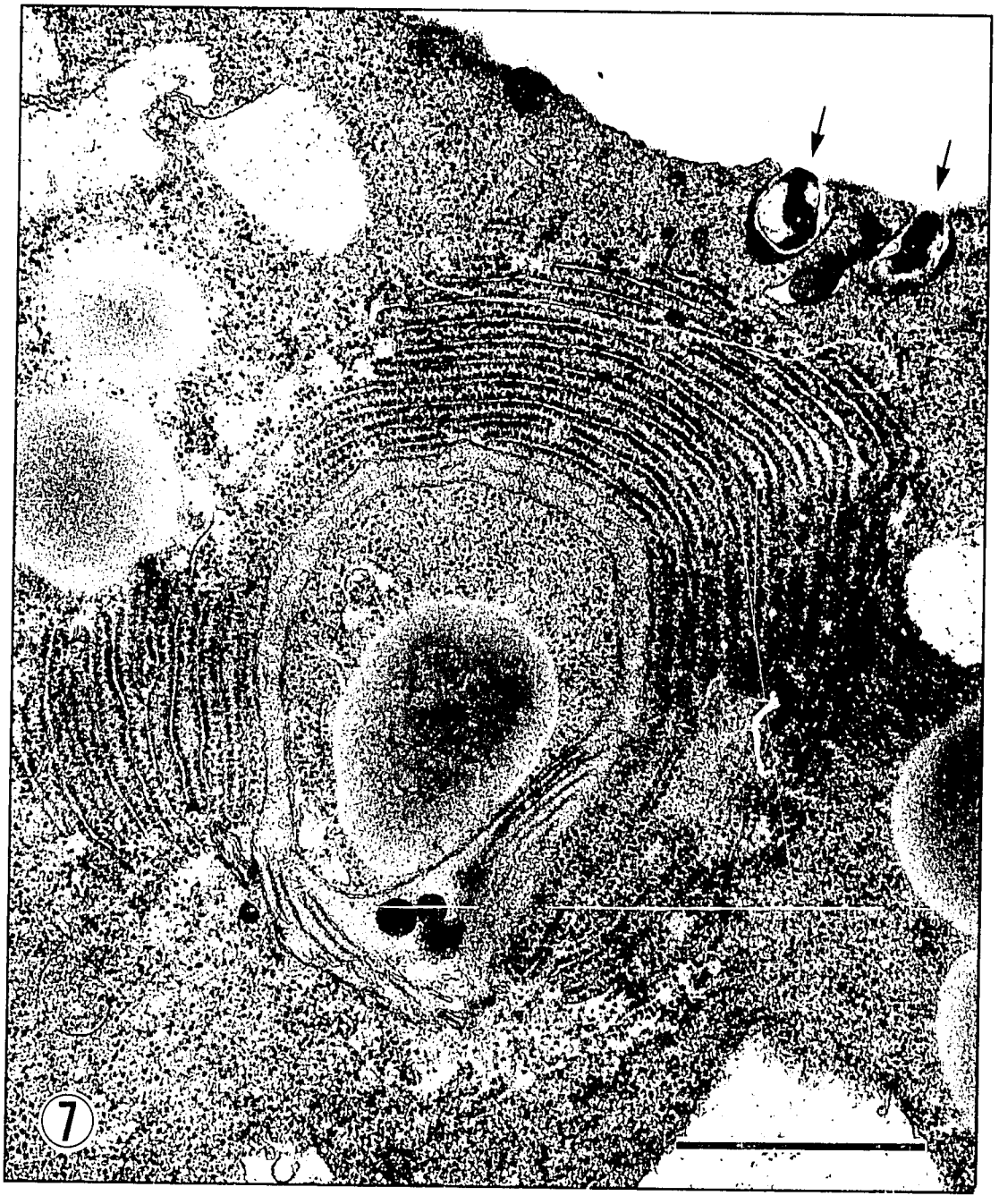


Fig 7 A membrane whorl of the rough endoplasmic reticulum appearing in cells near a vascular strand of rice scutellum attached with root (bar one μm). This suggests a high level of activity in protein synthesis. Arrows indicate the characteristic structure invaginated from the cell wall

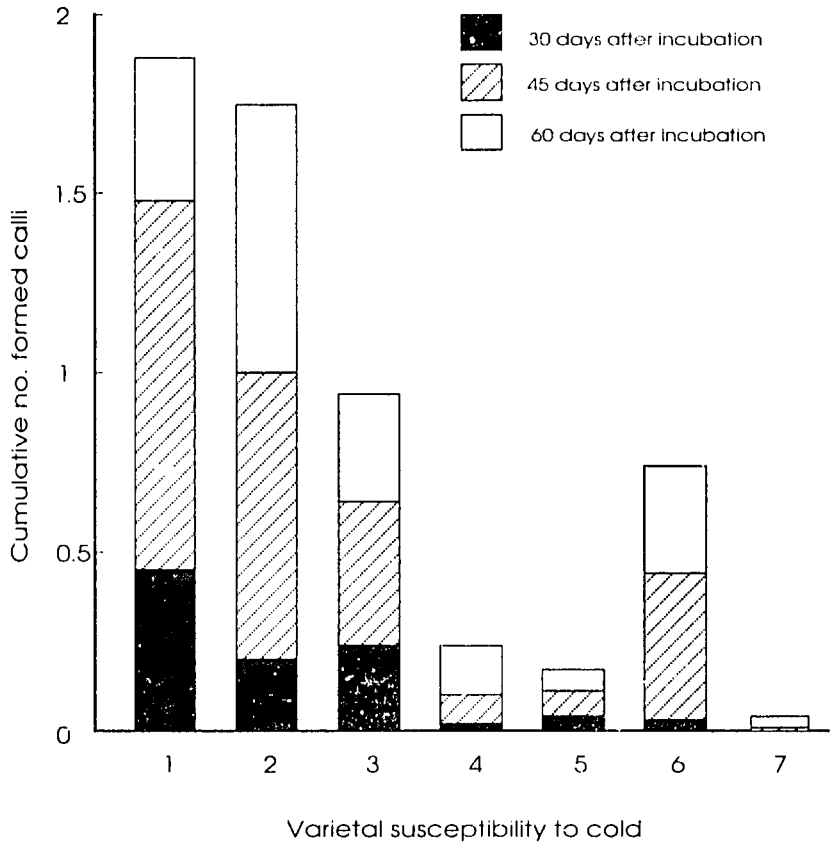


Fig. 8. Frequency of callus formation in the anthers of different cold resistant cultivars[2]. Degree of cold resistance: 1 and 2, cold sensitive; 3, 4 and 5, intermediate; 6 and 7, cold-tolerant cultivars

enough information about the exact cyto-histological significance of rice microspores, especially with relation to a high frequency of callus induction in anther culture.

REDIFFERENTIATION

A series of additional experiments was undertaken on the redifferentiation of rice callus cultures, all concerned with the cytological changes during early culture in a shoot-forming medium. When the proliferated callus cultures were transferred to this medium, many distinctive features developed on the callus surface as part of a sequence of developmental steps, leading to shoot-bud formation[11]. The callus surface gradually exhibited round knobs, green patches, milky white regions, necrotic regions, small protuberances, laminated or cylindrical leaf primordia, shoot apices, roots, root hairs, and root clusters. Thereafter, shoot buds developed in a non-synchronized fashion from the callus surface.

This gross morphology can easily be identified. Unfortunately, however, little is known about the cytological properties of the callus surface at various developmental stages. We cannot have a complete understanding of shoot-bud formation, without a detailed knowledge of surface topography in callus cultures. The variations in callus surface were thus followed with the aid of light and scanning electron microscopy.

Proliferated callus masses have a globular surface which lacks a epicuticular layer. This surface seems to be composed of polysaccharide membrane, which gives the appearance of a wall mat. This has a positive PAS reaction. The wall mat is soft and unstable in nature. It is continuously supplied by the surface cells, which change in appearance in a growing callus (Fig. 9). Sometimes this mat partly breaks down to form fibrillar materials, or even a bundle of fibrils. This unstable state is probably due to a discrepancy between polysaccharide production and growth of the individual surface cells. Enlargement and multi-

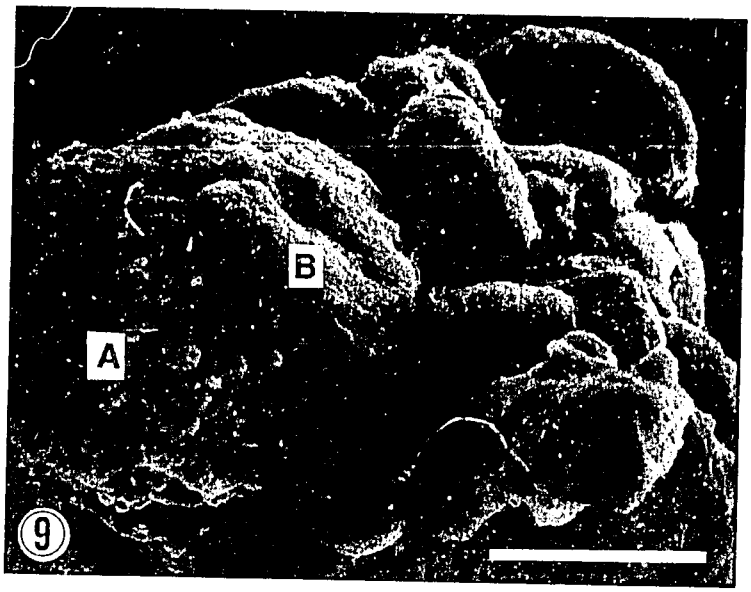


Fig. 9.
Microtopography of the callus surface in rice anther culture at an early stage (bar 400 μm)[2]. A: surface of growing callus, B: surface with signs of differentiation

plication of the surface cells tends to produce a break in the polysaccharide mat. The wall mat may be occasionally repaired through polysaccharide production by the surface cells themselves, in an actively, growing callus. Thus, the mat is itself a continuous surface, in fact the functional surface covering the callus mass. It usually has a moist appearance, suggesting that it uses the medium as a nutrient supply. The wall mat probably supports the movement of nutrients through the callus surface.

Complete repair of the callus surface and more advanced stabilization at the early growth stage are the first signs of the regeneration of an epidermis-like layer, consisting of isodiametric cells. After this early event, the cells become slightly elongated, and aligned in parallel, and appear to be of a stabilized epicuticular nature. By the time the epidermal layer is produced by surface cells, the callus mass already has a vascular system inside. At this stage, the callus structure is covered partially or completely with epidermal cells, which are coated with water-impermeable materials. The callus masses accordingly begins to show a marked reduction in the loss of water from its surface.

The early development of the epicuticular surface is assumed to take place at particular sites, in view of the occurrence of localized green patches. These are usually located on the callus periphery, and are in physical contact with the shoot-forming medium. They occur as early as three days after transfer. The relevance of greening as a predictor for shoot-bud formation has already been proved[1]. The sites at which green patches appear differs in various cultivars, and is also influenced by the culture conditions used. In some cases, the patches appear on the upper portion of a callus culture but not on the periphery. It is postulated that the appearance sites are regulated by a close interaction between the nature of the callus tissues and the nutrients supplied. Maeda and Thorpe[9] showed that the earliest shoot primordia which appeared in tobacco callus came from the protrusions which had developed close to the tissue-medium interface.

Since the physiological balance producing the green patches seems to be regulated by the nutrient supply, and is controlled by the microenvironment surrounding the particular sites of the callus, it is probable that callus masses with epicuticular cells and a vascular system are in a favorable state for organizing a shoot apex. Callus masses, finally, are able to organize themselves into a miniature of a whole plant.

These results show clearly that changes in the callus surface must be analyzed, if we are to maximize shoot-bud formation. With more basic information of this kind, it should be possible to obtain a higher frequency of shoot differentiation. We hope that one of the attractive aspects of *in vitro* morphogenesis is that surface topography is closely correlated with callus growth, ultimately leading to shoot-bud formation. In this respect, it is very important to reveal the cytological differentiation of the inner cells intimately associated with surface topography. Surface microtopography and cell cytology of rice callus cultures are now in progress in our laboratory, in order

to elucidate the consecutive events associated with shoot-bud formation.

In conclusion, it seems that the epithelial cells of rice scutellum are obliged to diverge in their act[illegible] under different circumstances, and may, for example, degenerate promptly, maintain their haustorial nature, or actively proliferate. The cells have a characteristic structure and function in rice plants, and probably also in other cereal species. Much additional work remains to be done on the interaction between microtopography and the internal structure of callus cultures.

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DISCUSSION

Q. (H. Uchimiya)
I know that some years ago, you discovered the effect of ABA on the regeneration of rice. How do you explain this effect, in terms of the morphological changes?

A. ABA does seem to be effective in encouraging a high frequency of shoot formation. I think this effect can be considered from two aspects: cytokinin metabolism and water stress. As far as cytokinin metabolism is concerned, one series of experiments on sweet potato showed that a high concentration of ABA lowered a high cytokinin level. The water stress may be the result of a high sucrose concentration.

Q. (M.T. Senawi)
Could you please comment on the interaction between genotype and basal media, with respect to morphogenesis? This is a great problem in our work on Indica species.

A. Indica species produce a very different type of callus than Japonica ones. For example, "Raikei" cultivars develop elongated cells, but give rise to shoot buds from meristematic tissue occurring in these elongated cells.

Comment: (F.J. Zapata)
At IRRI, we have found that callus induction is a recessive character, governed by four or five genes. We crossed Japonica and Indica rices, and produced some Indica types which have a slightly higher regeneration rate than most Indicas. However, since the character is recessive, it is difficult to transfer it. We are trying to do this by back-crossing, but every time we cross, we lose 50% of the genes. It may be necessary to study the different genotypes among the Indicas, and try and find varieties with a different genome for plant differentiation.

Q. (H. Uchimiya)
Generally speaking, cultures which are cultured over many years cannot be regenerated into plants. As you point out in your paper, this can now be done using chemicals such as ABA. Are there any other materials which can create regeneration ability in long-term cell cultures?

A. One of my students is now working on this, examining the application of ABA. It seems that it can regenerate very long-term rice cultures. The work is for a Ph.D. thesis, and has not yet been written up.

Q. (A. Komamine)
We have never succeeded in regenerating long-term cultures of carrot, although Dr. Ojima of Tohoku University has succeeded in doing this. He prepared a protoplast from a long-term culture which could divide and differentiate. It seems possible that some cell-to-cell interaction is involved in differentiation, but I have no idea what it is.

Comment: (E. Maeda)
I have read some papers by a group of Indian scientists working with ABA and wheat. They were able to take a long-term callus and regenerate it, using ABA. This medium seems to be applicable to a number of species.

Comment: (J.D. Chung)
A similar phenomenon is seen with cultures of woody plants, and also some vegetables. I understand that Dr. Nagashima some years ago worked with sweet potato and tried the induction of morphogenesis with ABA.

Comment: (K. Oosawa)

A heavy concentration of sucrose gives a high level of normal embryos. We were able to produce embryos by several methods, but could not regenerate a viable plant. When we added 0.5 mg ABA, no embryos were normal.

Comment: (K. Ohyama)

We have been working on long-term cultures of the Japonica cultivar Tainan 5. Another line has now been introduced from Mainland China which is an Indica type, called "Hai-Nan-Tsou". This is a highly differentiated line, and about 90% of cultures regenerate at the first stage. After subculturing this percentage decreases, but there is still a very high regeneration rate.

In contrast, Tainan 5 has a very poor regeneration rate. However, after nearly one year of culturing, if the cultures are subjected to even moderate stress, such as changing the agar concentration from 0.8% to 0.7%, the regeneration rate increases.

EMBRYOGENESIS

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ABSTRACT

Zygotic and somatic embryos (embryoids) from entirely different origins have a strikingly similar sequence of embryo formation: a globular stage, a heart stage and a torpedo stage. The origin of a somatic embryo may be a single cell or even a protoplast, but it can also develop from multiple cells derived from the same origin.

Somatic embryos can be initiated in a wide range of inorganic macro- and micro-elements, provided that the concentration of potassium ions ranges between 12 and 40 mM, and a level of ammonium ions not lower than 10 mM is maintained. Organic supplements such as casein hydrolysate, glutamine and alanine are reported to have a beneficial effect on somatic embryogenesis. Generally, auxin is not required for initiation of the embryo, but it is needed for the growth of callus prior to this stage if embryogenesis cannot be induced directly from the tissue of the explant. Even the physical environment is not generally critical for somatic embryogenesis, but some conditions are said to promote better growth and development than others.

Somatic embryos are likely to become a very useful plant material in the future, if initiation development and synchronization can be regulated successfully. Using this type of material yields a large number of uniform embryoids per volume of medium, which means that they are amenable to mechanical handling.

INTRODUCTION

Embryogenesis *in vivo* has been studied for several decades, but Haberlandt realized that one could successfully cultivate artificial embryos from somatic cells as early as 1902. The concept of embryogenesis was accepted a long time ago, but the first practical results occurring *in vitro* were not obtained until 56 years later (in 1958) by Steward, when he was studying the behavior of phloem explants excised from carrot roots in a suspension culture supplemented with coconut water (liquid endosperm). Subsequent studies by Reinert showed that the young plants emerging from carrot callus had a size and shape similar to zygotic embryos. At present, detailed studies of other plants by many scientists show that under certain conditions, callus and cell suspensions obtained from different sources of the explant can be induced to form roots, shoots and embryo-like structure (generally called embryoids).

Somatic embryogenesis is of great potential value to biology as well as agriculture since it provides an important tool for the study and analysis of molecular and biochemical events. Some examples are the study of the formation and development of mutants in carrot culture by Breton and Sung[8], and of somatic embryos of *Brassica napus*[11] with embryo-specific storage proteins. In agriculture, it can be used for the clonal propagation of inherently difficult groups of plant such as orchids, some ornamental cultivars and even some species used in reforestation. Somatic embryos of some clones can be obtained in large numbers (e.g. 60,000 embryoids per liter of medium[4]) at nominal cost. These can be subjected to any subsequent manipulation, such as scaled-up embryogenesis or artificial seed production, for convenience of handling. Other uses of somatic embryogenesis could be to establish pathogen-free planting stock, genetic crop improvement, and for germplasm storage and dissemination.

In this paper, the emphasis is placed on somatic embryogenesis occurring in *in vitro* culture.

THE CELLULAR ORIGIN OF SOMATIC EMBRYOS

Somatic embryogenesis may arise directly from explants or through an intermediary callus, but some observations have revealed that organogenesis occurs in place of embryogenesis. There have been some studies concerned with the origin of organs or embryoids that pinpointed whether all the cells originated from a single cell or not. There have also been studies on the site of organogenesis which ascertained whether this could arise independently, or must be restricted to preexisting organ initials[21].

The development of somatic embryos or organs does not generally proceed directly from a single cell, although the single cell origin of callus embryo has been documented for many species. In several cereal callus cultures, the differentiation of embryoids and adventitious shoots is confined to partially organized regions. Such regions were carried over from embryo explants, or were associated with aberrant roots that differentiated *in vitro*[21, 36]. In orchids, Kanchanapoomi[16] studying tissue of *Vanda* revealed that a single-cell origin occurred in callus induced from the lateral buds of the mature plant. The same pattern of development was found in earlier work on dicotyledonous plants such as carrot, endive and *Ranunculus*[19, 35]. Single cells or even sole protoplasts have been induced to grow and form embryos[7-10]. These studies enable us to study embryogenesis *in vitro*, by following the detailed development patterns of a particular species.

Variation in the cell morphology of cell aggregates derived from callus in suspension cultures has also been studied[24, 34]. Two major types of cell are regularly observed in *Oryza sativa*, *Saccharum officinarum* and *Rauwolfia serpentina*. These are:

- the non-embryogenic type, which are long, tubular cells, with large vacuoles occupying most of the space within the cytoplasm: this type of aggregate would not differentiate into embryoids or shoot primodium.
- the embryogenic type, which has a proportionately large nucleus and dense cytoplasm, and is similar to cells in the shoot primodium or in the natural embryogenic cells at an early stage. This type of aggregate could be expected to have the potential to differentiate into embryoids, or at least shoot primodium, in a suitable environment.

In conclusion, it is certain that either isolated single somatic cells or cell initial can be called totipotent, but even in the most suitable environment only an extremely small proportion of these differentiate into embryoids which can go through all stages of development to become a complete plant.

SOMATIC EMBRYOGENESIS

Two categories of embryos have been classified by Kohlenbach[17]. These embryos may arise normally, or occasionally *in vivo*, or under a controlled environment *in vitro*.

- **Zygotic embryos**, formed from a zygote resulting from regular fusion of the egg.
- **Non-zygotic embryos**, formed by cells other than the zygote.
  - Parthenogenetic embryos, formed from unfertilized eggs, or a fertilized egg without karyogamy.
  - Androgenetic embryos, those formed from microspores, microgametophytes or sperm.
  - Somatic embryos (embryoids), formed by somatic cells either *in vivo* or *in vitro*.

Somatic embryos formed in cultures have generally been called "embryoid", but other names such as accessory embryos, adventitious embryos, and supernumerary embryos are also found in the literature. The first case of somatic embryogenesis was reported by Steward and co-workers in 1958, a green carrot plant emerging from a torpedo-shaped embryo among groups of aggregates in a suspension culture. A more detailed study was carried out[27, 28] on embryos arising from callus grown on agar medium. Since then, many species have been studied in some detail, including *Ranunculus sceleratus*[18], *Citrus reticulata*[30] and *Nicotiana tabacum*[5, 6]. Up to now, over 30 families have been used in experiments on somatic embryogenesis[23, 26], see also Table 1.

Development of Zygotic Embryos

The pathway of zygotic embryo development found in many plant species is shown in Fig. 1. The following section deals with zygotic embryogenesis of the carrot. The stages of

Table 1. Some examples of plants in which somatic embryogenesis has been induced under *in vitro* conditions

Plant species	Reference
Anagallis arvensis L.	Baiaj and Mader (1974)
Asclepias curassavica L.	Prabhudesai and Narayanaswamy (1973)
Asparagus officinalis L.	Wilmar and Hellendoorn (1968)
	Takatori *et al.* (1968)
Atropa belladonna L.	Konar *et al.* (1972)
Brassica oleracea var. Borytis	Pareek and Chandra (1978)
Carum carvi L.	Ammirato (1974)
Citrus sinensis var. "Shamonti" orange	Kochba and Button (1974)
Coriandrum sativum L.	Steward *et al.* (1970)
Coffea arabica L.	Söndahl and Sharp (1977)
Daucus carota L.	Steward *et al.* (1958)
	Reinert (1959)
	Halperin and Wetherell (1964)
	Homes (1967)
Foeniculum vulgare Goert.	Maheshwari and Gupta (1965)
Lotus corniculatus L.	Nizeki and Grant (1971)
Macleaya cordata R.Br.	Kohlenbach (1965)
Nicotianum tabacum var. Samsun	Lorz *et al.* (1977)
Petunia inflata R. Fries	Handro *et al.* (1973)
Pinus ponderosa Dougl.	Moore (1976)
Rananculus sceleratus L.	Nataraja and Konar (1970)
Solanum melongena L.	Yamada *et al.* (1967)
Vanda x Miss Joaquim	Kanchanahoomi (1976)

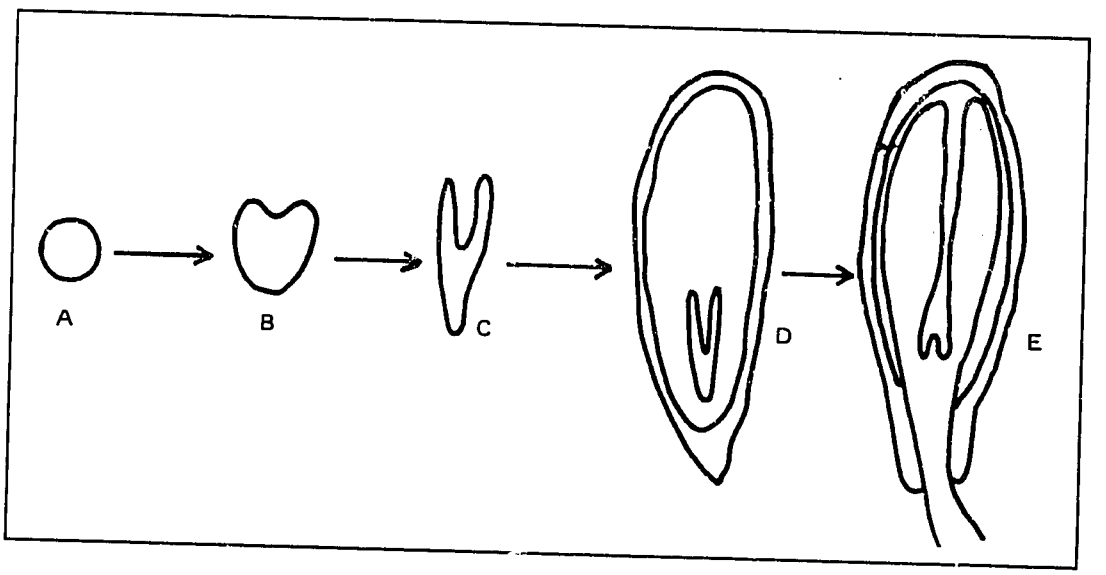

Fig. 1. Key stages in zygotic embryogenesis in carrot
(A) globular proembryo
(B) heart-shaped stages with initiation of cotyledonary primordia
(C) torpedo stage with shoot and root meristem and procambium
(D) mature seed with small embryo surrounded by cellular endosperm
(E) germinating seed at about five days
(Modified from Ammirato 1987)

embryogenesis begin with most of the cells dividing actively and increasing in size, but retaining their spherical shape (the globular proembryo). At this stage, the primary meristem (protoderm, ground meristem and procambium) becomes visible. Following this stage, the callus continues to divide and differentiate into a heart-shaped stage embryo, with initiation of cotyledon primordia. As the cotyledons develop, the embryo passes into the torpedo-shaped stage. The cells inside the cotyledonary ring divide to form shoot and root apical meristem, and procambium differentiation takes place. At this point, the carrot embryo becomes quiescent, with a cessation of mitotic division accompanied by a decrease in water content and other events associated with the formation of the mature seed. At maturity, the embryo is small and surrounded by a mass of cellular persistent endosperm[7]. In carrot and other umbellifers, the formation of the mature embryo is characterized by extensive cell division without substantial cell enlargement or cell differentiation[3, 15]. The cells of the mature embryo are thus far smaller than those of the subsequent seedling, and have few of the structural features associated with differentiated cells.

Development of Somatic Embryo

In carrot and other plant species, the induction of embryogenic growth can be done in one of two ways. Certain cells may only need placement on a simple basal medium to begin embryogenesis, while others may require a more complex medium with an exogenous auxin supply[4]. Somatic embryos are initiated in callus from superficial clumps of cells associated with highly vacuolated cells which do not take part in embryogenesis[15]. In carrot, the embryoid-forming cells are characterized by their dense cytoplasmic content, large starch grains and relatively large nucleus. These phenomena are very similar to those found in *Saccharum officinarum* and *Rauwolfia serpentina*. Staining techniques reveal that the carrot embryogenic cells have a high protein and RNA content. These cells also exhibit high dehydrogenase activity with tetrazoliam staining[30, 33]. Each proembryoid cell is capable of passing through the same sequence as zygotic embryo formation (globular stage, heart stage and torpedo stage (see Fig. 2).

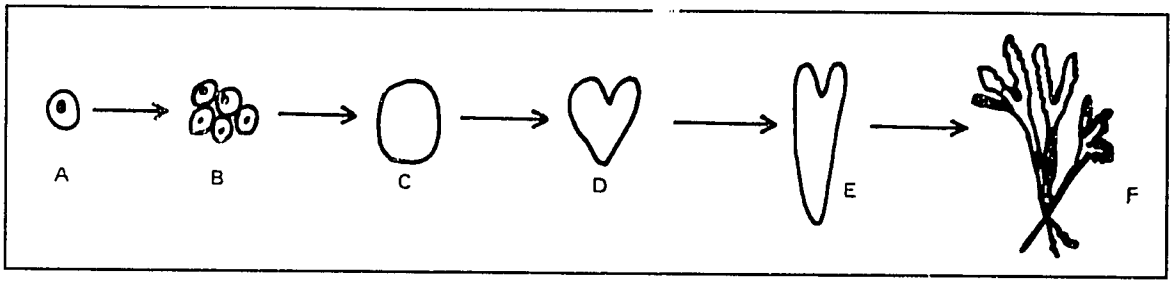

Fig. 2. Stages of somatic embryogenesis
(A) single cell stage
(B) small cell aggregate
(C) globular stage
(D) heart-shaped stage
(E) torpedo stage
(F) young plantlet

The expression of totipotency in single cells was studied in detail in *Ranunculus sceleratus* by Konar and Nataraja[15]. They cultured various floral parts as well as somatic tissue in a medium containing 10% coconut water, with or without IAA. Numerous embryoids appeared on the callus within three weeks. An ultrastructural comparison has been made of epidermal and callus cells undergoing embryogenesis, and the embryoids appear to originate from both peripheral and deep-seated cells of the callus (see Fig. 3). Each cell in a given embryoid is said to inherit from a single cell, and the developing pattern takes a course similar to zygotic embryogenesis. A characteristic feature of the embryogenic cells of different origin is that they contain large number of vesicles in their

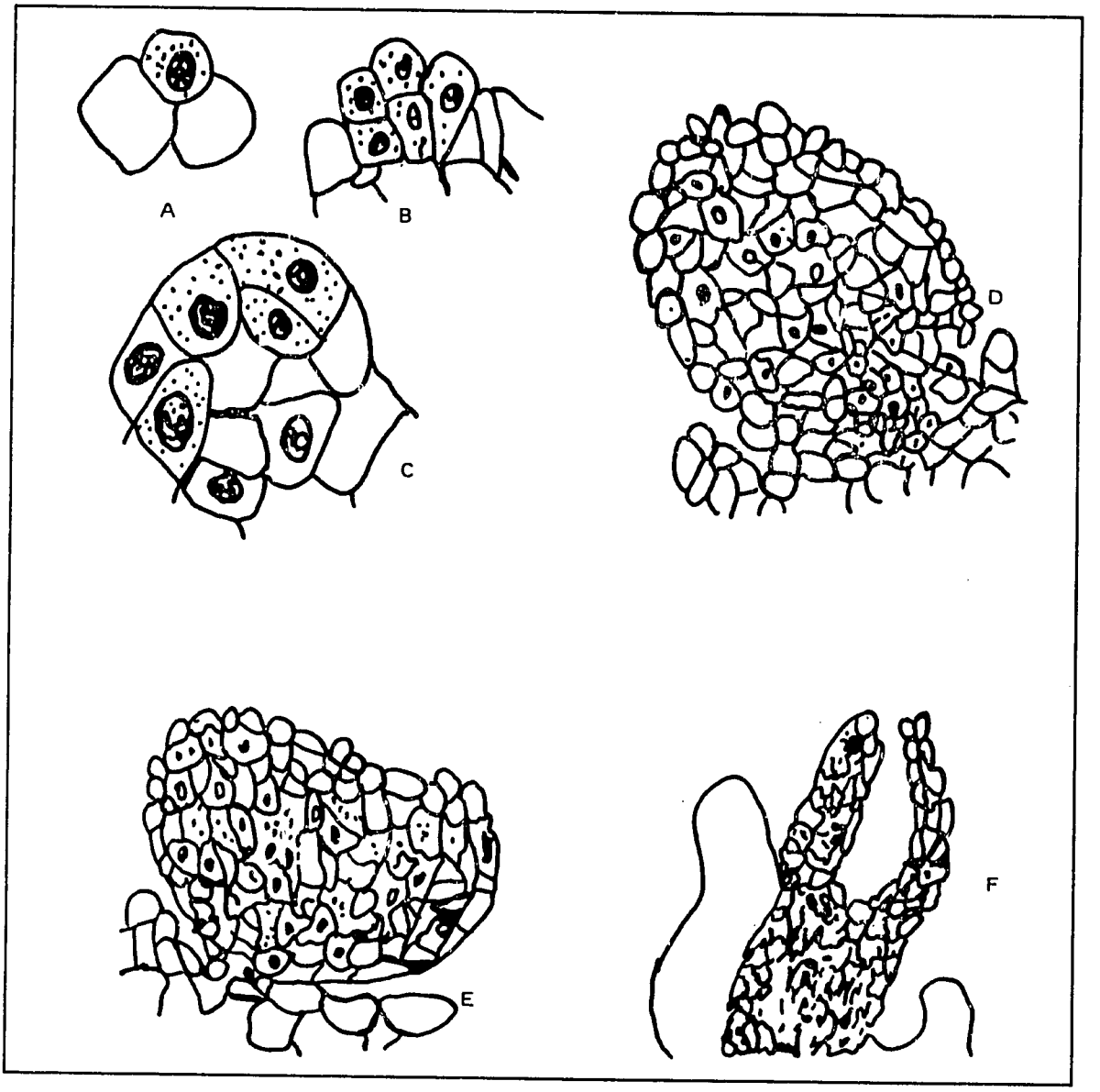

Fig. 3. Stages in the development of embryoids from epidermal cells of the hypocotyl of *Ranunculus sceleratus*

cytoplasm, adjacent to the cell wall. In cells of developing epidermal embryoids, there may be so many as to give the cytoplasm a honeycomb appearance. It has been suggested that these vesicles contain lipid reserves important for the growth of embryoids into plantlets, or that they may be important in the transport of substances from the cell surface into the cell. After protoplasmic continuity is served, such a metabolism could provide a means of transporting essential metabolites from the stem or from the callus aggregate into the developing embryoids.

Zygotic embryogenesis of orchids is worth noting, because the embryo itself is not fully developed when the seed matures: it comprises only a mass of parenchymatous cells without any perceived differentiation. Development of the embryo takes place where the seed falls on the bark of a tree or on the soil surface. Typically, the developing embryo takes the form of a protocorm, which initiates shoot apex and continues to form stems and leaves, but not roots. If a protocorm is cultured in a medium supplemented with coconut water, or in a combination of coconut

water and auxin, it divides and forms a cluster of protocorms, and occasionally callus arises from this structure.

Somatic embryogenesis seems to follow the pattern of zygotic embryogenesis, except in the initial stages. When an axillary bud of *Vanda* x Miss Joaquim was cultured in a medium supplemented with 0.1 ppm NAA, a cell in the epidermis or hypodermis of leaf primodia began to divide, forming a tissue mass similar to that of a zygotic embryo[36]. The development thereafter followed the pattern of zygotic embryogenesis. In a study of other genera of orchids in our laboratory, we found that proliferation of the protocorm could be regulated by the level of auxin in the presence of 10% coconut water. At a suitable level, proliferation seemed to go on indefinitely. Separation of the protocorms into smaller clusters encouraged more proliferation. Some protocorms developed further in the presence of auxin, into complete seedlings with leaf, stem and root. When a cluster of protocorms were transferred onto a medium void of auxin, most of protocorms developed into seedlings, but some still proliferated further for several subcultures, due to their ability to synthesize endogenous auxin. An anatomical study of the callus that arose occasionally in the culture was made, and we found that small cells with large nuclei and dense cytoplasm were forming among loose parenchymatous cells. These groups of cells later formed a shoot apex, and subsequently became a protocorm.

The rapid clonal propagation of orchids is based on the behavior of somatic embryogenesis described above, which was first suggested by Morel in 1963.

REGULATION OF SOMATIC EMBRYOGENESIS

The chemical and physical environment has a profound effect on somatic embryogenesis. Somatic embryos have been grown on a wide range of media, from the relatively dilute White's medium to the more concentrated formulae of Murashige and Skoog or Schenk and Hildebrandt[22, 31, 38]. The most important chemical factors involved in the induction of somatic embryogenesis are the exogenous auxin content of the medium, and the composition of nitrogen compounds added as nutrients. Reinert[29] suggested that induction required the presence of auxin and an increased ratio of nitrogen to auxin.

At present, it is widely accepted that auxin is not involved in the development of embryoids from callus, although it is needed for callus growth. Embryoids will generally form when the callus is transferred from an auxin-supplemented medium to another with a lower concentration or even one without auxin. Nomura and Komamine[25] have suggested a useful model of pathways and conditions leading to various developmental stages in carrot culture. This model is summarized schematically in Fig. 4. Embryonic development appears to be initiated by culturing 12 μm cells in a medium with a low auxin level, where these cells divide to generate proembryogenic masses (PEMs). When PEMs are transferred to an auxin-free medium, active somatic embryogenesis results.

Gibberellins are found to have a negative effect on both embryogenesis and organogenesis of carrot and other plants[12, 14].

Besides auxins, the forms of nitrogen compound present in the medium have a significant effect on somatic embryogenesis. Wetherell and Dougall[37] reported that in the culture of wild carrot, the addition of 10 mM NH_4Cl to an embryogenic medium already containing KNO_3 (12-40 mM) produced a near-optimal number of embryoids. Glutamine may replace NH_4Cl as a supplement to KNO_3. Halperin and Wetherell[15] demonstrated that the presence of reduced nitrogen was critical only in the induction medium, so that if callus were raised on a medium with KNO_3 + NH_4Cl, it would form embryoids irrespective of the presence of NH_4Cl in the differentiation medium.

For embryogenesis to occur in cultured cells of carrot, the presence of a minimal amount of NH_4^+ is essential. However, if it is not convenient to use NH_4^+ in combination with NO_3^- for embryogenesis, casein hydrolysate, glutamine and alanine may serve as reliable substitutes[37].

Brown and co-workers[9] have reported that high potassium (20 mM) is required for maximum yield in the embryogenesis of carrot, whereas a 5 mM level is sufficient for cell proliferation.

There have been some reports that several substances are released by tissue culture which inhibit morphogenetic activity as well as cell growth. One example is the release in citron cells of ethylene and ethanol, which are both potential repressors of somatic embryogenesis. However, it is possible to reduce the effects of some inhibitors by adding activated charcoal to the medium[21].

Physical factors, such as temperature, light intensity, photoperiod, gas, vessel, state of the medium and speed of the shaker, have also been reported to affect somatic embryogenesis. The

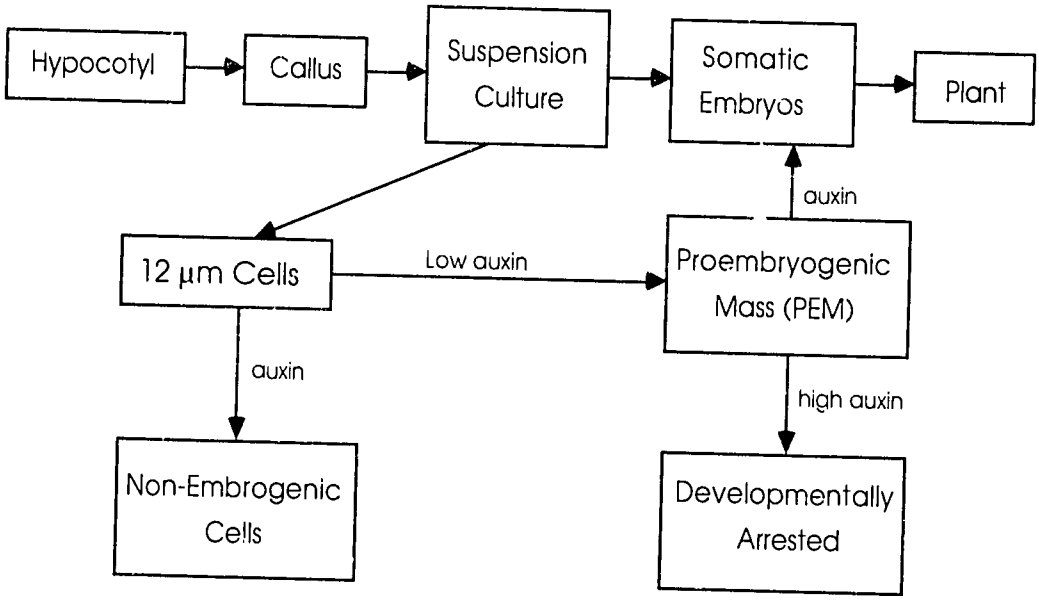

Fig. 4. Schematic pathways and conditions leading to embryogenic and non-embryogenic states in carrot cultures
Source: Nomura and Komamine, 1985[25]

optimum temperature may be specific for various species and stages of development. Heat or cold treatment at certain stages may improve embryogenesis and the germination of somatic embryos and other propagules to complete development.

POSSIBLE APPLICATIONS OF SOMATIC EMBRYOGENESIS

Embryoids derived from cell cultures have many distinctive features, some of which are useful and may be of great value in the future. An embryoid is a complete plant in itself. It has both shoot and root, and little effort is required to induce the seed to germinate and grow. In contrast, an organogenetic plant, which produces only a shoot at the early stages, needs considerable work in changing the media to suit each stage of development before it becomes a plantlet. If synchronization of growth can be readily controlled, somatic embryogenesis will be amenable to mechanical handling, since it usually produces a large number of embryoids per volume of medium which float freely.

Dormancy occurs in the zygotic embryos of some groups of plants. If this could be induced in somatic embryos, the possibility arises that they could be incorporated into artificial seeds, either by coating or encapsulation. These artificial seeds could be handled like natural seeds, but could be expected to be more uniform genetically. If the cost of production could be made comparable to that of natural seeds, this would be a very worthwhile subject for future research.

Some secondary metabolites normally found *in vivo* are reported to be absent in actively dividing cells in callus or suspension culture. Through mutation selection, one might obtain such chemicals, or even new chemicals which never appear *in vivo*. Alternatively, the synthesis of such a metabolite might begin again in early embryogenesis so that the mature embryo ends up containing a high level of it[1]. The discovery of this possibility seems certain to lead to the future improvement of techniques for obtaining secondary metabolites *in vitro*.

At present, the frequency of somatic embryogenesis still varies appreciably from variety to variety, clone to clone or even from experiment to experiment[2]. These variations may be largely due to our lack of a complete understanding of the controlling factors, but it makes it difficult to apply somatic embryogenesis to industry. Research on the factors controlling synchronization of embryogenesis, dormancy and the synthesis of secondary metabolites are needed in due course, but one should not overlook the value of basic research in growth, differentiation and development.

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DISCUSSION

Q. (M.T. Senawi)
I am curious about the effect of shaking tissue cultures on the embryogenesis of protocorms. Does it improve the aeration? Does it help with the problem of vitrification?

A. Yes, it may, but in some plant species a low concentration of oxygen stimulates a high regeneration rate. It has to be tested for each species, and in the majority of cases this has not yet been done.

PROTOPLAST TECHNIQUES IN TISSUE CULTURE

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ABSTRACT

Protoplast techniques have developed rapidly during the last decade. Enzymes of good quality, such as cellulase Onozuka R-10, macerozyme R-10, driselase, cellulysin and pectolyase Y-23, are commercially available, and highly viable protoplasts can be isolated with them.

More efficient culture methods, such as the liquid drop culture, nurse culture, microchamber culture and semisolid agarose plating culture, have been devised in order to increase the rate of cell division and cell colony formation.

Various fusion methods have been suggested and improved. Of these, the PEG-high pH-high Ca^{++} method has been the most widely used. An interface of sucrose-glucose PEG method which has recently been modified is expected to give even more efficient protoplast fusion.

Electrofusion and microinjection method are also believed to be useful for protoplast fusion, but the equipment for these is very expensive.

Visual selection through a microscope, using a micromanipulator, is the most accurate way to select hybrid cells fused between foreign protoplasts on a 1:1 basis, but it is inefficient. Mechanical selection with FACS is a highly efficient method, if better equipment becomes available. Because other methods such as complementation and drug resistance are limited to hybrid cell lines which have specific characteristics, it is difficult to apply them if a wide range of genetic resources is to be selected.

Compositions and concentrations of media, 8p-KM, V-KM, NT, B_5 and MS are formulated, and revised for the culture of protoplasts. Phytohormones to supplement the basal media are identified, in accordance with culture stage, cell division, callus formation and organogenesis from protoplast culture. Of these media, 8p-KM medium containing NAA, 2,4-D and BA is best for initial protoplast culture, and MS medium with 2ip and/or zeatin, for organogenesis.

Petunia, Lycopersicon, Solanum and Brassica species are easily regenerated from protoplasts. Somatic hybridization has been achieved through inter- and intra-specific fusion of species or genera belonging to the Solanaceae and Brassicaceae. Of the woody species, Prunus, Citrus, Hibiscus, Malus and cherry protoplasts have been cultured. Of these, plantlets were regenerated from cultures of Citrus, and fusion products between Citrus sinensis and Poncirus trifoliate were cultured and somatic hybrid produced.

INTRODUCTION

The *in vitro* breeding system can introduce a broad range of desirable genetic material into field crop species, and can solve various problems which are impossible to solve by conventional breeding systems. These are:

- the development of hybrid plants of distantly related species through embryo culture;
- the establishment of triploid plants through endosperm culture, and
- the production of selfing seeds from *in vitro* production of haploids, to shorten the time needed for plant breeding.

However, none of these suggest the possibility of obtaining hybrids from cross incompatible plant species[19].

Since 1970, there have been many attempts to introduce desirable genetic material into protoplasts. At first, the selection of mutants from protoplast culture, and the production of somatic hybrids from fusogen-treated protoplasts, were studied. The plant protoplasts had the remarkable property of being able to take up various organelles and specific genetic material such as DNA, mitochondria, chloroplast, and chromosomes. Furthermore, there was the future prospect of introducing the symbiotic nitrogen-fixing bacterium "*Rhizobium*", and free nitrogen-fixing bacteria such as "*Azotobactor*", into plant protoplasts[4]. The use of Ti-plasmid of *Agrobacterium*, as a vector for the genetic transformation of plant protoplasts by uptake, or of subprotoplast by fusion, was demonstrated.

In this paper, I should like to suggest a basic theory of protoplast isolation, fusion and culture, and some potential applications to horticultural crop improvement of plant protoplast culture.

PROTOPLAST ISOLATION

Source of Protoplasts

Protoplasts can be isolated from various kinds of tissues and organs in horticultural plants, including the leaf[76, 89, 105], shoot[73], shoot tip[14], epidermal cell[68], root[47], hypocotyl[7], cotyledon[2, 22], somatic embryo[7], stem[27] and fruit[52, 80]. In addition, protoplasts have also been isolated from callus[51] and suspension cells[5, 43, 46, 92]. Both callus and suspension cells have certain advantages, in that the requirements for their growth and morphogenesis are already known. Mesophyll tissues from leaves are, however, the most commonly used source of protoplasts, apart from those plants, *Hibiscus*[51] and *Amaranthus*[22], for which it is impossible or ineffective to isolate protoplasts from mesophyll tissue.

Environmental Conditions

Since the physiological condition of the source tissue influences the yield and viability of isolated protoplasts, stringent control of the growth of plant material assists in optimizing release and maximizing protoplast growth during subsequent culture. Calcium pectate often accumulates especially in the tissue of plants grown under high temperatures. As a result, cell wall degradation is difficult and healthy protoplasts cannot be released[17].

Controlled environment chambers alleviate some of the variation associated with field and greenhouse grown plants of *Solanum tuberosum*[45] and *S. melongena*[6, 83]. Likewise, Sink and Niedz[94] reported the influence of environmental preconditioning factors of planting efficiency (PE) in the tomato. Planting efficiency was greatly increased when cultured protoplasts were isolated from CEC plant at 27°C DT/22°C NT regime. Increasing the temperature to 32°C DT/27°C NT did not increase PE, but decreasing the temperature decreased the PE. This result indicates that the physiological status of the source material has a marked effect on the survival of cells and cell division capability in culture (Table 1).

Table 1. The influence of environmental preconditioning factors on plating efficiency of *L. esculentum*[43]

Treatment	Plating efficiency (%)
Green house	22.6
CEC: DT 22 C/ 16 C NT	35.5
DT 27 C/ 22 C NT	50.4
DT 32 C/ 27 C NT	49.7

Material: The 1st leaf of 4-5 weeks old plant (green house) or 29 days-old plant in a controlled environmental chamber (CEC). DT: Day temperature. NT: Night temperature

Preferred environmental conditions such as temperature, humidity and light intensity vary in different plant varieties. Suitable temperatures range from 17°C to 28°C, humidity, 60 to 90%, light intensity, 2500 to 12000 lux and a photoperiod of 12-18 hours[93, 98, 101].

When *in vitro* cultured plants are used as protoplast sources, Murashige-Skoog's medium[70], with or without phytohormone, is widely used as a nursery medium. The range of temperature and photoperiod are approximately the same as in *in vivo* culture, but the light intensity is comparatively low[14, 75, 82, 87].

Higher plating efficiency has often been indicated in cultures of protoplasts which originate from mesophyll tissue cultured *in vitro* rather than *in vivo*. Comparison tests of two species, *Solanum melongena* var. fructualbo and *Brassica oleracea* var. *acephala* DC. *forma tricolor* Hort., are shown in Fig. 1. Both species show a higher percentage of cell division and cell colony formation from cultures of protoplasts which originate from *in vitro* cultured mesophyll tissue.

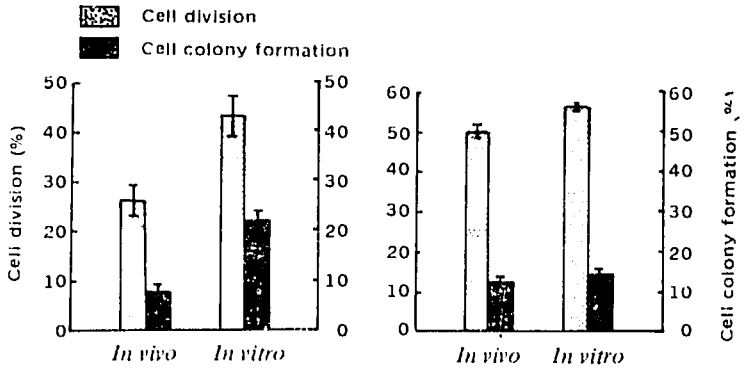

Fig. 1.
Plating efficiency of mesophyll protoplasts in *Solanum melongena* "fructualbo" (left) and *Brassica oleracea* (right). Vertical bars represent standard error[23, 26]

Control of the growth of the source material is most easily achieved with cultured cells. Suspension cultures have been used routinely by many workers[5, 13, 46, 92], with optimal protoplast formation in the early log phase of the growth cycle from batch propagated suspensions[100].

Cell doubling time depends upon the species, but the optimal time for easy release of protoplasts and improved viability of protoplasts is seven to ten days after subculture[15, 25, 29].

Trends in cell division and cell colony formation according to the number of days of subculture are shown in Fig. 2. In *Hibiscus syriacus*, cell division and cell colony formation increased slightly on the 10th day after subculture, but subsequently decreased, while in *Hibiscus rosa-sinensis*, both increased on the 7th day after subculture and subsequently decreased rapidly. However, no difference in protoplast yield and viability during the subculture period was observed in either species. The period of subculture seems to have much more effect on cell division capability than on protoplast yield.

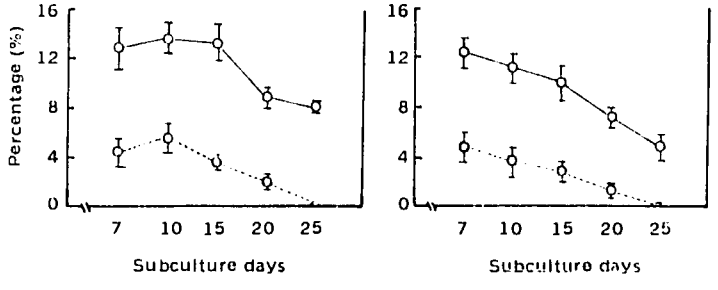

Fig. 2.
Effect of no. days subculture on cell division (o–o) and cell colony formation (o--o) from callus derived protoplast of *Hibiscus syriacus* (left) and *H. rosa-sinensis* (right). Vertical bars represent the standard error[51]

Mechanical Maceration of Source Plants

To assist the penetration of enzymes into leaf tissues, the lower epidermis is peeled off[56, 79], or the leaves are sliced longitudinally or transversely[21, 76, 33]. Sometimes, pricking leaves with a needle[40], brushing[5], or brushing and slicing the leaves[59], promotes easy release of the protoplasts, and as a result, protoplast yield increase during a short period of enzyme digestion. Methods best suited to the characteristics of the material used should be chosen for maximum protoplast release.

Pretreatment of Source Material

All plant tissues are physiologically stressed during incubation in enzyme solution. To minimize physiological stress, preplasmolysis, or stabilization of the source material prior to enzymatic digestion, is required, to force protoplasts to shrink and to seal the plasmadesmata connecting adjacent cells.

This prevents spontaneous fusion, and reduces the amount of enzyme taken up into the cytoplasm by endocytosis during the initial period of protoplast contraction. Sealing the plasmadesmata also prevents leakage of the cell contents, particularly of ribonuclease which can initiate senescence and

protoplast lysis (for a detailed description, see Potrykus *et al.* 1983)[30]. Since stabilization at room temperature followed by cold temperature treatment decreases the rate of accumulation of starch grains, it helps to produce healthy protoplasts and stimulates cell division[88]. The effect of preplasmolysis or pre-cold treatment on protoplast yield is shown in Fig. 3. When leaf tissues of *Solanum melongena* var. fructualbo were pre-plasmolyzed after peeling in 0.7 M mannitol solution containing CPW inorganic salts at room temperature for one hour, protoplast yield increased markedly.

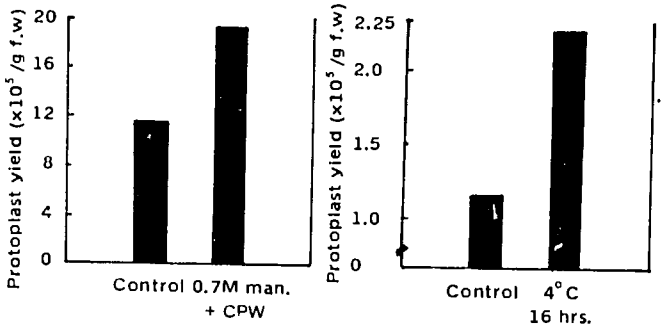

Fig. 3.
Effect of preplasmolysis in *Solanum melongena* "fructualbo" (left) and pre-cold treatment of *Nicotiana sanderae* (right) on protoplast yield[21, 25]
man.:mannitol

Protoplast yield also increased tremendously when sliced leaf tissue of *Nicotiana sanderae* was placed in a solution containing 1.0 mg/*l* NAA and BA, 1 mM $CaCl_2 2H_2O$, and 1mM NH_4NO_3 for 16 hrs at 4°C.

Enzymatic Digestion

Cocking[27] first used enzymes to release plant protoplasts by applying an extract of hydrolytic enzymes isolated from tomato root tips. The most frequently used are hydrolytic enzymes such as cellulase, hemicellulase and pectinase, which are partially purified from microorganisms and now commercially available.

Enzyme mixtures useful for the isolation of protoplasts in several horticultural plants are shown in Table 2. Cellulase Onozuka R-10, macerozyme R-10 and driselase are good hydrolytic enzymes for isolating protoplasts from the mesophyll tissue of *Solanum melongena* var. fructualbo, the cotyledon of *Amaranthus tricolor*, the stem tissue of tomato "Tiny Tim" and the callus of *Hibiscus syriacus*. However, different materials require different optimal combinations and concentrations of enzymes and incubation time in the enzyme solution. Similarly, methods suitable for isolating viable protoplasts from one particular type of plant may be unsuitable for another genus or species.

Table 2. Enzyme mixtures for the isolation of protoplasts in several horticultural plants[21, 22, 23, 51]

	Species				
	S. melongena var. fructualbo		Tomato "Tiny Tim"	*Hibiscus syriscus*	*Amaranthus tricolor*
	Source				
	mesophyll		stem	callus	cotyledon
	Growing condition				
Enzyme mixture	*In vivo*	*In vitro*	*In vitro*	*In vitro*	*In vitro*
Macerozyme R-10 (%)	0.2	0.2	0.5	0.5	0.3
Cellulase R-10 (%)	1.5	1.0	2.0	6.0	2.0
Driselase (%)	–	0.3	–	–	0.3
Mannitol (M)	0.6	0.4	0.4	0.6	0.3
MES (mM)	0.01	0.01	0.01	0.01	0.01
BSA (%)	0.2	0.2	0.2	0.2	0.2
Inorganic salts	CPW	CPW	CPW	CPW	CPW
pH	6.3	6.3	6.3	5.8	5.8
Digestion time (hrs)	3–4	2	3	4	2.5
Temperature (°C)	28	30	30	29	28

Osmotic Regulation

Mannitol can be used in enzyme solutions as an osmotic regulator. Optimal concentrations are 0.6 M for callus tissue of *Hibiscus rosa-sinensis*, 0.4 M for the stem tissue of tomato "Tiny Tim", 0.3 M for the cotyledon of *Amaranthus tricolor*, and 0.5 M for *in vitro* cultured mesophyll tissue of *Brassica oleracea* var. *acephala* DC. *forma tricolor* Hort. (Fig. 4). The molarity of osmotica varies in different tissues or organs. Optimizing the osmotic condition is very important in obtaining viable protoplasts. In general, this is easily accomplished by the inclusion of an osmotica stabilizer, such as 0.3-0.8 M mannitol and/or sorbitol, glucose or sucrose in the solution, or by a combination of ionic as well as nonionic osmotica[1, 48, 55].

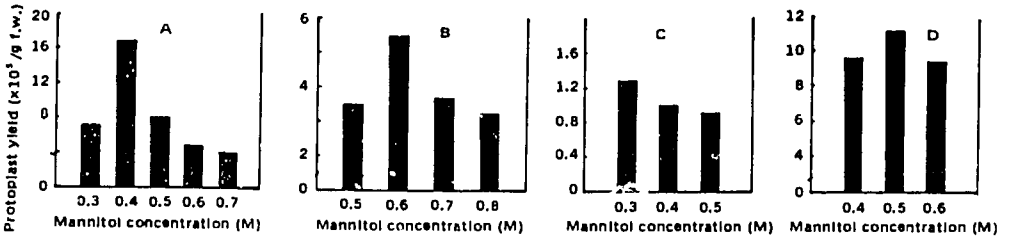

Fig. 4. Effect of mannitol concentration in enzyme solution as osmoticum on healthy protoplast yield of tomato "Tiny Tim" (A), *Hibiscus syriacus* (B), *Amaranthus tricolor* (C) and *B. oleracea* (D). Healthy protoplast yield (■, x10⁵/g f.w)[21, 23, 26, 51]

The hydrogen ion concentration in the enzyme solution is also critical, and optimal values range from 5.8 to 6.3[22, 23, 26, 51].

As with concentration and combinations of enzymes and osmoticum regulators, digestion time also influences protoplast yield and viability.

In *Brassica oleracea* L. var. *acephala* DC. *forma tricolor* Hort., total protoplast yield increased continuously for 6 hours, but healthy protoplast yield increased linearly for only 5 hours After this, the viability of the protoplasts began to decrease rapidly. It is considered that enzymatic digestion for 5 hours is suitable for obtaining viable protoplasts (Fig. 5).

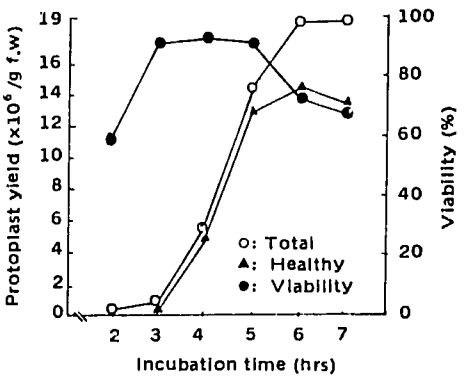

Fig. 5. Effect of incubation time in enzyme solution on protoplast yield and viability of *Brassica oleracea* L. var. *acephala* DC. *forma tricolor* Hort.[26]

The Addition of Organic or Inorganic Compounds to the Enzyme Solution

Various kinds of compounds, alone or in combination, can be added to the enzyme solution. They help in obtaining healthy and viable protoplasts by stabilizing them, and prevent deteriolation in isolated protoplasts. The addition of such compounds to the enzyme solution has a marked influence on protoplast yield and viability. In tomato "Tiny Tim", when stem tissue was digested in enzyme solution containing BSA, MES and PVP, alone and/or in combination, protoplast yield increased noticeably. In callus tissue of *Hibiscus syriacus*[51], DTT added to the enzyme solution led to the release of many more protoplasts compared with other solutions (Fig. 6).

PURIFICATION OF PROTOPLASTS

As soon as enzymatic digestion of the material is completed, the protoplasts must be removed from the enzyme solution. In most cases, the debris can be satisfactorily eliminated by filtering it through a stainless steel sieve or nylon filters (40-

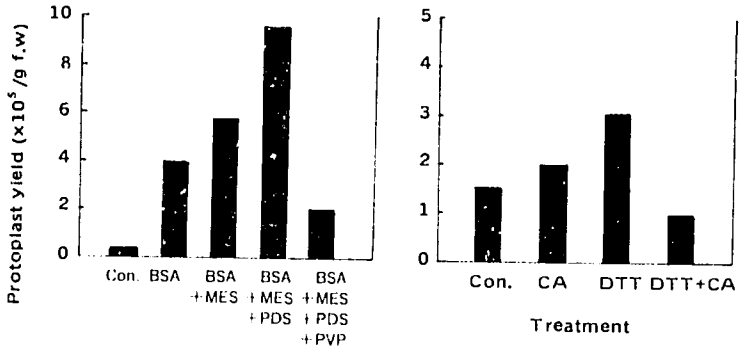

Fig. 6.
Effect of additives to enzyme solution on protoplast yield of tomato "Tiny Tim" (left) cultured *in vivo* and *Hibiscus syriacus* (right). Healthy protoplast yield (■, x10⁵/g f w)[23, 51]

BSA: Bovine Serum Albumin
MES: (N-Morpholino) ethanesulfonic acid
PDS: Potassium Dextran Sulfate
PVP: Polyvinylpyrrolidone
CA: Citric acid
DTT: Dithiothreitol

100 μm pore size), followed by a short period (3-10 min) of low-speed centrifugation (40-80 g) to yield the protoplasts[89, 105, 109]. The concentration of sucrose varies between 0.3 M to 0.6 M sucrose and/or mannitol[31,89].

The sucrose flotation method results in less protoplast breakage, but may in some cases damage protoplasts due to osmotic shock. The most critical variables for protoplast isolation when this method is used are the concentration of sucrose, mannitol or sorbitol, and the speed of centrifugation: both affect the stability of fragile protoplasts.

Ficoll[42], percoll[2], the dextran-polyethylene glycol two-phase system[52] and Shepharose J-539 beads[57] are also used for purification of protoplasts (for handbook, see Evans and Bravo[35]).

Protoplast Viability

The viability of the freshly isolated protoplasts can be checked by a number of methods:

- observation of cyclosis or cytoplasmic streaming, as an indication of active metabolism,
- oxygen uptake, measured by an oxygen electrode, to indicate the respiratory metabolism,
- photosynthetic activity[7].

Methods mentioned above are physiological ways of determining protoplast viability. The most frequently used staining methods for chemically assessing protoplast viability are shown in Table 3.

Table 3. Chemicals to check for protoplast viability[7]

Chemicals	Final concns	Response	Check after staining
Fluoresceine diaceatate (FDA)	0.01%	yellow, green : viable	5 min.
Phenosafranine	0.01%	red : inviable	30 min.
Evans' Blue	0.1%	blue : inviable	5 min.
Calcoflour White (CFW)	0.1%	fluorescent: viable	–

Subprotoplasts

Subprotoplasts can be obtained by X-ray irradiation, plasmolutic induction and centrifugation, or may form spontaneously[63].

Microplasts, surrounded by an inner membrane of the cell (probably derived from the tonoplast) can be readily isolated in a number of plant species by rupturing auxin-induced highly vacuolated thin-walled callus cells[9]. Even if fragmentation of nuclear material cannot readily be achieved, the transmission of useful genes might be possible in such enucleate subprotoplasts and microplasts, in order to transfer a range of cytoplasmic factors by fusion with nucleated protoplasts.

A diagram of the transfer of cytoplasmic-based male sterility and herbicide resistance between sexually incompatible species is given in Fig. 7.

Fusion studies with subprotoplasts have been carried out on *Brassica napus*[103], *Allium cepa*[15], petunia nuclei and tobacco subprotoplasts, and petunia protoplasts with tomato subprotoplasts[10]. Cell division and segregation of plastids were observed in petunia and tobacco fusion products.

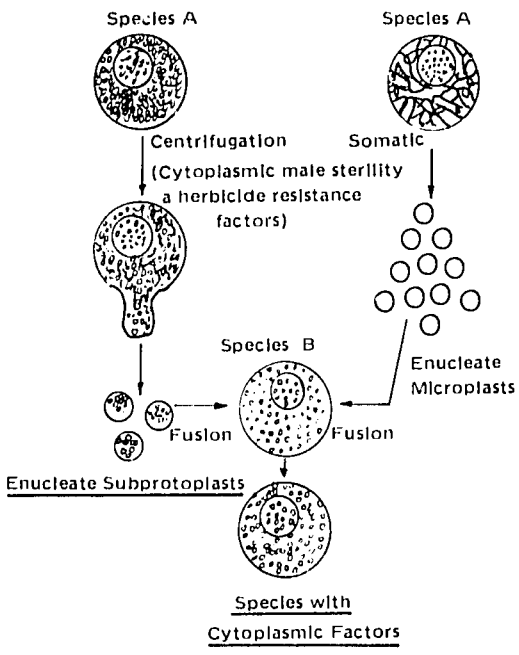

Fig. 7. Transfer of cytoplasmic factors by fusion[77]

PROTOPLAST FUSION AND SOMATIC HYBRIDIZATION

Spontaneous Fusion

During the enzymatic degradation of cell walls, some of the adjacent protoplasts fuse together, forming homokaryons. This type of protoplast fusion, called "spontaneous fusion", is generally ascribed to expansion and subsequent coalescence of the plasmadesmatal connections between the cells[106]. Enzymatically isolated meiotic protoplasts of *Lilium longiflorum* and *Trillium kamtschaticum* show one or more nuclei by spontaneous fusion[49, 50]. Spontaneous fusion has also taken place in protoplasts of *Datura innoxia*[91]. A longer treatment or increased concentration of the enzyme dramatically decreased their ability to fuse spontaneously.

Induced Fusion

The fusion of mechanically isolated protoplasts was reported as early as 1909 by Küster[61]. He tested several chemicals for their ability to induce fusion, and found calcium nitrate to be the most effective fusion agent, even though the frequency of fused protoplasts was low. During the last decade, a variety of fusogens have been tested: $NaNO_3$, artificial sea water, lysozyme, mechanically induced adhesion, gelatin, high pH-high Ca^{++}, antibodies, plant lectin Concanavalin A, Polyethylene glycol, Polyvinyl alcohol and phospholipid, 1,2-0-depenta-decyl-methylidine-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine, PEG-Ca^{++}, PEG-high Ca^{++}-high pH under high temperature, dextran and PEG-lipid(1-tocopherol). Of these, PEG-high pH-high Ca^{++} methods have been most successful (for a review see Visil[85]). Recently, Kao[54] has improved the PEG method, and proposed a revised fusion method, using the interface of a glucose and sucrose-PEG solution. Several methods which have been routinely used for photoplast fusion were tested with *Petunia hybrida* and *Solanum nigrum*, *Datura stramonium* or *Solanum melongena*. The PEG-high pH-high Ca^{++} method was identified as the preferred fusion method for all fusion partners (Table 4). Optimal conditions for efficient fusion between *Petunia hybrida* and *Solanum nigrum* were investigated. Fusion frequency increased as the PEG concentration became higher, while when other factors were tested, fusion frequencies increased markedly only when optimal conditions obtained (Fig. 8). However, conditions under which the fusogen was effecti ve were approximately similar to already established routine conditions. A slight modification of fusion condition will generally be enough to get good fusion efficiency, regardless of fusion partners.

The electrofusion method was first demonstrated by Senda *et al.*[86], and subsequently by

Table 4. Percentage of fusion frequency at different fusion methods in several combinations[24]

		Percentage of fusion frequency (%)		
			Petunia hybrida	
Chemical	Conc.	*Solanum nigrum*	*Datura stramonium*	*S. melongena*
PEG-high pH-high Ca^{++}	MW 6000 30%	16.8 ±2.0	15.8 ±1.9	16.8 ±1.0
	MW 1540 30%	14.3 ±2.5	17.2 ±0.2	10.1 ±1.2
Dextran	10%	9.3 ±1.1	7.8 ±2.1	7.4 ±1.5
PVA	15%	17.1 ±0.2	15.8 ±4.2	15.9 ±1.7

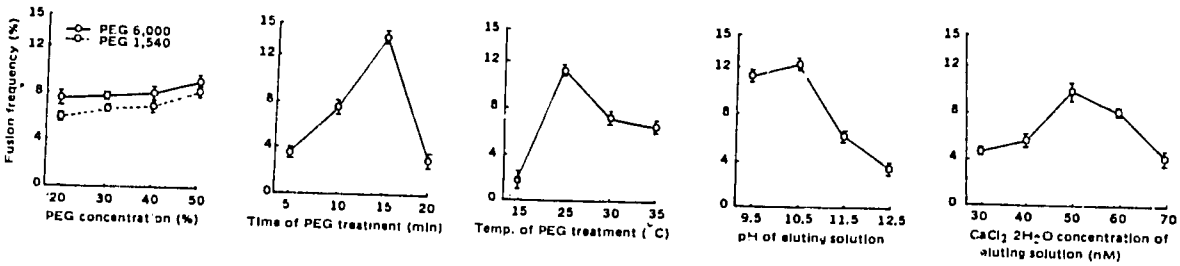

Fig. 8. Factors affecting fusion between two kinds of mesophyll protoplasts, *Petunia hybrida* and *Solanum nigrum*[47]. Vertical bars represent standard error
PEG: Polyethyleneglycol

Zimmermann and Scheurich[110]. Recently, a more efficient electrofusion method has been proposed by Spangebery[96].

Narton *et al.*[72] assayed the integrity of interspecific hybrids obtained by the electrofusion of vacuolate and evacuolate protoplasts (Table 5). About 90% of vacuolate and evacuolate protoplasts show the presence of fluorescein diacetate. Less than 80% of hybrids retained the ability to produce it (production depends on an intact plasmamembrane and the activity of cytosolic esterase). In contrast, assay on the basis of photosynthetic oxygen evolution generally showed a lower level of integrity.

Table 5. Integrity of vacuolate and evacuolate protoplasts of tobacco mesophyll and of interspecific hybrids obtained by electrofusion[72]

Type of protoplast	Integrity (%) FDA	According to bacterial assay
Vacuolate protoplast	90.0 ±5.7	77.4 ±3.3
Evacuolate protoplast	85.6 ±12.4	64.5 ±4.9
Interspecific hybrids	76.5 ±9.2	59.4 ±13.3

More recently, methods of immobilizing plant protoplasts for microinjection have been demonstrated by several workers[29, 44, 62, 69, 90, 97]. However, the efficient transformation of plant cells by microinjection has yet to be demonstrated. If microinjection techniques become more advanced, and as reliable as techniques of gene transmission, it will become a promising method for the transformation of expected gene sources into plant cells. Usually, a high percentage of karyoplasts survive after microinjection. In one series of experiments, a number of microinjected karyoplasts were electrofused with cytoplasts, and approximately 30% of the fusion products survived (Table 6). More recently it has been found that survival does not depend completely on fusing the microinjected karyoplast with a cytoplast. In fact, the microinjected karyoplast is capable of regenerating cytoplasm and a cell wall rather soon after microinjection, and also undergoing cell division. It therefore behaves like a microinjected protoplast. The major difference is that intranuclear microinjection into a karyoplast is much easier than into a protoplast.

Table 6. Microinjection of individually selected protoplasts karyoplasts and cell reconstructions with microinjected karyoplasts[44]

Microinjected cell or fragment	% Survivors[z]
Protoplast	82 ±6
Karyoplast	83 ±8
Karyoplast + cytoplast	22 ±9

z Mean values ± standard error from 8 independent experiments are given

Cell division and callus formation have also been observed in microinjected protoplast cultures of *Nicotiana tabacum*[62] and *Medicago sativa*[99].

Selection of Somatic Hybrids

A variety of different products, including homokaryotic and heterokaryotic fusion products, may be formed in fusion experiments involving protoplasts from two different species. The selection of true somatic hybrid products is therefore a key

problem in somatic hybridization. At the present time, one of the most difficult problems is to identify and promote the growth of the desired fusion product.

Some selection methods have been developed[27, 98]. These include:

- visual selection through a microscope, using a micromanipulator[54, 76],
- complementation selection, as for chlorophyll synthesis or auxotroph mutants[28, 66, 85],
- drug or nutritional resistance, including resistance to antibiotics and herbicides, and nutrition such as amino acid analogues and nitrate reductase deficient cell lines[64, 65, 74, 104, 107],
- growth patterns[16, 84, 95],
- morphological differences[12, 40, 41, 60, 67], and
- mechanical selection with fluorescence activated cell sorting[81].

PROTOPLAST CULTURE

Culture Methods

Methods used for protoplast culture are basically the same as those employed for tissue and cell culture, but the fragility of protoplasts, and the need to adjust osmolarity, should be considered when protoplasts are cultured in a liquid or semisolid medium. Cultural methods should also be devised to improve planting efficiency. Protoplast culture methods developed so far comprise:

- culture in liquid media, including liquid culture, drop culture, microchamber culture, multiple drop array technique and microdroplet culture,
- culture on semisolid media, including agar, agarose, and alginate as gelling agents,
- a combination of liquid and solid media, including gel-embedded protoplast culture and semisolid media for liquification, and
- various feeder techniques, including feeder layers, nurse cultures, reservoir media, filter paper discs on agar media and agar drop techniques.

Of these methods, liquid and drop culture methods are most commonly used for protoplast culture. Agarose as a gelling agent has given the best results, in terms of retention of viability and secondary product production[34].

Basal Medium

As the nutritional requirements of cultured cells and protoplasts are very similar, modified cell culture media are usually employed for protoplast culture. Modified MS[70], 8p-KM[56], V-KM[14], B_5[38] and NT[71] media have all been used for protoplast culture. Characteristically, the formulations of these media have a low concentration of ammonium ions, and a low concentration of iron and zinc, while they tend to contain a higher concentration of calcium ions than cell culture media (two to four times higher). In addition, protoplast media are abundantly enriched with sugars, sugar alcohols and vitamins.

When protoplasts of *Petunia hybrida*, *Solanum melongena* var. fructualbo and *Hibiscus syriacus* were cultured in three media, NT, V-KM and 8p-KM, cell division and cell colony formation were found to be stimulated most by the 8p-KM medium (Fig. 9).

Glucose seems to be the best carbon source added to the initial culture media for most protoplasts[37], although other carbon sources may be preferred for the initial culture of some species[56]. Sucrose is suggested as the best carbon source for protoplast-derived callus culture, and for organogenesis as in tissue or cell culture[21, 26, 51].

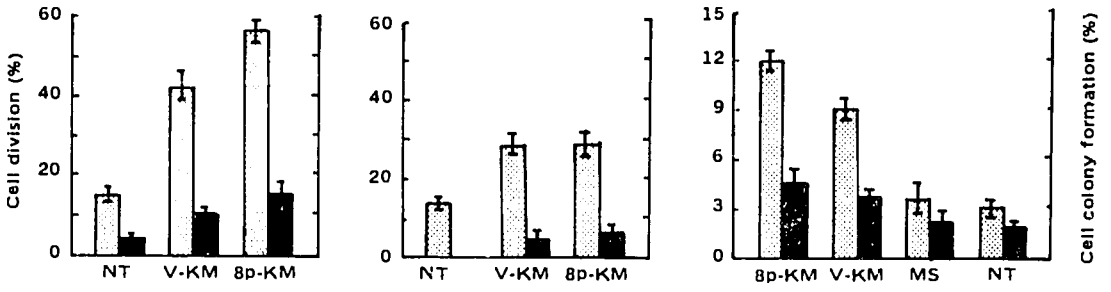

Fig. 9. Effect of culture media on cell division and cell colony formation from mesophyll protoplasts of *Petunia hybrida* (left), *Solanum melongena* "fructualbo" (middle) and *Hibiscus syriacus* (right)[21, 51] □ cell division ■ cell colony formation

When protoplasts of *Hibiscus syriacus* were cultured in 8p-KM medium supplemented by various combinations of sugars, such as glucose, sucrose and mannitol, cell division and cell colony formation increased with a higher glucose concentration. This indicates that glucose is effective in promoting cell division and cell colony formation in protoplast cultures (Fig. 10).

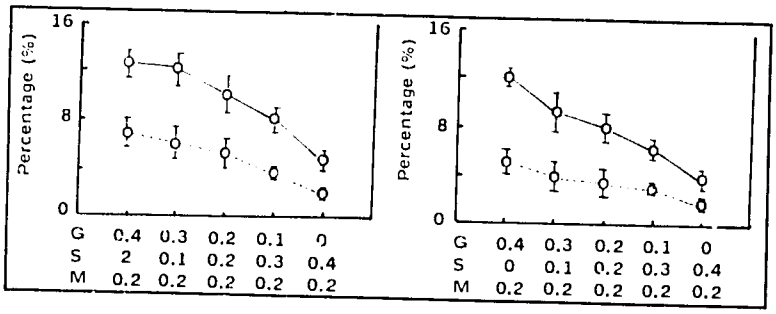

Fig. 10.
Effect of sugar concentrations in culture media on cell division (o–o) and cell colony formation (o--o) from callus derived protoplasts of *Hibiscus syriacus* (left) and *H. rosa-sinensis*[41]
G: glucose
S: sucrose
M: mannitol

However, most protoplast culture media contain a mixture of carbon sources. For tomato, a mixture of sucrose and glucose in a 2:1 ratio is used[100]. In many cases, non-metabolizible osmotica such as mannitol and sorbitol may be necessary[102].

Changes in the Nutritional Requirements of Protoplast Cultures

The successful culture of protoplasts demands regular dilution of the initial medium, in order to reduce gradually the level of osmoticum and ensure a continuous supply of nutrients for active growth.

This is a typical scheme: protoplasts (in 8 ml of medium:9% w/v mannitol zero time) $\xrightarrow{2\ \text{weeks}}$ addition of 2 ml of medium with 6% mannitol $\xrightarrow{2\ \text{weeks}}$ addition of 2 ml of medium with 3% mannitol $\xrightarrow[\text{as long as necessary}]{2\ \text{weeks}}$ addition of 2 ml of medium with 0% mannitol[50].

Simultaneously, a change in the exogeneous hormone regime may be advantageous to promote maximal cell division. In *Solanum tuberosum*, phytohormone concentration is changed according to the following procedure: Murashige-Skoog's medium with 2 mg// NAA and 0.5 mg// BAP and 2 mg// 2,4-D $\xrightarrow{4\text{-}5\ \text{days}}$ addition of newly prepared MS medium with only 5 mg// NAA in a 1:3 ratio $\xrightarrow{1\ \text{week}}$ transfer to 0.2% agar medium $\xrightarrow{3\ \text{weeks}}$ MS medium with 1 mg// BA and 0.2 gm// NAA $\xrightarrow{10\ \text{days}}$ MS medium with 0.01 mg// NAA and 1 mg// zeatin for organogenesis[95].

The procedures for protoplast culture of several horticultural crops are illustrated in Table 7.

CONCLUSION

Horticultural plant breeders appear to be skeptical of the potential for crop improvement by genetic manipulation through the use of protoplasts. The possibility most frequently referred to in protoplast research is to produce somatic hybrids between different plant species which cannot be hybridized by conventional means.

In terms of methodology, various techniques for the isolation, culture and fusion of protoplasts have been developed, but these techniques have been largely obtained from model systems using solanaceaous plants such as *Nicotiana*, *Petunia*, *Datura* and *Solanum*. However protoplast research has also been extended to include a number of different plant genera, including legumes, cereals and tree species.

Some new plant types from fusion product cultures of both closely and distantly related species have been produced, but no somatic hybrids with any advantages or superior characteristics compared with existing cultivars.

In order to use protoplasts for crop improvement, some problems must first be solved.

Firstly, how can we improve the regeneration ability from cultures of protoplasts in a wide range of economic crops? Secondly, how can we lessen the frequency of amphidiploids induced by multi-cell fusion? Thirdly, is there any way to prevent aneuploids occurring as a result of chromosomal elimination? Fourthly, how can we utilize protoplasts or cybrids* to introduce specific foreign genes such as nuclear RNA, DNA, chloroplasts or mitochondrial genomes? Finally, is there any

* Formerly known as cytoplasmic hybrids. Ed.

Table 7. Procedures for protoplast culture of several horticultural crops

Procedure	*Petunia hybrida*[z] *In vivo* mesophyll	*Solanum melongena*[z] *In vivo* mesophyll	*Solanum melongena*[z] *In vitro* mesophyll	*Hibiscus rosa-sinensis*[y] *In vitro* callus	*Hibiscus syriacus*[y] *In vitro* callus	*Brassica oleracea*[x] *In vitro* mesophyll	*B. oleracea*[w] + *B. napus* *In vitro* mesophyll
0 time	Initial culture medium	8p-KM 2,4-D 0.2mg/l NAA 1.0mg/l BA 0.5mg/l glucose 0.4M mannitol 0.1M pH 5.8		8p-KM 2,4-D 0.2mg/l NAA 1.0mg/l BA 0.5mg/l glucose 0.4M pH 5.8		8p-KM 2,4-D 0.3mg/l NAA 2.5mg/l BA 1.0 (or 2ip 4.0mg/l) GA 0.5mg/l glucose 0.4M mannitol 0.1M pH 5.8	8p-KM 2,4-D 0.2mg/l NAA 1.0mg/l BA 0.5mg/l glucose 0.2M mannitol 0.3M pH 5.8
7 days	Addition of fresh medium	mannitol 0.1M down	glucose 0.1M down			mannitol 0.1M down	
12 days				glucose 0.1M down			
14 days		MS+2,4-D 0.2mg/l NAA 1.0mg/l BA 0.5mg/l sucrose 30g/l pH 5.8					
21 days						MS+2,4-D 0.2mg/l NAA 1.0mg/l BA 0.5mg/l sucrose 30g/l pH 5.8	
25 days				MS+2,4-D 0.3mg/l NAA 2.5mg/l BA 1.0mg/l GA 0.5mg/l sucrose 30g/l pH 5.8			
5 weeks	Regeneration medium	MS-NAA 0.01mg/l 2ip 2.5mg/l sucrose 30g/l agar 7g/l pH 5.8	MS+2ip 8mg/l sucrose 30g/l agar 7g/l pH 5.8			MS+zeatin 4.0mg/l sucrose 30g/l agar 7g/l pH 5.8	MS-NAA 0.01mg/l zeatin 4.0mg/l sucrose 30g/l agar 7g/l pH 5.8
8 weeks							

[z] Chung and Lee, 1986[24]
[y] Jo, 1986[24]
[x] Chung and Jee, 1987[26]
[w] Unpublished

possibility of introducing cytoplasmic genetic information such as CMS, flower color and resistance into protoplasts? In addition, the mechanism of specific gene expression introduced into protoplasts should be identified. If protoplasts are fused with protoplasts, the cells become genetically unstable because they include two nuclei and two kinds of cytoplasmic genetic information, and they exhibit chromosomal and phenotypic variation as a result. Chromosomal elimination and disturbance of specific gene expression may follow.

In this respect, the availability of genetic variability in somatic plant hybrids also offers the opportunity of fusing protoplast-cytoplast, nuclear-cytoplast and protoplast-cell organelles, to help in the expression of specific desired genes.

I feel that the outlook for the use of protoplast technology, and concepts for gene transfer in crop improvement is very promising, especially for horticultural crops, once the problems indicated above are solved, which is likely to happen in the near future. This is the reason why there are so many scientists working in this field to make it possible to produce new and wonderful horticultural crops from protoplasts for the benefit of mankind.

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PROTOPLAST FUSION IN TOBACCO BREEDING

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ABSTRACT

Protoplast fusion has been used in an attempt to transfer valuable genes from Nicotiana wild species to tobacco. Somatic hybrids between tobacco and N. repanda, a wild species with valuable disease resistances, were produced, but incorporation of the resistance into the tobacco genome in the backcross generations was not successful. Utilization of these somatic hybrids for practical breeding requires further study. Protoplast fusion has also been used for producing cytoplasmic male sterile (MS) tobaccos within a short period of time. MS tobacco lines with a given nuclear genotype can be produced within only two generations by cytoplasm substitution, following protoplast fusion between tobacco and a X-irradiated cytoplasm donor parent. This method is much more efficient than the conventional backcross method, for which several years are required. Stable transmission of agronomic characteristics and male sterility of such MS lines have been confirmed in later generations. Production of MS lines by this method can therefore be incorporated into a practical F_1 hybrid breeding program.

INTRODUCTION

Tobacco (*Nicotiana tabacum*) is a crop of considerable economic importance. It has also played an important role in many advances in botanical research, because of its advantages as an experimental plant. Tobacco grows fast, is easy to culture, and flowers within a relatively short period. It is also easy to produce crosses of tobacco, and obtain a number of seeds from a single capsule. In recent years, tobacco has been the most noteworthy crop in studies of plant tissue and cell culture, and genetic transformation. Haploid plants can be obtained at a high frequency, and plants can be efficiently regenerated from protoplasts. Most of the techniques developed in plant cell culture have arisen from studies of *N. tabacum* or one of the wild *Nicotiana* species, and these techniques have also been applied to tobacco breeding.

PROTOPLAST FUSION

Protoplasts are plant material of single cells which are used for mutant isolation, production of somatic hybrids and incorporation of foreign DNA. Plant regeneration from protoplasts was first demonstrated in *N. tabacum* by Nagata and Takebe[9]. They cultured protoplasts isolated from leaf mesophyll cells by enzyme treatment, and succeeded in obtaining numerous calli. Shoots were differentiated from the calli, and whole plants were regenerated after rooting. The protocol for plant regeneration from tobacco protoplasts is now well established. Leaves and cell suspension cultures are mainly used as the source of protoplasts.

Protoplast fusion is considered a possible method of overcoming barriers to hybridization, and can supply valuable germplasm in the form of somatic hybrids between species normally cross incompatible. The first somatic hybrid in higher plants was produced in the genus *Nicotiana* by protoplast fusion between *N. glauca* and *N. langsdorffii*[3]. Since then, various hybrids between tobacco and related species have been reported[4].

SOMATIC HYBRID OF *N. TABACUM* AND *N. REPANDA*

N. repanda is a valuable source of germplasm because of its resistance to nine diseases of tobacco. Resistance to *Meloidogyne javanica* is particularly important, because *N. repanda* is more resistant than any other *Nicotiana* species[10]. Breeders have

long been interested in this species, and attempts were made to utilize its resistance in tobacco breeding by producing a bridge cross with *N. sylvestris*, or by direct hybridization of autotetraploids of *N. repanda* and tobacco. The resistance, however, has never been incorporated into a tobacco genome.

Nagao[5] isolated leaf mesophyll protoplasts from *N. repanda* and a chlorophyll deficient mutant Aurea of *N. tabacum* cv. Bright Yellow, and fused them with PEG treatment. After two months in culture, nine calli were observed, greenish white in color and with a compact appearance. Their color was inherited from the parental type calli, which are white or green, respectively. These were selected as putative somatic hybrid calli. Shoots differentiated from these nine calli, but root formation occurred in only three cases to result in the regeneration of whole plants.

The somatic hybrids appeared intermediate for numerous characteristics such as flower color, flower size, leaf shape and shape of petiole. The plants were all aneuploids (2n = 52, 58, 64), according to cytological analysis. Each had less than the 96 chromosomes of normal plants (48 from each parent).

N. repanda has resistance to tobacco mosaic virus (TMV) but has no nicotine in its leaves, whereas Aurea is susceptible to TMV but has nicotine. Since the somatic hybrids had resistance to TMV and nicotine in their leaves, their hybridity was confirmed. Although pollen fertility of these somatic hybrids was very low, one plant which showed relatively normal morphology was female fertile and produced seeds when crossed with *N. tabacum* pollen. Selection for resistance to *M. javanica*, however, in the backcross generations was not successful. Hence, utilization of this somatic hybrid for practical breeding requires further study (Nagao pers. communication).

PRODUCTION OF MALE STERILE LINES BY PROTOPLAST FUSION BETWEEN *N. TABACUM* AND *N. DEBNEYI*

From fusion between protoplasts that differ in their cytoplasmic organelles, cytoplasmic hybrids (now called cybrids) are produced. These organelles segregate during the process of plant regeneration to form novel cytoplasmic genotypes. Through this system, transfer of cytoplasmic factors has been achieved[1,2]. The application of X- or gamma rays has been shown to be effective in excluding the nuclear function of a cytoplasm donor parent[11].

Male sterility is a state of non-functional male gametes, and is a useful tool for reducing the cost of F_1 hybrid seed production, because there is no need to emasculate prior to pollination when male sterile (MS) plants are used as the female parent. In *N. tabacum*, male sterility is expressed when its nucleus is combined with the cytoplasm of certain *Nicotiana* species such as *N. suaveolens* or *N. debneyi*. MS tobaccos can be obtained by the conventional repeated backcross method, but it usually takes several years to produce a MS line of a given variety.

Kumashiro and Kubo[6] examined a protoplast fusion method in which the nuclei of the cytoplasm donor parent were inactivated by X-irradiation, as a possible alternative method for introducing male sterility into tobacco. *Nicotiana debneyi* served as the cytoplasm donor. A chlorophyll deficient tobacco line, Consolation 402, was used as the line into which male sterility was introduced.

Protoplasts of *N. debneyi* were exposed to various doses of X-rays. Ten kR was found to be effective in inactivating nuclear function. Following the fusion of the X-irradiated *N. debneyi* protoplasts and non-irradiated tobacco protoplasts, more than 300 calli were subjected to regeneration. Almost all the regenerates had the normal overall morphologies of tobacco except with regard to their flower, and had the normal somatic chromosome number of tobacco (2n = 48). Out of 318 regenerates, 30 possessed male sterility. These male sterile regenerates exhibited variation in flower morphology and were classified into four types (Table 1). These distinct flower morphologies were stably transmitted to the BC_1 progenies, although some minor deviation was observed. None of the progenies of the male sterile regenerates produced fertile plants, indicating that the transmission of male sterility was stable.

Kumashiro and Kubo[7] further examined the progenies in the BC_1–BC_3 generations for agronomic characters. They observed that the majority of the MS lines showed a field performance almost equivalent to that of the tobacco parent, while none of the MS lines produced any fertile plants. This observed stability of the MS lines, in terms of both agronomic traits and male sterility, showed that production of MS lines can be incorporated into a practical breeding program.

PRODUCTION OF MS TSUKUBA 1 BY PROTOPLAST FUSION

On the basis of the observation mentioned

Table 1. Frequency of male sterile plants in regenerates derived from protoplast fusion between tobacco and X-irradiated *N. debneyi*

	Male sterile				Self fertile	
	Type N	A	B	C	2n	4n
Petal split	No	Slight	Medium	Deep		
Anther dehiscence	No	No	No	No		
Pollen grain	Few	Trace	No	No		
No. plants	7	10	9	4	282	2

above. Kubo[5] tried to produce a practical MS line of a flue-cured variety, Tsukuba 1, for F_1 hybrid breeding. Since Tsukuba 1 is a high-yielding commercial variety and has resistance to five major tobacco diseases in Japan, it was expected to have high ability as a parent of F_1 hybrid varieties.

MS tobacco lines have been obtained by fusion between X-irradiated protoplasts of MS Burley 21 and those of Tsukuba 1. The former is an MS line possessing cytoplasm from *N. suaveolens*, with deformed stamens which have neither anthers nor pollen. Observations of the regenerated plants grown in a greenhouse are presented in Table 2. More than half the 207 regenerates showed male sterility, including MS Burley 21 type plants probably derived from protoplasts which escaped X-irradiation. Nearly 100 MS plants had the characteristics of Tsukuba 1, though many of them showed some abnormality in leaf shape. MS plants resembling Tsukuba 1 and showing normal morphology were selected. These plants were backcrossed, with Tsukuba 1 as the male parent.

Table 2. Regenerated plants from protoplast fusion between Tsukuba 1 and X-irradiated MS Burley 21

Flower	Plant type	Leaf shape	No. plants
Male sterile	Tsukuba 1	Normal	37
	Tsukuba 1	Abnormal	72
	MS Burley 21	——	26
Male fertile	Tsukuba 1	Normal	40
	Tsukuba 1	Abnormal	32
	MS Burley 21	——	0
Total			207

Thirty one BC_1 lines were evaluated in terms of their agronomic characters and their transmission of male sterility in a field experiment (Kubo *et al.* in prep.). Nine lines out of 31 were observed to have apparently abnormal leaf shape. Data on their agronomic characters were not collected. The other 22 lines showed normal growth, and their range of major characters is presented in Table 3.

Table 3. Variation in the BC_1 generation of Tsukuba 1 type MS lines

Character	Range of MS lines	Tsukuba 1 (control)	No. lines	
			Sig.[z]	NS[y]
Days to flower	70.4 – 76.8	71.0	2	20
Plant height (cm)	154 – 181	160	0	22
No. leaves	30.8 – 36.6	32.9	2	20
Leaf length (cm)	67.6 – 70.4	67.5	0	22
Leaf width (cm)	28.6 – 31.6	30.8	2	20
Yield (kg/ha)	2140 – 2820	2,610	0	22
Value (Y/kg)	950 – 1280	1,160	0	22
Total alkaloids (%)	2.0 – 2.6	2.1	0	22
Nornicotine/TA x 100	4.4 – 16.7	6.1	2	20
All characters			6	16

[z] Sig: Significantly different from Tsukuba 1 at the 5% level [y] NS: Non-significant

Sixteen out of 22 lines were not significantly different from Tsukuba 1 in any of the characters studied. The two best lines, with the same flower type as MS Burley 21 and comparable to Tsukuba 1 with respect to field performance, were selected.

These selected lines were further examined in the following backcross generations, BC_2–BC_4 and proved to have no significant difference from Tsukuba 1 in all characters studied throughout the four generations. This indicates that these lines were properly selected in the BC_1 generation, and hence were isogenic to Tsukuba 1 with respect to nuclear genome. In addition, the results also suggest that the cytoplasm from *N. suaveolens* has little effect on agronomic characters.

In the BC_1 generation, all plants of any line derived from MS regenerates showed male sterility, and no plant was recovered with anthers or pollen. The stable transmission of male sterility was maintained in all generations examined. Finally, a single line exhibiting the best performance was chosen as MS Tsukuba 1, for use in F_1 hybrid breeding in flue-cured tobacco. A comparison of the agronomic characters of Tsukuba 1 and MS Tsukuba 1 in the BC_4 generation is shown in Table 4.

Table 4. Agronomic characteristics of Tsukuba 1 and MS Tsukuba 1

Character	Tsukuba 1	MS Tsukuba 1	LSD .05
Days to flower	72.9	74.1	1.3
Plant height (cm)	151	155	8
No. leaves	33.2	33.0	0.7
Leaf length (cm)	52.8	54.7	2.0
Leaf width (cm)	24.4	25.6	2.0
Yield (kg/ha)	1,950	2,220	27
Value (¥/kg)	1,690	1,780	140
Total alkaloids (%)	2.0	1.9	0.7
Nornicotine/TA x 100	1.8	2.0	2.0

CYTOPLASMIC GENOME CONSTITUTION OF MS TOBACCO OBTAINED BY PROTOPLAST FUSION

In somatic hybrids, the constitution of cytoplasmic genomes other than male sterility is also of interest. Table 5 shows the chloroplast genome estimated by analysis of chloroplast coded traits of MS plants derived from protoplast fusion. In these three cases, no association was observed between male sterility and the chloroplast genome. These data are consistent with previously published results indicating that male sterility is independent of the chloroplast genome.

Table 5. Chloroplast genome*, estimated by large subunit of Fraction 1 protein or tentoxin sensitivity of MS plants regenerated following protoplast fusion

Combination of parents	No. regenerated MS plants			
	T	D	S	TS
T + D	11	15	–	0
T + S	3	–	22	6
T + S	13	–	18	1

* Chloroplast genomes are:
T: *N. tabacum*, D: *N. debneyi*, S: *N. suaveolens*, TS: mixture of *N. tabacum* and *N. suaveolens*

Chloroplast DNA (cpDNA) was extracted from ten MS plants belonging to the line Consolation 402, and was analyzed by digestion with restriction endonuclease. These plants possessed either one or the other parental chloroplast genome, and no unique bands were detected (Asahi *et al.* in prep.). Mitochondrial DNA (mtDNA), on the other hand, from MS plants with typical flower types all showed banding patterns which were quite different from those of the other plants or those of their parents. Each plant had new bands not observed in the parents, indicating that recombination occurred in the mitochondrial genome. No clear correlation, however, was observed between flower type and the ratio of restriction bands specific to the parents.

CONCLUSION

Protoplast fusion can make a potentially valuable contribution to tobacco breeding, by overcoming barriers to hybridization. Formation of a somatic hybrid between tobacco and related species has already been achieved for certain combinations. However, incorporation of a specific gene useful in commercial varieties is often much more difficult and time-consuming, as many examples have shown. More effort should be made to improve interspecific gene transfer in the future.

On the other hand, utilization of cybrids as a method to transfer male sterility does have

practical application for F_1 hybrid breeding. MS tobacco lines comparable to the original line and with a given nuclear genotype can be produced within two generations: one for regeneration of the cybrids, and the second for selection in the BC_1 generation. This method improves the efficiency of F_1 hybrid breeding, by shortening the period required for producing MS lines. Cytoplasmic hybridization is also likely to make possible the transfer of useful cytoplasmic genes other than male sterility, although only a limited number of such genes are available at present.

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DISCUSSION

Q. (M. Vajrabhaya)
When you tested the type of the cytoplasm of MS plants regenerated after protoplast fusion, why did you use tentoxin?

A. There are few marker genes associated with cytoplasm. Tentoxin binds to the coupling factor of chloroplasts and prevent chlorophyll formation in a sensitive plant. It is also reported that some species are sensitive to tentoxin, while others are not. The test is very easy to apply, and large numbers of plants can be handled at the same time, while only one week is needed to detect this activity. It is thus a very good test for the cytoplasmic genome.

Q. (E. Maeda)
What was your objective in creating the MS lines of tobacco?

A. To use in F_1 hybrid breeding. Over the last ten years, we have had a program to introduce F_1 hybrids of tobacco varieties into Japan. The first F_1 hybrid variety was used in Japan last year (1986), and now the acreage planted in this variety is increasing. If we are producing a range of F_1 hybrids, we need many MS lines, and if it takes too long to produce these, F_1 hybrid breeding cannot be efficient. That is why we use this method to produce MS lines.

Q. You observed that after irradiating protoplasts with X-rays, cell division was inhibited. Would it be correct to assume that the chromosomes were not completely destroyed, and after fusion the chromosome was transferred to new hybrid cells?

A. Yes, I think this is probably correct, although it depends on the dose of X-rays. This is one of the reasons why I carried out selection in the BC_1 generation. X-rays helped bring about degradation of chromosomes, but the effect may not be complete. That means we can use this method of partial protoplast fusion to transfer chromosomes from one species to another.

Section II.
Tissue Culture of Rice and Other Gramineae

CORN AND OAT TISSUE CULTURES AND GENETIC VARIATION IN REGENERATED PLANTS

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ABSTRACT

Corn and oat plant regeneration is possible from compact, organogenic/embryogenic and friable, embryogenic cultures initiated from immature embryos of specific genotypes in both species. Plants regenerated from these cultures exhibit high frequencies of tissue culture-induced genetic variation. Novel variation such as early maturity in corn inbreds indicates that new traits may be selected. Chromosome breakage is the most frequent cytogenetic event detected in regenerated plants. Activation of transposable elements, which cause single gene mutations, has also been observed. A hypothesis concerning the mechanism underlying chromosome breakage is discussed.

INTRODUCTION

The first well-described cases of plant regeneration from tissue cultures of corn and oats were achieved at the University of Minnesota in 1975[8] and 1976[5], respectively. The key features of these accomplishments were the identification of the immature embryo as an explant for the establishment of totipotent cereal cultures, and the recognition that for optimum culture establishment the genotype and the developmental stage of the embryo are important. Culture media composition is also critical, and specific media supplements, such as proline, were shown to induce formation of friable, embryogenic callus in the corn inbred A188[2].

Tissue culture-induced genetic variation observed in regenerated plants is paradoxically opposed to the use of plant tissue culture for cloning; nevertheless, the phenomenon is widely described (for review see 15, 22, 23, 26). Tissue culture variation is either a boon or a bane to tissue culturists, depending on the type of genetic manipulation pursued by the investigator. Recovery of novel variation through tissue culture may be a source of plants with improved agronomic performance, and provide variants for tissue culture selection experiments. On the other hand, manipulations such as micropropagation, germplasm preservation, somatic hybridization, and transformation suffer from the additional genetic variation which can result from a tissue culture passage. Investigations of the genetic basis of tissue culture-induced variation in corn and oats at Minnesota were initiated with the development of regenerable tissue cultures. Because of the comprehensiveness of these investigations, and the emphasis on cytogenetic analysis of meiotic rather than mitotic chromosomes, considerable insight into the underlying mechanism has been gained. Further knowledge of the genetic mechanism of tissue culture-induced variation may enable researchers to control levels of tissue culture-induced variation in regenerated plants.

This paper describes the evolution of corn and oat tissue culture to its present status and the analyses of tissue culture-induced variation conducted at the University of Minnesota.

CORN TISSUE CULTURES

Plant regeneration from corn tissue cultures was first reported by Green and Phillips[8]. Immature

embryos of specific corn genotypes, especially A188, were plated with the shoot-root axis in contact with modified Murashige and Skoog (MS) medium[20] containing 2,4-D as an auxin source[5]. This orientation retards embryo germination, and promotes proliferation of scutellar cells to produce regenerable callus. These cultures are compact, proliferating mixtures of callus tissue, shoot meristems, and scutellum-like structures[24]. Plant regeneration occurs by the organogenic formation of shoot meristems, from which elongated shoots are excised and rooted[5]. These cultures have been designated 'Type I' callus[2]. The term 'Type I' also has been used to describe compact, embryogenic corn cultures[26], indicating that compact callus in some cases supports both organogenesis and embryogenesis. It has been suggested that shoot meristems which lack any structural relation to root apices arise from the precocious germination of somatic proembryos in Type I cultures[26]. Type I cultures have been established from a large number of corn genotypes[22]. However, because both organogenic and embryogenic Type I callus are compact and slow growing, these cultures do not readily go into liquid suspension culture.

Friable, embryogenic corn callus cultures (Type II) were initially isolated from embryogenic sectors observed in A188 Type I callus in the laboratory of B.G. Gengenbach. This callus consists of somatic proembryoids and embryoids borne on suspensor-like structures, which in some cases are narrow at the base, suggesting a single cell origin[7]. Because of the friable or loose aggregation of this less differentiated callus type and its rapid growth rate, Type II callus is preferred for *in vitro* selection experiments. Armstrong and Green[2] later demonstrated that proline combined with N6 medium induced the formation of friable, embryogenic callus in A188, allowing routine establishment of this culture type. The efficacy of proline induction of friable, embryogenic callus is dependent on genotype and the size/age of the immature embryo explant[2]. Fewer genotypes give rise to friable, embryogenic callus, compared to the number which can produce Type I callus[22]. From genetic studies of the frequencies of Type I and II callus initiation, it has been demonstrated that regenerable callus initiation in corn is controlled by only a few genes[10,25]. Some recalcitrant genotypes, notably B73, have a very low frequency of response[25]. However, they may confer culture longevity when used as a parent in crosses with A188[6]. Even within A188, there seems to be variation in the initiation of friable, embryogenic callus. We have isolated lines of A188 regenerated from Type II cultures that exhibit elevated culture initiation frequencies compared with A188 lines that have never been through a culture cycle. Further investigations to determine the inheritance of this phenotype are under way.

Friable, embryogenic callus represents an improvement over Type I cultures for the establishment of liquid suspension cultures, because it grows rapidly and is comparatively dedifferentiated. Several groups have initiated suspension cultures from friable, embryogenic cultures[14,27]. These suspension cultures attain up to ten-fold increases in fresh weight during a seven-day culture period, and have retained plant regeneration capacity for more than one year. We have isolated and cultured protoplasts from these cultures but have not observed colony formation. However, colony formation[11], somatic embryoid formation[27], and plant regeneration (C. Rhodes, unpub. presentation to the 1987 TCA meeting) have been reported from similar cultures.

OAT TISSUE CULTURE

Interesting similarities exist in the development of tissue cultures in corn and oats. Regeneration of oats from tissue culture was reported in 1976 by Cummings *et al.*[5], who patterned oat culture procedures after those developed for corn. Oat cultures are established from immature embryos plated on MS medium supplemented with 2,4-D, and resemble Type I corn callus. Furthermore, regeneration is considered to take place via organogenesis, although this has not been studied in detail. Genotype, embryo age and culture medium are important in oat tissue culture establishment. Compact, embryogenic oat callus has also been described[9], indicating that oat callus also has organogenic and embryogenic potential.

Recently, we have established friable, embryogenic oat callus cultures based on our experience with friable, embryogenic cultures of corn. Embryogenic sectors of oat callus observed in the original compact Type I callus were isolated (Fig. 1). Continued selection for friable, embryogenic callus type during subculture resulted in a uniformly embryogenic culture very similar in appearance to Type II corn cultures. More recent experiments indicate that the establishment of friable, embryogenic oat callus is dependent on genotype and culture medium. Furthermore, friable, embryogenic cultures have been initiated directly from immature embryos and from the mesocotyl

of mature seed (P. Bregitzer, unpub.) (Fig. 2). These results indicate that recognition of the appropriate callus type is extremely important in establishing and maintaining friable, embryogenic callus in corn and oats. Furthermore, regenerable liquid suspension cultures have been established from the new Type II oat cultures.

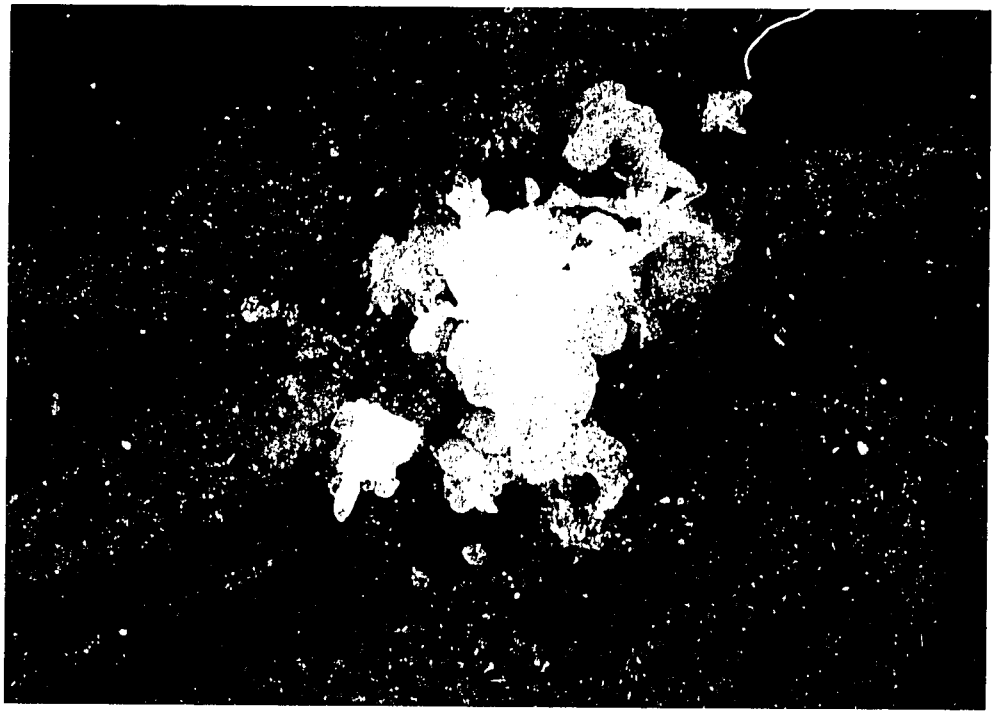

Fig. 1 Compact oat callus (Type I) with an embryogenic sector on the callus surface

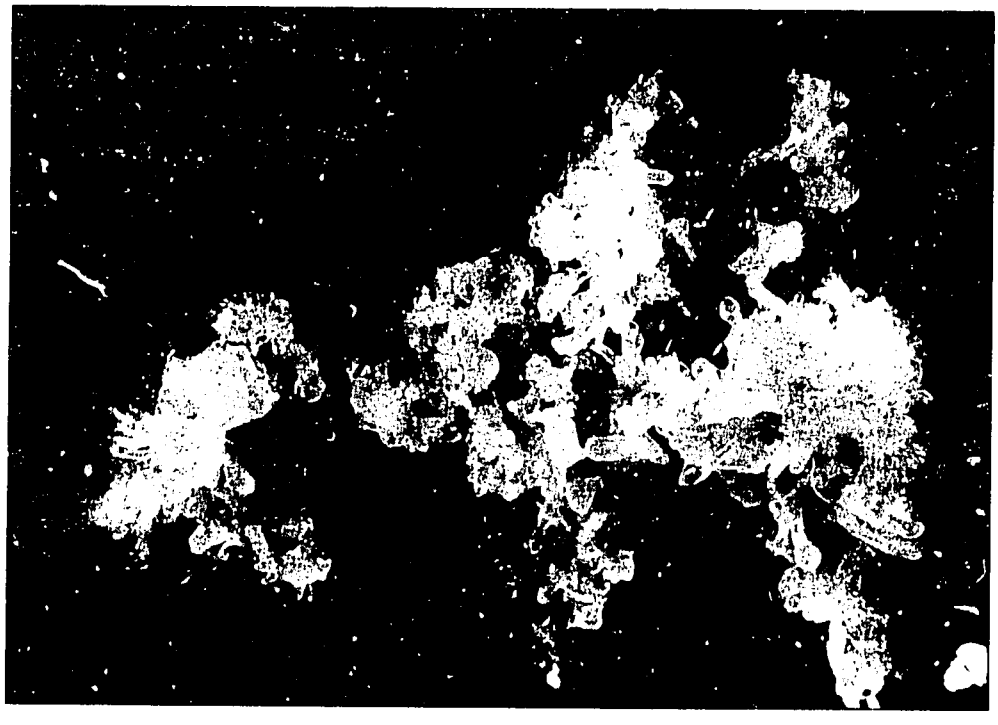

Fig. 2 Friable, embryogenic oat callus (Type II) initiated from the mesocotyl of a germinated seedling

TISSUE CULTURE-INDUCED GENETIC VARIATION IN OATS

The first systematic cytogenetic study of tissue culture-induced variation at Minnesota was conducted on regenerated oat plants[19]. The results of this analysis led to a fundamental hypothesis concerning the mechanism of tissue culture-induced genetic variation. Therefore studies of tissue culture variation in oats will be described first in this paper.

Plants regenerated (R_0) from the original Type I oat cultures exhibited a high frequency of genetic and cytogenetic variation, indicating that oat tissue cultures, like all other plant tissue cultures, are subject to genetic instability. Variants observed included heritable changes such as striped leaves, fatuoids, shortened plant height and delayed maturity[5,23]. The high level of variation observed among regenerated plants and their progeny suggested that selection for improvements in agronomic traits might be possible among tissue culture-induced variants of oats. However, preliminary tests of progeny lines derived from 53 Lodi R_1 plants and 123 Tippecanoe R_1 plants indicated that grain yield of the progeny of all regenerated plants was less than, or equal to, the parent cultivar[24].

In a systematic investigation of tissue culture-induced cytogenetic variation, McCoy *et al.*[19] demonstrated the maxims that variation increases with culture age and is subject to genotype effects. Cytogenetic analysis of meiosis in R_0 regenerated plants indicated that the predominant chromosome aberrations resulted from chromosome breakage (Table 1). Over 85% of the defined cytological abnormalities were due to breakage, and often involved partial chromosome loss, *i.e.* telocentric or near-telocentric chromosomes.

The prevalence of near-telocentric chromosomes as the primary cytogenetic abnormality led McCoy *et al.*[19] to postulate that in oats, there may be late replicating heterochromatin flanking the centromeres of chromosomes, and that late replication of this heterochromatin may be involved in tissue culture-induced chromosome breakage. This hypothesis is based on the observation that heterochromatin normally replicates late in the cell cycle, and that perturbation of the cell cycle in tissue culture cells might cause heterochromatin to be even later in replicating. If pericentromeric heterochromatin had not completed replication at anaphase, the chromosome bridges formed would lead to chromatid breakage near the centromere (Fig. 3). The premise of this hypothesis, *i.e.* that pericentromeric heterochromatin occurs in oat chromosomes, has been demonstrated by Johnson *et al.*[12], and, in fact, this is present in all 21 chromosomes. Furthermore, these heterochromatic regions were shown to be late replicating in root-tip cells[12]. Whether the pericentromeric heterochromatin replicates even later in oat tissue culture cells has yet to be tested. However, the hypothesis suggests that treatments that affect the cell cycle of tissue culture cells, and perhaps DNA replication efficiency, may alter levels of tissue culture-induced variation.

Table 1. Chromosomal alterations in Tippecanoe and Lodi oat plants regenerated from tissue culture. Data from McCoy *et al.*[19]

Type of alteration	Aberration frequency among plants	
	Tippecanoe	Lodi
	------ %	-----
Polyploidy	0	0
Aneuploidy	3.1	6.9
Structural alterations		
Interchanges	3.7	7.5
Inversions	0.3	0.2
Telocentrics	20.6	23.8
Total plants with alterations[a]	22.4	32.4

[a]Some plants contained more than one alteration

The genetic and cytogenetic variation in plants regenerated from Type II embryogenic oat tissue cultures are uncharacterized at this time, because of the recent development of this culture type. The availability of these cultures will provide the opportunity to compare levels and types of variation arising from the different culture systems.

TISSUE CULTURE–INDUCED VARIATION IN CORN

Corn has some additional advantages that are useful in investigations of tissue culture-induced genetic variation compared with allohexaploid oats. Corn is diploid which allows ready detection of recessive mutations in progeny of R_0 plants. It is also a monoecious but diclinous plant, in which

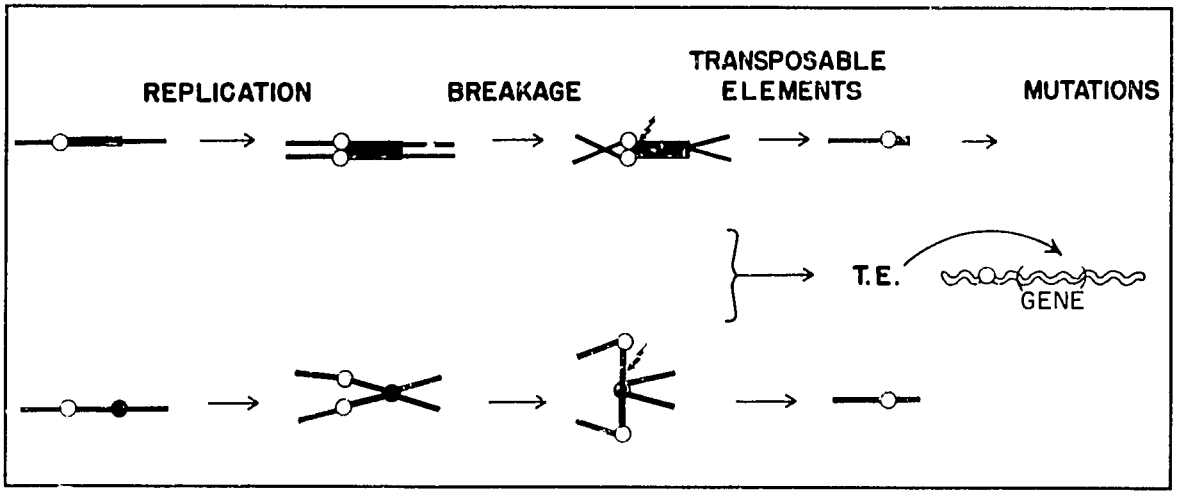

Fig. 3. Schematic representation of the proposed mechanism for chromosome breakage and activation of transposable elements (T.E.) in corn and oats. Oat chromosomes (upper panel) have pericentromeric heterochromatin, whereas corn chromosomes (lower panel) have knob heterochromatin. Heterochromatin is indicated by the shaded structures

cells for the tassel and ear are set apart in the embryo of mature kernels[13]. Spatial separation of cells that form the stamens and pistils during embryo development may result in genetic nonconcordance between the tassel and ear of a R_0 plant. During organogenic shoot meristem formation, culture cells carrying different mutations may be recruited into the separate cell lineages that form the tassel and the ear[1,22]. Similar events may occur in somatic embryoids formed from multiple rather than single cells, or mutations may occur in the male or female cell line after embryoid formation, as a result of genetic instability caused by the tissue culture passage. Chimeric plants with a recessive mutation in the tassel or the ear can be detected by observing the segregation of a trait in the R_2 generation that did not segregate in the R_1 generation. Superimposed on these advantages are the extensive knowledge of genetics and cytogenetics which make corn an elegant tool for basic genetic studies.

Over 50 different phenotypes controlled by single gene mutations have been observed in the progeny of regenerated corn plants[22]. These mutations have readily observable phenotypes, such as albinos and kernel defectives, and therefore the frequency of observed mutations is an underestimate of the total extent of tissue culture-induced variation. Variation in quantitative traits has also been documented. For example, increased earliness has been observed among progenies of R_0 plants, and in some cases specific regenerants outyielded their non tissue cultured parental line[17]. Increased earliness is a trait not often observed in corn after chemical or physical mutagenesis, indicating that some portion of the variation observed in progenies of R_0 corn plants is novel.

Genetic and cytogenetic investigations of tissue culture-induced variation in corn indicate that the frequency of variation increases with tissue culture age, and that the frequency of chimeric plants which are regenerated from both organogenic and embryogenic cultures decreases with time in culture[1,4,16]. These two observations may seem contradictory, but pedigree analysis of clonal subcultures has provided some insight. The number of different variants detected in progenies of R_0 plants increases with increasing culture age, indicating that the mutation rate is quite constant[4]. Mutations that occur early in the culture process proliferate as increasingly larger sectors within the callus during subsequent subculture. If plants are regenerated at the early stage of the culture, the ratio of mutant cells to normal cells is small, and the probability of recruitment of both mutant and normal cell lines into the lineages giving rise to tassel and ear initials is increased compared with several passages later, when the mutant sector represents a larger proportion of the callus, and mutant cells participate in both male and female cell lines[3]. The similarity in this trend of organogenic and embryogenic cultures argues that the majority of somatic embryoids have a multicellular origin, even though a single cell origin is indicated by the frequent observation of a narrow

suspensor supporting the embryoid[1]. These observations also refute the possibility that persistent genetic instability in cells of regenerating structures produces the majority of the chimeric plants, because the frequency of chimeras would be expected to remain constant, or increase with increasing culture age. In other studies of tissue culture-induced variation in monocot species regenerated via somatic embryogenesis, comparatively low frequencies of genetic and cytogenetic abnormalities were observed[26]. In contrast to these observations, the frequency of variants and cytogenetic abnormalities in embryogenic regenerants was about the same as, or even greater than, the variation in plants regenerated from organogenic cultures (Table 2). The rapid growth rate of the Type II callus compared with Type I callus may have more to do with the greater instability of the Type II cultures than the mode of plant regeneration[1,22].

Table 2. Comparison of tissue culture-induced variation frequencies in corn plants regenerated from organogenic and embryogenic cultures. Data from Armstrong and Phillips[1]

Abnormality	Frequency of Abnormalities			
	4-month cultures		8-month cultures	
	organogenic	embryogenic	organogenic	embryogenic
	%			
Cytological aberrations	0	12	6	15
Phenotypic variants	22	19	38	42
Sectoring for phenotypic variants	60	50	33	21

Cytogenetic analyses of R_0 corn plants indicate that, as in oats, the most frequently observed chromosome abnormalities (interchanges and deletions/duplications) arise from chromosome breakage (Table 3). In corn chromosomes, however, the break point resides between the centromere and heterochromatic knobs that are found on most corn chromosomes[22]. This observation again evokes the possibility that late replication of heterochromatin in the chromosome knobs causes a chromosome bridge at anaphase, leading to chromosome breakage between the knob heterochromatin and the centromere (Fig. 3). According to this hypothesis, genotypic differences in knob heterochromatin or heterochromatin specific DNA sequences may result in genotypic differences in tissue culture-induced variation.

High frequencies of chromosome breakage observed in both corn and oats do not necessarily account for the high frequency of single gene mutations observed in the progeny of regenerated plants. One possible explanation is that tissue culture-induced genetic variation includes the activation of transposable elements, which in turn cause single gene mutations. This is a likely hypothesis, in the light of McClintock's observation that genome shock imposed by chromosome breakage activates transposable elements[18]. Activation of *Ac* has been demonstrated in the progeny of R_0 plants by Peschke *et al.*[21]. Tests of 1200 plants, representing 301 R_0 plants from 94 embryos, indicated *Ac* activation in cultures initiated from 3 immature embryos from different source plants. Plants grown from seed of the same plants used for embryo isolation and culture initiation were negative for *Ac* activity. *Ac* is only one of a number of transposable elements in corn, indicating that 3% activation probably underestimates the overall frequency of the transposable element activation which must account for some of the single gene mutations observed in regenerated plants[22].

Table 3. Chromosomal alterations found in plants regenerated from corn tissue cultures. Adapted from Phillips *et al.*[22]

Alteration	Aberration Frequency		
	Benzion[3]	Lee and Phillips[16]	Armstrong[1]
	%		
Chromosomal alteration			
Polyploidy	2	0.7	0.7
Aneuploidy	0	0.4	0.7
Structural alterations			
Interchanges	6	17	5
Inversions	0.3	0.4	0
Deletions/Duplications	4	22	0
Total plants scored	370	267	142

CONCLUSION

Oat and corn culture systems are very similar. Regenerable callus may be compact and organogenic/embryogenic (Type I) or friable and embryogenic (Type II). The latter callus type is suitable for liquid suspension culture initiation in both species. The explant source, genotype, and culture media are critical for the initiation of regenerable cultures, particularly for Type II cultures. High frequencies of genetic and cytogenetic variation is observed in progenies of corn and oat plants regenerated via organogenesis and embryogenesis. Some of the variation may prove to be useful in cultivar improvement, but for the most part the variation is deleterious. The frequency of variants increases with culture age, and is affected by genotype. The prevalent cytogenetic events are chromosome abnormalities caused by chromosome breakage. The breakpoints in oat chromosemes are in pericentromeric heterochromatin, whereas corn chromosomes are broken in regions between the centromere and knob heterochromatin. These observations have led to the hypothesis that delayed replication of late replicating heterochromatin in tissue culture cells results in chromosome breakage. Chromosome breakage in turn activates transposable elements which may cause the high frequency of single gene mutations observed in progenies of regenerated plants. Future research to determine the metabolic basis for the perturbation of the cell cycle that may delay replication of heterochromatin may provide the means for controlling levels of tissue culture-induced variation.

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DISCUSSION

Q. (A. Komamine)
You mentioned two types of culture, organogenic and embryogenic. What is the important factor in deciding which takes place?

A. First of all, I doubt whether the organogenic type of callus is truly organogenic. There are scutella-like bodies in the callus, and these are embryogenic structures. However, it has also been shown that there are quite well-defined shoot meristems in the culture. It might be that these are only the precocious germination of somatic embryos, but nevertheless, the important fact is that there can be two distinct types of cultures established from one genotype. The establishment of friable, embryogenic, Type II callus, is very specific, usually to A188 although there are a few other proprietary inbreds that respond. The culture medium of the organogenic is MS, with 2,4-D at 2 mg per liter, while that of the embryogenic is N6 with 1 mg of 2,4-D per liter, plus proline, which is very important. Thus, the medium is apparently the major factor. However, there are a number of other factors as well, such as the physiological state of the embryo. Type II callus must be initiated from embryos ten to twelve days old; Type I is more flexible.

Q. (F.J. Zapata)
The classical proof of embryogenesis is usually sectioning, followed by examination with a microscope or scanning electron microscope. Have you done this?

A. Yes, we have demonstrated this for maize. We are still in the process of demonstrating this for oats, but we have scanning electron microscope results that show the whole structure of the embryo i.e. the coleoptilar notch etc., which are fairly convincing. Although I am interested in the mode of regeneration, my primary interest is the establishment of oat suspension cultures.

Q. (H. Uchimiya)
You have told us that *Ac* is activated during tissue culture. This is probably due to the demethylation of DNA. Can you determine the specific stage of tissue culture at which DNA is demethylated?

A. This is a very interesting idea. We don't know when methylation patterns are altered. However, in the pedigree study of subcultured cell lines we observed cytogenetic abnormalities in regenerated plants. These changes (chromosome breakage) occur early in the callus initiation process, and proliferate throughout the callus during subsequent subcultures. The rate of appearance of the alterations is constant during callus maintenance, implying that the callus stage of these cultures is when chromosome breakage occurs. The relationship between chromosome breakage and demethylation in cultured cells is not yet understood.

Q. (F.J. Zapata)
It has been demonstrated that in rice, specific types of tissue culture-induced variation appear in regenerated plants in sequence. Have you observed this in cultures of maize at four and eight months, or in cultures of oats at four and twenty months?

A. We haven't yet looked at our data in this way, but it would be very interesting to go back to our studies and see if this trend exists. In corn regenerates, we usually see a loss of male fertility. This may be associated with rearrangements of the mitochondrial DNA or chromosomal changes which occur at high frequency in corn tissue culture.

Q. (H. Uchimiya)
Is there any difference in basic response between corn and oats?

A. This is a very difficult question. Tissue culture is considered a highly scientific procedure, but in fact it proceeds mainly by trial and error. There are certainly some close parallels between the two systems, for example with regard to genotype. We don't really understand the genetics of culture initiation. At least in maize, initiation appears to have some dominance, but we don't really know how many genes are involved. Once we begin to understand systems, such as the genetics behind the tissue culture, we may be able to make more progress.

One aspect that troubles me with regard to protoplast culture in maize, is that we have cultures from only a few genotypes. This could perhaps be a genetic restriction or genotype effect on another level. Perhaps this occurs at all levels of tissue culture, in callus initiation, suspension cultures, protoplasts etc.

RICE ROOT CULTURE

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ABSTRACT

In order to understand the root physiology of different rices, root tips were excised and cultured aseptically in improved R1, R2 and nitrogen modified media. The results revealed that the excised roots of Indica rices, including native (short-grain) and IRRI varieties (long-grain and medium-grain), grew well in the synthetic media. Their root diameters and growth rates were much greater than those of Japonica rice. However, the roots of Japonica rice grew better when urea or casamino acids were the nitrogen source. The difference in nitrogen absorption and metabolism between Japonica and Indica rices was also studied. It was found that the nitrate uptake by the roots of Japonica rice was apparent in the initial stage of culture, but then almost stopped. The nitrate consumption of Indica rice, on the other hand, increased gradually as the length and the weight of the root increased. The activity of enzymes such as nitrate reductase (NR) and glutamate dehydrogenase (GDH) in the process of nitrogen assimilation has also been determined. The activity of NR and GDH enzymes was higher in Indica rice roots than in Japonica rice. These results confirm that the nitrogen metabolism of Japonica and Indica rice roots is significantly different. The reducing ability of Indica rice root with regard to inorganic nitrogen is greater than that of Japonica rice roots. The respiration rate of roots is apparently higher in Japonica type rices than in Indica type rices. An assay of peroxidase, catalase, and glycolate oxidase also showed a higher enzyme activity in Japonica rices than in Indica ones. The results obtained indicate clearly that a physiological difference exists between the roots of Japonica and Indica type rices. The significance of these differences is discussed, from the viewpoint of the cultivation and breeding of rice varieties.

INTRODUCTION

Most rice cultivars are either an Indica or a Japonica type[6, 8]. In general, Japonica cultivars are cultivated in temperate regions, while Indica cultivars are cultivated in tropical and subtropical ones. As Taiwan is situated on the edge of the subtropical zone, both subspecies are widely cultivated. The so-called 'Ponlai' rices in Taiwan belong to the Japonica (or Keng) group, while native rices belong to the Indica (or Seng) group. Since the traditional Indica rices in Taiwan can maintain their productivity in rather infertile paddy soils, they were widely grown many years ago when almost no chemical fertilizer was available. Japonica rices became more popular as the fertilizer supply increased. After the establishment of modern Indica cultivar Taichung Native No. 1 (Ti-Chueh-Wu-Chien x Tsai-Yuan-Chung), which possesses a short, stiff culm, lodging resistance, positive nitrogen response and active tillering activity, the cultivation of Indica rices was also extended to the more fertile paddies. Indica type rice plants often show symptoms of early senescence i.e., their lower leaves turn yellow faster and wither sooner than those of Japonica type rices, during the maturing stage[18]. This tendency is more pronounced when the paddy soil is in a reducing condition[13].

This phenomenon may be related to the way the roots of Japonica and Indica type rices adapt to the soil environment[13]. However, little information is available on the differences between their various root characteristics.

As this information is important in improving

culture techniques, and in breeding new cultivars, this paper introduces some recent experiments on the differentiation of physiological characters of rice roots, mainly by *in vitro* culture methods.

IMPROVED CULTURE MEDIA R1, R2 FOR EXCISED ROOT CULTURES

The culture of excised roots is an useful technique for studying the physiology and behavior of root growth, without any interference from shoots or various environmental factors. The excised root culture of rice has been studied since the early 1950's[4, 9, 10]. However, it was not until recently, when the author and his coworker improved the culture media for Indica rice, that further progress was made in this field. Lai and Lee[11] have shown that the excised seminal roots of Indica cultivar Taichung Native 1 could grow well in either R1 or R2 improved culture medium. R1 type improved culture medium contains modified White's inorganic salts (containing 4.0 mg/l of Fe in the form of ferric citrate), 3% sucrose, and 0.1 mg/l each of Thiamine-HCl, Pyridoxine-HCl, Nicotinic acid and 0.3% Vitamin-free casamino acids (acid hydrolyzed casein). The pH value is adjusted to 5.0 (Table 1). The R2 type improved culture medium contains 5% sucrose and, except that it has no casamino acids, has the same composition as the R1 type while its pH value is adjusted to 4.0 (Table 2). However, neither of these two media was suitable for the growth of excised roots of Japonica cultivar Taichung 65. This suggests that there may be characteristic physiological differences between the roots of Japonica and Indica rices.

Table 1. Improved R1 type culture medium

Component	Concentration (mg/l)
$MgSO_4 \cdot 7H_2O$	734
$Ca(NO_3)_2 \cdot 4H_2O$	290
Na_2SO_4	200
KNO_3	80
KCl	65
$FeC_6H_5O_7 \cdot nH_2O$	20
$NaH_2PO_4 \cdot 2H_2O$	18.7
$MnSO_4 \cdot 4H_2O$	6.7
$ZnSO_4 \cdot 7H_2O$	2.7
H_3BO_3	1.5
KI	0.75
$CuSO_4 \cdot 5H_2O$	0.0025
MoO_3	0.00015
Thiamin·HCl	0.1
Pyridoxine·HCl	0.1
Nicotinic acid	0.1
Sucrose	30000.0
Vitamin free Casamino acids	3000-4000
pH	5.0

Table 2. Improved R2 type culture medium

Component	Concentration (mg/l)
$MgSO_4 \cdot 7H_2O$	737
$Ca(NO_3)_2 \cdot 4H_2O$	290
Na_2SO_4	200
KNO_3	80
KCl	65
$FeC_6H_5O_7 \cdot nH_2O$	20
$NaH_2PO_4 \cdot 2H_2O$	18.7
$MnSO_4 \cdot 4H_2O$	6.7
$ZnSO_4 \cdot 7H_2O$	2.7
H_3BO_3	1.5
KI	0.75
$CuSO_4 \cdot 5H_2O$	0.0025
MoO_3	0.00015
Thiamin·HCl	0.1
Pyridoxine·HCl	0.1
Nicotinic acid	0.1
Sucrose	50000.0
Vitamin free Casamino acids	0
pH	4.0

THE GROWTH RESPONSE OF EXCISED ROOTS OF JAPONICA AND INDICA RICES

Japonica rice varieties Taichung 65, Tainan 5 and Tainung 61, and Indica rice varieties Taichung Native 1, Ti-Chueh-Wu-Chien and Tuan-Kung-Hua-Lou were used as materials. The excised roots were cultured in both R1 and R2 medium for three weeks. In another experiment, Taichung Native 1 and Tuan-Kung-Hua-Lou (short-grain Indica), IR 22 (long-grain) and IR 3 (medium-grain) were used. The excised root tips were cultured in R2 medium for four weeks. The root lengths and weight were determined. The excised roots of Japonica rices. Taichung 65, Tainan 5 and Tainung 61 showed poor growth. The excised roots of Indica rices Taichung Native 1, Ti-Chueh-Wu-Chien and Tuan-

Kung-Hua-Lou grew better than those of Japonica rices, whether the culture medium was R1 (Table 3) or R2 (Table 4)[14].

One interesting fact was that the excised roots of Japonica rices cultured in R1 medium (containing nitrate and casamino acids as a nitrogen source) grew better than those in R2 (containing nitrate as the sole source of nitrogen).

The excised roots of IR 8 and IR 22 grew as well as those of Taichung Native 1 and Tuan-Kung-Hua-Lou (Table 5). The results indicated that the excised roots of Indica rices can grow well in synthetic culture media, regardless of the variety's grain length, while their growth rate was much higher than that of Japonica rices[14]. It was noted that the excised roots of Japonica rices showed a high growth rate only during the first week of culture. After this, growth was rather limited, while the diameter of the root tips gradually decreased over the culture period. The excised roots of Indica rices, on the other hand, showed a rapid growth rate, while the diameter of the root tips remained the same size.

Table 3. Growth of excised roots of Japonica and Indica rices cultured in R1 medium (average of 14 roots)

	Variety	Root length (cm) 7th day	14th day	21st day	Dry weight* (mg)
Indica	Taichung Native 1	8.1 ±0.8	20.4 ±1.9	24.4 ±2.2	4.05
	Tuan-Kung-Hua-Lou	7.6 ±1.3	13.7 ±1.2	14.5 ±0.8	3.59
	Ti-Chueh-Wu-Chien	7.4 ±0.6	12.6 ±1.9	13.5 ±1.1	3.89
Japonica	Taichung 65	5.5 ±0.6	7.2 ±1.4	6.9 ±1.0	0.97
	Tainan 5	5.0 ±0.6	7.0 ±0.7	7.7 ±1.2	0.94
	Tainung 61	6.3 ±0.4	7.5 ±1.0	7.5 ±0.6	1.00

* 21st day only

Table 4. Comparison on the growth of excised roots between Japonica and Indica rices cultured in R2 medium (average of 14 roots)

	Variety	Root length (cm) 7th day	14th day	21st day	Dry weight* (mg)
Indica	Taichung Native 1	7.2 ±1.2	20.1 ±1.5	33.2 ±1.5	4.50
	Tuan-Kung-Hua-Lou	7.8 ±1.8	17.0 ±3.0	20.8 ±1.9	2.66
	Ti-Chueh-Wu-Chien	6.8 ±0.4	16.8 ±1.8	20.5 ±1.5	2.69
Japonica	Taichung 65	3.5 ±0.7	4.5 ±1.0	5.6 ±1.4	0.75
	Tainan 5	3.7 ±0.3	4.3 ±0.9	5.8 ±1.4	0.84
	Tainung 61	3.8 ±0.4	4.3 ±0.6	4.5 ±0.7	0.73

* 21st day only

Table 5. Elongation of excised roots of Indica rices cultured in R2 medium

Variety		Root length (cm) 7th day	14th day	21st day	28th day
Taichung Native 1	(Short-grain)	8.2 ±2.8	23.2 ±2.5	38.4 ±3.9	47.8 ±7.0
Tuan-Kung-Hua-Lou	(Short-grain)	8.2 ±0.8	21.2 ±0.9	24.7 ±1.6	27.4 ±2.1
IR 8	(Medium-grain)	8.0 ±0.5	18.4 ±2.0	24.7 ±2.6	32.8 ±4.9
IR 22	(Long-grain)	9.1 ±1.2	22.2 ±2.2	35.0 ±1.5	51.7 ±4.5

THE GROWTH OF EXCISED ROOTS CULTURED IN NITROGEN MODIFIED MEDIA

As these results demonstrate, Japonica rices cultured in R1 and R2 medium grew better in the former, but the Indica rices did not. This implies that the root responses of these two subspecies to nitrogen might be different. To study this problem, the following experiment was carried out.

Japonica rice Taichung 65 and Indica rice Taichung Native 1 were used. Excised root tips were cultured for four weeks in R2 medium, with nitrate or urea as the nitrogen source. In order to prevent biuret formation during autoclaving, the medium was sterilized with 450 nm pore size millipore film. The nitrogen concentration was 45.6 ppm. The growth rates of different rice roots in media containing nitrate or urea are given in Table 6. The data shows clearly that in either of the two media, the excised roots of Taichung Native 1 grew much better than those of Taichung 65. The excised roots of Indica rice grew faster in medium containing nitrate than in one containing urea (Table 6A), while the roots of Japonica rice showed a better growth response in the medium with urea (Table 6B).

Table 6. Comparison of the growth rate of excised roots of Japonica and Indica rices cultured in R2 medium with nitrate or urea

Rice variety	Root growth rate (cm)			
	7 days	14 days	21 days	28 days
A. Nitrate-containing R2 medium				
Taichung 65	7.5 ±0.4	8.8 ±0.4	8.8 ±1.4	9.6 ±2.0
Taichung Native 1	10.3 ±2.3	25.7 ±1.4	42.4 ±3.1	46.6 ±7.8
B. Urea-substituted R2 medium				
Taichung 65	11.5 ±0.9	14.3 ±0.9	15.0 ±1.5	17.0 ±1.0
Taichung Native 1	11.1 ±1.7	23.6 ±2.1	26.0 ±2.7	25.0 ±4.7

The roots of Japonica rice grew better when the nitrogen source was in a reduced form, such as urea. However, no such effect was seen with Indica rice. Kawata *et al.*[9] and Lai and Lee[11], have reported that the growth rates of the excised roots of Japonica rice Norin 29 and Taichung 65 were accelerated if casein was added to the modified White's medium, with nitrate as the sole nitrogen source. These results indicate that the roots of different rices may have a different nitrogen metabolism. The root tissue of Indica rice may contain certain enzymes which can utilize various nitrogen sources for growth while such a metabolic process may be incomplete in Japonica rice roots.

NITROGEN ABSORPTION AND EXCISED ROOT GROWTH

Nitrogen, which is essential to the plant as a constituent of protein and other components, is generally taken up by the roots in the form of nitrate or ammonium. In order to understand the different responses of cultured excised roots of Indica and Japonica rices to nitrogen sources, excised roots were cultured in R2 medium containing nitrate as the s le nitrogen source.

Japonica rice Taichung 65, and Indica rice Taichung Native 1, were used as the experimental materials. Four root tips of each rice cultivar were excised and cultured for two weeks in a 125 *ml* Erlenmeyer flask containing 20 *ml* R2 liquid medium. Four flasks were sampled every 48 hours after inoculation. The roots were taken from the flasks, and their length and dry weight were measured. The medium in the flasks was kept in an aseptic condition, and stored in a refrigerator until the end of the experiment. The nitrate concentration was then analyzed, using Conway's microdiffusion analysis method[2]. The nitrate absorbed by roots was estimated according to the difference in nitrate content between the control medium and the root inoculated one. As indicated

in Figs 1 and 2, root length and dry weight of Taichung Native 1 were greater than those of Taichung 65[15]. The growth of the excised roots of Taichung Native 1 remained vigorous throughout the two-week culture period, while that of Taichung 65 gradually declined. This was in good accordance with results obtained previously. In this experiment, a total of four roots of each cultivar were cultured in 20 *ml* R2 medium containing 45.6 ppm nitrate-N. On the second day of culture, the total nitrate consumption of Taichung 65 and Taichung Native 1 was approximately 3 ppm and 1 ppm, respectively. However, the nitrate uptake by Taichung 65 almost ceased after the fourth day. During the 14 day period, the total N consumption of Taichung 65 did not exceed 4.7 ppm, which was about one-tenth of the total nitrate-N present in one flask of R2 medium. The nitrate consumption of Taichung Native 1, on the other hand, increased gradually as the length and the weight of the roots increased (Fig. 3)[15]. This result shows clearly that the root of Taichung Native 1 had a far more effective nitrate uptake and nitrogen metabolism than the root of Taichung 65.

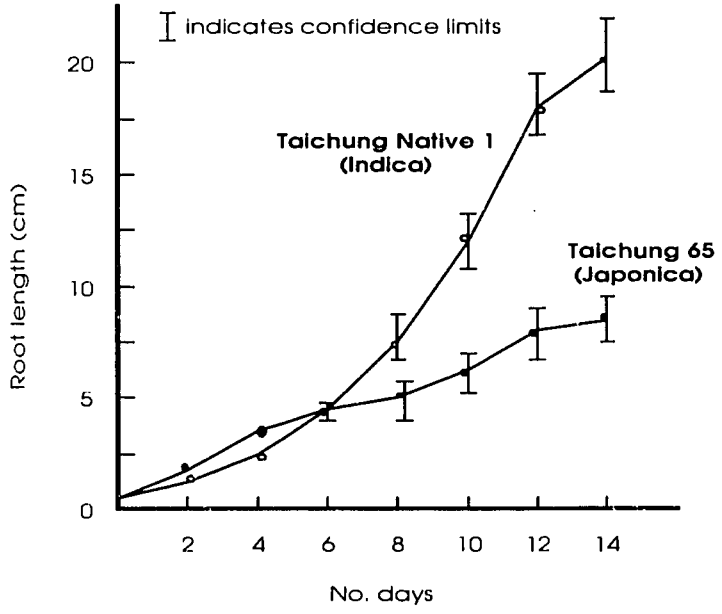

Fig. 1. The elongation of cultured rice roots of Japonica and Indica rices (average of 8 roots)

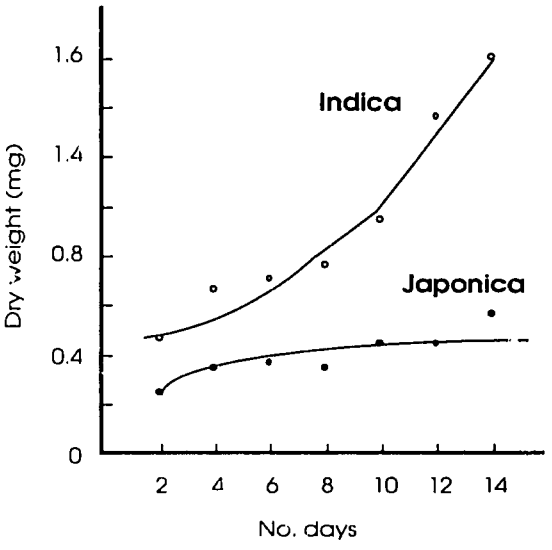

Fig. 2. Dry weight of cultured rice roots of Japonica and Indica rices

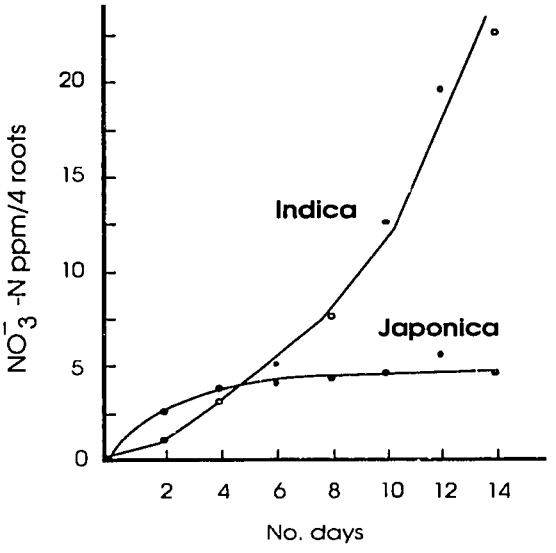

Fig. 3. The total nitrate absorption of roots of Japonica and Indica rices (four roots were cultured in each flask containing 20 ml R2 medium with 45.6 ppm NO_3^--N)

NITRATE REDUCTASE AND GLUTAMATE DEHYDROGENASE IN THE ROOTS OF JAPONICA AND INDICA RICES

The relationship between the growth of cultured roots and their nitrogen absorption, as well as the key enzymes in nitrogen assimilation, were investigated.

The seeds of Taichung 65 and Taichung Native 1 were sterilized, and germinated on a 2% agar bed. About 40 aseptic root tips were excised, and each was cultured in a 125 *ml* Erlenmeyer flask containing 30 *ml* R2 liquid medium, under aseptic conditions. In order to control the induction time of Nitrate Reductase (NR) with accuracy, the root tips thus excised were first collected in a flask containing 15 *ml* of 3% sucrose solution. After all the roots had been prepared, the other components of R2 medium were poured into the flask to make a full 30 *ml* of R2 medium. The pH value of the medium was first adjusted to 5.0 with 1 N NaOH or HCl solution. The root materials were sampled at 1, 3, 5, 6, 12, 18 and 24 hours; and at 24, 48 and 72 hours after being transferred to the R2 medium. They were spread on cheesecloth, washed in tap water and deionized water, then blotted dry and weighed.

NITRATE REDUCTASE (EC 1.6.6.2.) ASSAY

One gram of fresh roots was homogenized in a cold porcelain mortar in 10 *ml* of 0.1 M tris-HCl buffer (pH 7.5), containing 1 mM cystein. The homogenate was centrifuged at 10,000 g for 20 minutes. The supernatant was used as a crude enzyme. Extraction procedures were carried out at 0-4°C. NR was assayed, using the following reaction mixture: 1.0 *ml* of 0.1 M potassium phosphate (pH 7.5), 0.4 *ml* of 5 mM KNO_3, 0.2 *ml* of 1 mM NADH, and 0.4 *ml* of crude enzyme. The samples were incubated in a water bath at 28°C for 30 minutes. Six replications were prepared for each sample. The reaction was stopped by adding 2 *ml* of 1% (W/V) sulfanilamide in 3 N HCl solution. The color was developed with 2 *ml* of 0.02% (W/V) N-(1-naphthyl)-ethylendiamine dihydrochloride solution, 30 minutes being allowed for color development. The sample was then centrifuged at 2,000 rpm for five minutes, and the absorbance read against zero time at 540 nm[3, 19, 21]. The nitrate content was obtained from a standard curve. The activity was expressed as μ moles NO_2^-/g fr. wt./hr.

GLUTAMATE DEHYDROGENASE (EC 1.4.1.3.) ASSAY

One gram of fresh root tissue was froze with dry ice, and ground in an ice-chilled mort with 5 *ml* of 0.2 M phosphate buffer (pH 7.8 containing 1 mM cystein. After being squeeze through a layer of nylon cloth, the homogenat was centrifuged at 20,000 g for 20 minutes at 0 4°C. The supernatant was used for GDH assa at 340 nm, by measuring the progress of the NADH oxidation over the first two minutes at about 30°C using a Shimazu spectrophotometer Model UV 200. The assay system consisted of the following components[1, 7]:2.0 *ml* of 0.2 M tris-HCl buffer (pH 7.8), 0.2 *ml* of 0.2 M α-Ketoglutarate solution 0.2 *ml* 1.5 M $(NH_4)_2SO_4$ solution, 0.5 *ml* of 1 mM NADH and 0.1 *ml* of enzyme solution. The reaction was begun by adding the enzyme solution. Activity was expressed as μ moles NADH/g fr. wt./min.

Nitrate reductase activity increased over time from 1 to 24 hours, during the enzymic induction period. The NR activity induced in the root tissues of Taichung Native 1 was always higher than in Taichung 65. The former was 1.2-1.7 times higher than the latter (compared at the same induction time, 1-72 hours after induction (Fig. 4)[15].

The glutamate dehydrogenase activity of the two cultivars showed that Taichung Native 1 was more active than Taichung 65, no matter how the culture medium was prepared. The former was about 1.2-1.5 times higher than the latter in R2 medium, and about 1.0-1.2 times higher in deionized distilled water. It was also noted that the GDH activity of roots cultured in R2 medium was higher than those cultured in deionized distilled water (Table 7)[15].

RESPIRATORY CHARACTERISTICS OF ROOTS AND RELATED OXIDATIVE ENZYMES

These experiments show clearly that the growth and nitrogen absorption of excised roots were different in the two rice subspecies. This implies that the respiratory characteristics of rice roots of Japonica and Indica rices may also be different[23].

RESPIRATORY CHARACTERISTICS OF ROOTS

The respiration rate and respiratory quotient of rice roots cultured in R2 medium, with or without

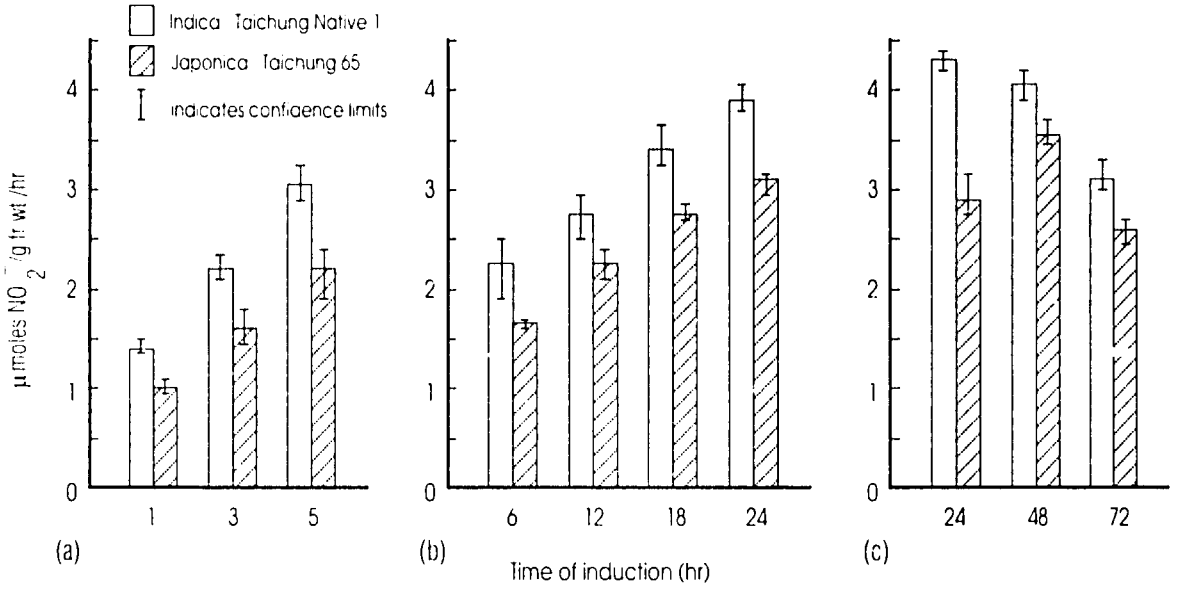

Fig. 4. Nitrate reductase activity of cultured rice roots of Japonica and Indica rices (the NR activity was induced separately with nitrate-containing R2 medium for 1-5 hours (a), 6-24 hours (b), and 24-72 hours (c)

Table 7. Comparison of the glutamate dehydrogenase (GDH) activity of Japonica and Indica rice roots cultured in R2 and water medium

Rice variety	Glutamate dehydrogenase act. (μ moles NADH/g. fr. wt./min) Culture medium R2	Water
Taichung 65	1.53	1.27
(Japonica)	1.27	1.27
$\bar{X}_1$	1.40	1.27
Taichung Native 1	1.80	1.67
(Indica)	1.67	1.33
$\bar{X}_2$	1.73	1.50
$\bar{X}_2/\bar{X}_1$	1.23	1.18

nitrate, were determined[12]. The results showed that the QO_2 of Taichung 65 (Japonica) was significantly higher than that of Taichung Native 1 (Indica). The respiratory quotient (RQ) of excised roots of Japonica and Indica rice cultivars cultured in R2 medium showed that, no matter which cultivar was used, the QO_2 and QCO_2 were markedly increased by treating the roots with a nitrate-containing medium. Moreover, the QO_2 and QCO_2 of the roots of Taichung 65 were significantly higher than those of Taichung Native 1. The RQ of Taichung 65 was about 1.0 in either nitrate-free (Table 8) or nitrate-containing R2 medium (Table 9). These results were in agreement with the respiratory characteristics obtained by using sucrose as the main respiratory substrate (i.e. sucrose was the sole respiratory substrate in R2 medium). The RQ of the roots of Taichung Native 1 was also approximately 1.0 when they were cultured in nitrogen-free medium (Table 10). However, the increment of QCO_2 was much greater than that of QO_2 in nitrate-containing medium, and consequently its RQ was always greater than 1.0 (Table 11).

Table 8. The QO_2, QCO_2 and RQ of excised rice roots of Taichung 65 (Japonica) in nitrate free R2 medium

Time (hr)	QO_2 (μlO_2/dry wt.g./hr)	QCO_2 (μlCO_2/dry wt.g./hr)	R.Q.
1–2	6,080	6,310	1.04
2–3	5,780	6,030	1.04
3–4	5,096	6,030	1.01
4–5	6,780	6,960	1.03
Average	6,150	6,333	1.03

Table 9. The QO_2, QCO_2 and RQ of excised rice roots of Taichung 65 (Japonica) in nitrate-containing R2 medium

Time (hr)	QO_2 (µlO /dry wt.g./hr)	QCO_2 (µlCO /dry wt.g./hr)	R.Q.
1–2	7,500	8,120	1.08
2–3	7,650	8,080	1.05
3–4	7,650	7,990	1.04
4–5	7,690	8,050	1.05
Average	7,635	8,060	1.06

Table 1. The QO_2, QCO_2 and RQ of excised rice roots of Indica cultivar Taichung Native 1 in nitrate-containg R2 medium

Time (hr)	QO_2 (µlO_2/dry wt.g./hr)	QCO_2 (µlCO_2/dry wt.g./hr)	R.Q.
1–2	4,240	7,105	1.68
2–3	6,185	9,450	1.53
3–4	5,565	8,820	1.58
4–5	5,565	8,920	1.60
Average	5,389	8,574	1.60

Table 10. The QO_2, QCO_2 and RQ of excised rice roots of Indica cultivar Taichung Native 1 in nitrogen-free R2 medium

Time (hr)	QO_2 (µlO /dry wt.g./hr)	QCO_2 (µlCO /dry wt.g./hr)	R.Q.
1–2	4,170	4,665	1.12
2–3	4,930	4,920	1.00
3–4	4,560	4,665	1.02
4–5	5,173	5,310	1.03
Average	4,708	4,890	1.04

CYTOCHROME (EC 1.9.3.1.) OXIDASE ACTIVITY OF ROOTS

Terminal enzymes are enzymes which control the final electron transfer to molecular oxygen in the respiration chain. Cytochrome oxidase is believed to be the main terminal enzyme in the respiration of most aerobic living organisms[5]. Cytochrome oxidase assay showed that the cytochrome oxidase activity of Taichung 65 was higher than that of Taichung Native 1 (Table 12)[16].

Table 12. Cytochrome oxidase activity in the cultured excised roots of Japonica type and Indica type rices (measured with polarographic method)

Rice variety	(a) Crude enzyme sol. only (µlO2/fr.wt.g./hr)	(b) Crude enzyme sol. + Cytochrome C (µlO_2/fr.wt.g./hr)	(b) – (a) Cytochrome oxidase activity (µlO_2/fr.wt.g./hr)
Taichung No. 65 (Japonica type)	277	830	563
Taichung Native No. 1 (Indica type)	277	628	351

CATALASE (EC 1.11.1.6.) AND PEROXIDASE (EC 1.11.1.7.) ACTIVITY OF ROOTS

Catalase and peroxidase possess the ability to oxidize a variety of substrates, using hydrogen peroxide as an oxidant. Both enzymes occur widely in tissues of higher plants. They remove H_2O_2 from plant cells, in order to prevent destructive oxidation. Catalase activity assay showed that the roots of Japonica cultivar Taichung 65 had significantly more activity than those of Indica cultivar Taichung Native 1 (Table 13).

Peroxidase activity was assayed with the same enzyme extracts used to determine catalase activity. The level of activity was estimated by the change in absorbance (ΔA) at 470 nm, during the formation of red purpurogallin from the reaction of peroxide and pyrogallol, which was catalyzed by peroxidase. The enzymic activity in the roots of Japonica cultivar Taichung 65 was significantly higher than that found in Indica cultivar Taichung Native 1 (Table 14)[16].

Table 13. Catalase activity of seminal roots of Japonica and Indica type rices

Rice variety	Enzyme fraction	Catalase activity (ml $KMnO_4$/g.fr.wt./min) Crude enzyme (*ml*) 0.1	0.2	0.3
Taichung 65 (Japonica)	supernatant	55.5	43.1	34.0
	ppt.	4.8	4.6	4.2
	Total (A)	60.3	47.7	38.2
Taichung Native 1 (Indica)	supernatant	33.0	30.0	26.0
	ppt.	7.8	6.8	6.0
	Total (B)	40.8	36.8	32.0
	(A)/(B)	1.5	1.3	1.2

Enzyme fractions divided by centrifuging at 15,000 g for 30 min.
$KMnO_4$ solution is 0.05%

Table 14. Peroxidase activities of seminal roots of Japonica and Indica type rices

Rice variety	Fraction	Peroxidase act. (ΔA/g.fr.wt./min) Duplications 1	2	3	Average
Taichung 65 (Japonica)	supernatant	21.88	23.02	26.33	23.74
	ppt.	1.37	1.37	1.49	1.41
	Total (A)				25.15
Taichung Native 1 (Indica)	supernatant	16.66	18.68	19.72	18.35
	ppt.	1.82	1.81	1.84	1.82
	Total (B)				20.17
	(A)/(B)				1.25

Buffer pH value 7.0

GLYCOLATE (EC 1.1.3.1.) OXIDASE ACTIVITY

Glycolate oxidase (GAO) also directly catalyzes the consumption of oxygen in an oxidation process. This oxidase is reported to be rarely found in the roots of upland plants, but to be present in the roots of lowland plants and in the green leaves of various kind of plants[16]. The GAO activity was assayed with excised aseptic seminal roots on the 7th day, using Warburg's manometer. The reaction vessel contained 20 aseptic root tips in 1.2 *ml* of 0.1 M phosphate buffer (pH 7.5), containing 20 ppm chloramphenicol. 0.2 *ml* of 10% KOH solution was added on the filter paper striped in the center well, while 0.2 *ml* of 0.2 M sodium glycolate solution (substrate) was added at the side arm. In the control vessels, the substrate was replaced by distilled water. The O_2 uptake over four hours was calculated. The sodium glycolate and water at the sides were poured into a phosphate buffer solution on the third hour. The GAO activity was estimated on the basis of the difference in O_2 uptake between roots given a sodium glycolate treatment and those given only distilled water. The results showed that GAO activity in the roots of Taichung 65 was much higher than in Taichung Native 1 (Table 15)[16].

Table 15. Glycolate oxidase activity in 7-day old seminal roots excised from Japonica and Indica type rices

Rice variety	(a) Excised root only (μlO_2/dry wt.g./hr)	(b) Excised root + Na-glycolate (μlO_2/dry wt.g./hr)	(b) – (a) Glycolate oxidase activity (μlO_2/dry wt.g./hr)
Taichung 65 (Japonica type)	3,185	3,890	705
Taichung Native 1 (Indica type)	3,555	3,760	205

Measured with Warburg manometry method

CONCLUSION

- Excised roots of Indica rice varieties can grow well in a simple synthetic medium. It is likely that the roots of Indica rices have a more independent growth system than those of Japonica ones[22], and can carry out nitrogen metabolism without the help of other parts of the plant. The root growth of Japonica rices, on the other hand, may depend to a certain extent on the scutellum[10, 17] and shoot[14]. The excised roots of Japonica type rices cultured *in vitro* did not grow well. This fact may be related to the incomplete operation of nitrogen metabolism. The activity of nitrogen metabolic enzymes, such as nitrate reductase and glutamate dehydrogenase, in the roots of Japonica rices, are of a markedly lower level than in Indica rices. It is likely that the functional differentiation of the plant into roots and other parts is more apparent in Japonica rices than in Indica ones.
- The absorption of nitrogen compounds is energy dependent. The fact that the cultured roots of Taichung 65 absorbed more nitrate than those of Taichung Native 1 during the first two days confirms that the ability for active nutrient absorption is higher in Japonica rices than in Indica ones. It is in good accordance with the results obtained in the present study, that the cytochrome oxidase and QO_2 of Japonica rices are significantly higher than in Indica rices. The culture medium used in this study was R2, containing sucrose as the sole carbohydrate. Sucrose is an available energy source which can be absorbed and utilized by rice roots. In general, if sucrose serves as a respiratory substrate and is completely oxidized, the volume of O_2 taken up by the tissue balances exactly the CO_2 released from the cells. The RQ of the roots of Taichung 65 was about 1.0, regardless of whether nitrate was present in the culture medium. This clearly indicates that nitrate does not influence the ratio of QCO_2/QO_2 in this rice cultivar. The RQ of the roots of Taichung Native 1 was also close to 1.0 in a nitrogen-free medium which used sucrose as the respiratory substrate. However, in this case, a high RQ was observed when nitrate was present in the culture medium. It is clear that the respiratory mechanism of Taichung Native 1 was modified to some extent under this treatment. These results imply that a reduction in nitrate may be linked with the TCA cycle system, with nitrate partially replacing oxygen as a hydrogen acceptor. This possibility has also been suggested and discussed with relation to barley roots by Willis and Yemm[23], and to various microorganisms by Sato and Egami[20]. This suggestion is further supported by the fact that the roots of Taichung Native 1 have a higher nitrate reductase activity but a lower cytochrome oxidase activity than those of Taichung 65, in nitrate-containing R2 medium.
- The level of activity of glycolate oxidase and catalase in the root tissues of Japonica rice is much higher than in Indica rice. The activity of peroxidase, which catalyzes the decomposition of H_2O_2, in the root tissues of Japonica rice is also higher. Japonica rice thus has a generally greater oxidizing ability. The activity of related enzymes found in the root cells of Indica rice was lower, and their oxidizing ability was weaker. It is likely that the oxidizing ability of Indica rices is not great enough to maintain an optimal potential or protect the plant from damage from reduced substances in a paddy soil. This character might be closely connected with the fact that Indica rices typically have more rotten roots, faster leaf senescence, and more

brown spots on the leaves, than Japonica rice varieties[13, 18].

- These results indicate that a clear differentiation in the physiology of the roots exists in Japonica and Indica type rices. The characteristics of this differentiation are summarized in Table 16.
- The fact that roots of Indica rices have a higher reducing ability, while the roots of Japonica rices have a higher oxidizing ability, might be an expression of their inherent character. The breeding of new cultivars with a particularly high oxidizing ability, which are able to adapt to the adverse environment of a reduced paddy soil, is very important. It is beyond doubt that Japonica rices with a high root oxidizing ability might be used as an important gene source for such a purpose.

Table 16. Differentiation of physiological characteristics of rice roots

Characteristic	Japonica	Indica
Behavior of root system in paddy soil[13]	Normal and healthy	Rotten roots apparent
Growth response of excised root culture	Poor	Good
Activity of NR and GDH	Lower	Higher
RQ in nitrogen-free medium	About 1.0	About 1.0
RQ in nitrate-containing medium	About 1.0	Over 1.0
Cytochrome oxidase activity	Higher	Lower
Catalase activity	Higher	Lower
Peroxidase activity	Higher	Lower
Glycolate oxidase activity	Higher	Lower
α-naphthylamine oxidizing ability[13]	Higher	Lower

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DISCUSSION

Q. (D.A. Somers)
Your data indicate genotypic effects on the root growth among cultivars of the two subspecies. Have you investigated the genetics involved in this variation in root growth?

A. I could not study the genetic differences in root behavior of Japonica and Indica, because of the sterility problem which exists in the crossing of Indica and Japonica type rices.

Comment: (F.J. Zapata)
You have shown that the elongation of roots in Japonica was affected by whether nitrogen was in a reduced form. We could go further, and suggest that this might be an interesting method of screening for drought resistance in rice varieties.

Q. (A. Komamine)
You concluded that respiration activity is higher in Japonica than in Indica type rices, but that nitrogen reduction activity is higher in Indica than in Japonica. Does this imply that nitrogen reduction activity limits the growth of roots?

A. This is true to some extent, but if the nitrogen source is changed from nitrate to more reduced forms such as urea, Japonica rice gives a better response. The respiration rate is fundamentally related to the respiration quotient (release of carbon dioxide and absorption of oxygen). The respiration rate is higher in Japonica rices, but the respiration quotient is the same, in nitrate free or nitrate

containing media. In other words, the respiration rate of Japonica rices increases in nitrate containing media, but the respiration quotient remains almost the same. In Indica rice, on the other hand, the respiration quotient becomes higher. In this sense, nitrate is reduced in the normal respiration cycle of Indica, whereas this phenomenon is not observed in Japonica rices.

Q. (A. Komamine)

You used a high concentration of sucrose in your root cultures. Does this reflect the nitrogen metabolism in intact rice plants? I think that photosynthetic activity can be a limiting factor in the growth of roots. What is your opinion of the relationship between C-metabolism and N-metabolism in the growth of roots?

A. I think this is a very important question, but because time was limited, I did not make a detailed study of this. In our research, we developed two types of improved culture media, the R1 and the R2. The sucrose concentrations are 3% and 5%, respectively. The sucrose concentration is higher in the R2 type, because it contains no casamino acids.

The excised roots cultured in R2 type medium must synthesize amino acids and protein from nitrate contained in the medium, so the energy consumption may be higher in this reducing process. On the other hand, excised roots in R1 type medium can utilize the directly reduced nitrogen of the casamino acids, so the energy requirement is comparatively lower than in the R2 type medium. I think this is the reason for the sugar concentrations in the R1 and R2 improved media of 3% and 5%, respectively.

Q. (E. Maeda)

Do you think the activity of the enzymes is concentrated in the root apex?

A. Yes, I think the activity is most vigorous in the root apex. However, the other parts of the root cannot be neglected.

Q. (K. Ohyama)

What is your source of carbohydrate? Do you use only sucrose?

A. The carbohydrate contained in the R1 and R2 type improved media for rice root culture was sucrose only.

TISSUE CULTURE PROGRAM AT IRRI

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ABSTRACT

The International Rice Research Institute has for the last eight years employed innovative breeding techniques, such as anther and pollen cultures, somatic cell culture and protoplast culture, to complement conventional breeding techniques in the production of new rice varieties or the improvement of existing ones. Basic and applied research projects are being undertaken. Basic research includes the improvement of callus production and plant regeneration efficiencies through manipulation of culture conditions and genetic factors, while applied research involves the generation of somaclonal and gametoclonal variants and immediate fixation of homozygosity in F_1 hybrids for faster production of varieties.

INTRODUCTION

When the International Rice Research Institute (IRRI) was established in 1960, one of the first projects set in motion by the Institute was the dramatic improvement in yield by means of breeding programs utilizing the best available parents from many nations. Thus, after 26 years IRRI has successfully come up with numerous improved varieties, one of which, IR36, is now grown on more than 10 million ha of the world's rice land[27]. Undoubtedly, conventional breeding techniques have done much to improve crop productivity. In view of the rapid rate of world population increase, however, other crop improvement techniques may have to be used to hasten the breeding and selection process. Plant breeders themselves have recognized the limitations of sexual hybridization, and pointed out ways in which innovative breeding techniques such as tissue culture can help conventional breeding to meet its objectives.

Some areas have been identified in which further improvement through conventional breeding techniques is difficult, or where the efficiency of conventional methods is inadequate, and therefore novel techniques such as biotechnology are needed[14]. Rice tissue culture is part of the biotechnology program at IRRI, and has geared its efforts specifically towards:

- breeding for increased biomass and therefore yield;
- breeding for protein quantity and quality;
- breeding for disease resistance;
- selecting for tolerance to soil stresses;
- increasing genetic variability and inducing novel variability by gametoclonal and somaclonal variation and by protoplast culture and fusion;
- clonal propagation using cell culture techniques for application in artificial hybrid seed production;
- rejuvenating germplasm by embryo rescue.

Tissue culture in rice began at IRRI eight years ago, and initially involved only anther culture. However, since we recognized that other tissue culture methods could open new opportunities of rice improvement, research at the Tissue Culture Laboratory now also includes pollen culture, somatic cell culture and protoplast culture. Practical application of these techniques has taken the form of collaborative projects at IRRI itself, and with other countries, including Korea and Peru.

ANTHER CULTURE

The advantages of anther culture have been discussed fully by Snape[26]. These include:

- shortening the breeding cycle – haploid

systems are the quickest method of advancing heterozygous breeding lines to homozygosity;

- increasing selection efficiency, because of better discrimination between genotypes within any one generation or field test, and better response to selection across generations.

In addition, anther culture can also be an effective way of producing variation, and allows the early expression of recessive genes.

Since the successful plant regeneration through anther culture in rice in 1968 by Niizeki and Oono[18], anther culture has proved to be a useful tool in rice breeding programs. The successful production of 81 varieties and strains in China[8] and more recently, the release of two varieties "Hwaseongbyeo"[15] and "Hwacheongbyeo"[7] in Korea, are proof that anther culture is indeed a vital part of rice improvement. This does not mean, however, that the use of anther culture is not without certain disadvantages and constraints. Two disadvantages have been pointed out. Firstly, because a random, unselected sample of genotypes is produced, a high proportion are likely to be discarded for failing to meet simple agronomic criteria for characters which have high heritabilities. Secondly, there are limited opportunity for recombination between the parental genomes in cases where F_1 crosses are used.

Some of the other constraints are the high proportion of albino plants produced, and the low efficiency of callus production and plant regeneration, especially in Indica subspecies. Basic studies to solve these problems and improve culturability are still needed.

Pathways of Pollen Development in Rice

Knowledge of the pathways of pollen development makes possible the manipulation of conditions to increase anther culturability, and direct the desired pathway of plant regeneration either through embryogenesis or organogenesis.

Several pathways of pollen development from a unicellular to a multicellular callus have been observed, using the variety Taipei 309 planted at uninucleate and binucleate stages[17].

Pathway I. Initiation occurred in the uninucleate pollen grain by equal division of the single nucleus. By further mitotic division, a multinucleate and multicellular pollen grain was produced. This was the most frequent pattern (Table 1).

Table 1. Number of androgenic pollen grains observed and the frequencies of pollen development in the three pathways of androgenic initiation

Total no. of androgenic pollen grains	No. of pollen grains showing early division			No. of pollen grains in advanced stages
	Pathway I	Pathway II	Pathway III	
697	128	43	7	519
	(18.4%)	(6.2%)	(1.0%)	(74.4%)

Pathway II. Initiation occurred in the binucleate pollen grain. The vegetative nucleus divided to give rise to multicellular pollen, while the generative nucleus remained inactive.

Pathway III. Initiation occurred in the binucleate pollen, but the generative nucleus divided first, followed by the division of the vegetative nucleus: both contributed to the microspore callus. This was the least frequent pattern.

A fourth possible pathway, reported by Qu and Chen[19], is callus formation exclusively from the generative nucleus. However, conclusive evidence for its occurrence was not obtained in our studies.

Increase in Callus Induction and Plant Regeneration Efficiency by the Use of Abscisic Acid

Abscisic acid (ABA) given for short periods increased pollen embryogenesis in tobacco anthers[9], and stimulated shoot bud and plantlet formation in rice somatic callus cultures[10]. These observations prompted us to investigate the effect of ABA in

two different studies – one on callus production and the other on plant regeneration. In the first experiment, on the effect of ABA on callus induction by stressing the anthers for short periods, the hormone was added to the callus induction medium (liquid and semisolid) at 0-30 mg/l for 1, 2, 3 or 4 days. The optimum concentration was 2.5 mg ABA/l in both types of media. However, in semisolid and liquid media, the best duration of exposure was for 2 and 3 days, respectively[13].

Likewise, ABA increased the frequency of calli producing green plants in Taipei 309 and the average green plant regeneration in the three varieties tested. Anther derived calli were grown in a preculture medium with ABA for 4 weeks before finally transferring them into the regeneration medium without ABA[30].

Heat Treatment to Increase Callus Induction Efficiency in Anther Culture

As mentioned above, the major constraint in the use of Indica varieties is their low level of efficiency in callus production and green plant regeneration. It has been a standard procedure to subject anthers to cold treatment (8°C for 8 days), as this greatly enhances their culturability. Heat treatment, on the other hand, has been widely used in the *Brassica* genus.

The effect of heat treatment on IR42 was determined. IR42 is one of the most important IR varieties, because it is resistant to many major insect pests and diseases, and has high yielding ability. Heat treatment at 35°C was given to the florets for 0, 5 or 15 minutes. Heat treatment increased callus induction in two media tested (Table 2), with an optimum duration of 15 min. As with cold shock, the application of heat may promote synchrony and viability, and also activate some of the enzymes necessary to induce the pollen grains toward vegetative development (in this case, callus production) instead of their normal generative function[38]. These results are in agreement with earlier reports[20,25] on increased callus induction and plant regeneration following heat treatment.

Table 2. Effect of heat treatment on callus induction efficiency in IR42 anther culture

Treatment	Anthers inoculated (no.)	Calli produced (no.)	% callus production
		E10 medium	
H_0	345	34	9.8
H_5	623	76	12.2
H_{15}	439	90	20.5
		ER medium	
H_0	565	64	11.3
H_5	706	102	14.4
H_{15}	377	84	14.6

Gamma Ray-Induced Green Plant Regeneration in Basmati 370

Breeding studies to improve the agronomic characteristics of Basmati rices have taken place over a long period, but without much success. Anther culture was attempted, in order to generate gametoclonal variants such as short stature and lodging resistance without altering the superior grain quality of Basmati 370. However, in preliminary studies using three callus induction media, only albino plants were regenerated[36].

Irradiation was therefore employed, in the hope of producing genetic alterations in the cell which would not only improve callus induction and promote green plant regeneration, but eventually increase positive variation in the regenerated plants. Basmati 370 seeds were subjected to irradiation dosages of 15, 20 or 25 krad at an application dose rate of 1.2 krad/min from a Cobalt 60 gamma cell, and anthers were collected from plants of irradiated seeds. Both callus production percentage and albino plant regeneration were decreased by the application of irradiation stress; the most inhibitory was 20 krad. However, it was also only at this irradiation dosage that green plants were regenerated (Table 3).

Table 3. Effect of irradiation on plant regeneration of Basmati 370 in G4 medium

Irradiation dosage (krad)	Calli inoculated (no.)	Calli with green plants (%)
0	10	0
15	61	0
20	23	26.1
25	84	0

Isozyme Analysis to Detect Recombination and Variability in Anther Culture-Derived Plants

Two important questions in the use of F_1 hybrids in anther culture are whether the anthers come from F_1 plants (i.e. hybridization is successful), and whether the development of some pollen grains is more likely than others.

Isozyme patterns can be used to clear these doubts. It would also save time if a correlation could be established between isozyme patterns and observable agronomic characteristics, since screening could then be done in the laboratory for characteristics of interest, without the tedious process of growing the plants in the field.

Recombination among 10 isozyme loci in 24 anther culture lines of the cross Tatsumi mochi/BG90-2 and 5 lines of the reciprocal BG90-2/Tatsumi mochi was observed[6]. This indicates that hybridization was successful, and that all the anther culture lines originated from pollen grains of F_1 hybrids.

The isozyme patterns likewise showed that sister lines have the same recombination patterns. Different isozyme patterns from sister lines was also observed. There may be two possible explanations for this. One is that the supposed sister lines may have actually originated from two distinct pollen grains which adhered and grew together as one callus. The second is that mutation in some portions of the callus may have taken place, similar to that observed by Fukui[5].

In some cases, there is the possibility of the calli being broken into several pieces prior to being plated in the regeneration medium. This may have occurred in some plates, since lines coming from what have been regarded as several separate calli have the same isozyme patterns.

The results of this study demonstrate that variability in lines coming from different calli, as confirmed by isozyme patterns, indicates that the plants developed from different pollen grains. Although the number of lines observed may be too limited to use as concrete evidence, the variability in the different recombination patterns does suggest that *in vitro* pollen selection may be absent during anther culture.

Observational Yield Trial of Basmati 370

As mentioned above, green plants were regenerated only from anthers collected from irradiated Basmati 370. These plants were grown in 3-row plots in observational yield trials for two seasons.

The general effect of irradiation was to shorten the days to flowering, reduce plant height and increase the number of tillers. The fertility of the irradiated plants was about the same as that of the control irradiated at 20 krad (Table 4). A seasonal effect was also observed. Because of more favorable conditions during the dry season, plants grown at this season required fewer days to flower, and had a shorter plant height and a greater number of tillers. As regards fertility, it cannot be assessed whether there was any increase in this during the dry season, since the plants were severely affected by a typhoon during the wet season.

AC 6166 and its three sister lines have been elevated to 10-row plots and will be screened for other characteristics, particularly grain quality, which is the best attribute of Basmati rices. All the 19 anther culture lines were sent to Pakistan for further evaluation under field conditions.

Replicated Yield Trials

A few of the anther culture-derived lines which were found promising in the 3-row observational yield trial and 10-row plots were further screened in replicated yield trials for three consecutive seasons. In most cases, the anther culture lines yielded higher than either parent, or gave the same yield as the better parent (Table 5). The yield component mainly responsible for this increase in yield of the anther culture lines was their higher tiller numbers resulting in higher harvest indices (Table 6). Anther culture lines 800 and 800-1 flowered 2 weeks earlier (70 DAS) than the earlier-flowering parent, Suweon 290 (83 DAS).

Collaborative Projects

Collaborative projects for the production by anther culture of homozygous lines which are tolerant to adverse conditions have been established. The general schematic diagram for these programs is shown in Fig. 1.

At present there are several collaborative projects with other IRRI scientists and between tissue culture laboratories in national programs. These programs include the production of cold tolerant lines (with the Rural Development Administration, Korea), lines adapted for Latin American conditions (with the Peruvian National Rice Program and the National University Pedro

Table 4. Agronomic characteristics of anther culture-derived lines from irradiated Basmati 370. IRRI observational yield trials. 1986WS and 1987DS[z]

Designation	No. days to flowering		Plant height (cm)		Tiller no.		Fertility (%)	
	1986WS	1987DS	1986WS	1987DS	1986WS	1987DS	1986WS[y]	1987DS
Basmati 370, ck	92	85	152	145	11	12	73	74
Basmati 370 (irradiated, 20 kr)	86	81	149	127	15	14	43	79
Anther culture lines								
5821	88	83	147	122	9	12	53	75
5821-2	90	83	139	126	11	12	30	77
5821-3	90	83	139	122	9	12	41	71
5821-4	92	85	144	129	15	15	52	68
5821-5	88	83	147	127	14	14	42	71
5821-6	90	84	155	127	10	13	58	71
5821-7	88	84	145	125	9	13	46	69
5824	90	84	150	132	14	10	62	82
5824-3	90	83	153	131	15	13	62	77
5824-4	90	84	153	135	9	13	47	80
5830	90	84	154	131	12	11	38	77
5830-1	90	86	153	128	9	13	39	72
5830-2	92	86	153	128	9	13	49	74
5835	92	86	155	130	13	13	63	72
5835-1	92	83	152	132	9	12	49	76
6166	90	81	147	124	13	15	54	81
6166-1	90	81	149	120	11	18	41	81
6166-2	88	81	141	122	11	15	39	83
6166-3	86	79	138	119	15	17	25	78

WS: Wet season DS: Dry season [z] Average of 12 plants per entry [y] Severely affected by typhoon

Table 5. Grain yield (mt/ha) in replicated yield trials of anther culture-derived lines over three seasons. IRRI, College, Philippines

Entry	Season		
	1985 wet season	1986 dry season	1986 wet season
IR36, ck	4.79 bcd	5.76 ab	3.90 defg
IR42, ck	4.15 b	5.06 bcde	3.41 gh
IR48[z]	4.97 bc	5.03 bcde	3.61 fgh
Suweon 290[z]	2.92 e	5.60 abcd	4.50 bc
AC 800[y]	5.43 ab	5.64 abc	4.65 ab
AC 800-1[y]	5.87 a	6.25 a	5.15 a
AC 1182[y]	4.60 cd	5.41 bcde	4.43 bcd
IR48[z]	4.97 bc	5.03 bcde	3.61 fgh
BG90-2[z]	2.86 e	4.87 defg	3.20 h
AC 655[y]	4.43 cd	4.84 efg	3.80 efg
AC 721-1[y]	4.35 cd	4.65 fg	3.65 fgh
IR58[z]	4.69 cd	5.73 ab	5.11 a
IR19792-15-2-3-3-3[z]	4.82 bcd	4.22 g	4.32 bcd
AC 833[y]	5.05 bc	4.71 efg	4.09 bcdef
AC 833-2[y]	4.77 bcd	5.09 bcde	3.85 defg
IR30[z]	4.10 d	4.75 efg	3.68 fgh
AC32B18[y]	4.73 bcd	4.93 cdef	3.96 cdefg

Note: In each column, means followed by a common letter are not significantly different at the 5% level by DMRT

[z] Female x male parents [y] AC: anther culture lines derived from each cross

Table 6. Harvest index of anther culture-derived lines and their parents in replicated yield trials over two seasons

Designation	Season	
	1986 dry season	1986 wet season
IR36, ck	0.49	0.46
IR42, ck	0.39	0.25
IR48	0.38	0.32
Suweon 290	0.44	0.37
AC 800	0.53	0.50
AC 800-1	0.53	0.51
AC 1182	0.44	0.45

Ruiz Gallo), and lines for upland conditions and salt tolerance with IRRI plant breeders. The total number of green plants we have so far regenerated in each project is shown in Table 7.

SOMATIC CELL CULTURE

Since the demonstration of the totipotency of plant cells, tissue and cell cultures have been employed as a possible method for the mass propagation of crops. At the start of the decade, however, the variation generated from tissue culture could no longer be ignored. This technique therefore became recognized as an agent to widen the genetic base in plants without resorting to hybridization. This phenomenon has been termed somaclonal variation, and refers to the enhanced genotypic variability in plants regenerated from tissue culture. Somaclonal variation in several crops has been reviewed[3, 16, 21, 22, 23]. Apart from spontaneous variability as a reaction of the exposure of cells

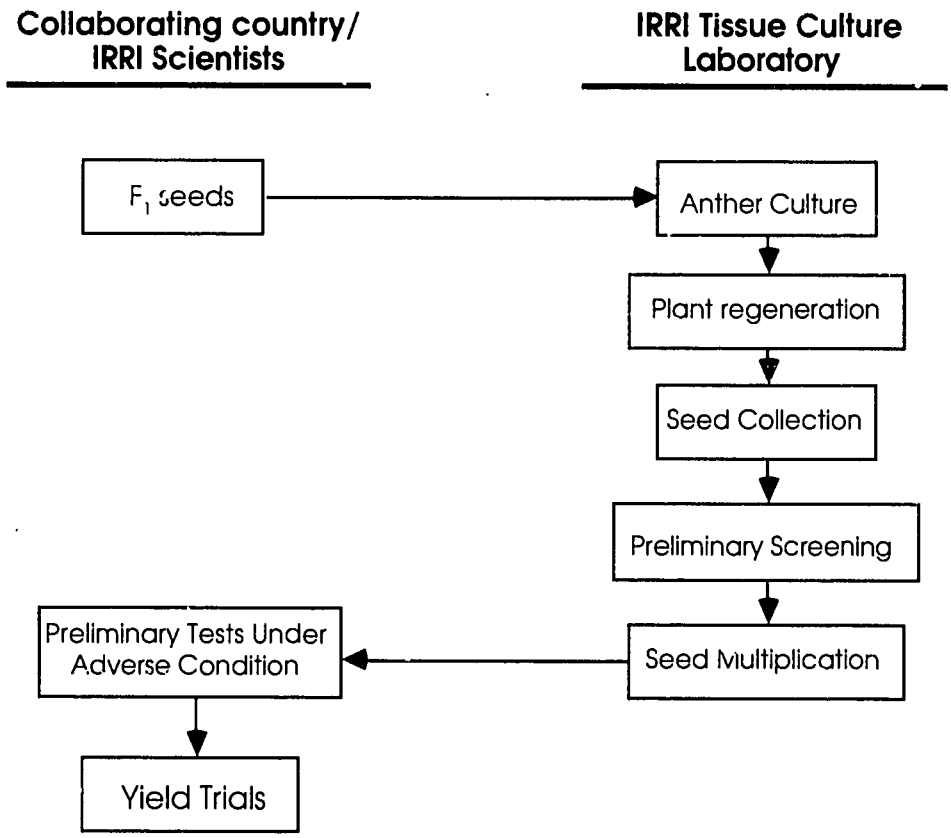

Fig. 1. Schematic diagram of collaborative programs for the production of homozygous lines tolerant to adverse conditions through anther culture

Table 7. Summary data on the rate of green plant regeneration in various collaborative projects concerned with the production of homozygous lines tolerant to adverse conditions through anther culture

Adverse condition bred for	Crosses inoculated for anther culture (no.)	Calli producing green plants (no.)	Green plants produced (no.)
Cold tolerance	32	1,898	11,450
Latin American conditions (particularly rice blast)	25	70	781
Upland conditions	107	361	1,988
Salt tolerance	101	119	1,622

to stress during *in vitro* culture, variation can be more or less directed by exposure of cells to supra-optimal concentrations of chemicals or environmental conditions which could screen and select mutant cells. These mutant cells will presumably give rise to mutant plants.

Although somaclonal variation may be a novel method of getting improved genotypes, this may not be desired in operations requiring clonal uniformity. Similarly, the genetic integrity of germplasm conserved by *in vitro* techniques is at a risk, because of somaclonal variation[21].

It is obvious that in some cases, a researcher would prefer to have a uniform phenotype from the *in vitro*-derived plants. In another situation, variability may be desired. Recent developments seem to indicate that plants regenerated through organogenesis show wide variability, while plants regenerated through somatic embryogenesis are true-to-type. Basic studies to understand fully the basis for somatic embryogenesis have been emphasized in our studies on somatic cell culture.

Effect of the Specific Gravity of Seed on the Efficiency of Somatic Cell Culture

One of the problems in basic research into somatic cell cultures is the wide variation of results among replications, even in instances where the seeds used as explants come from the same batch. One reason for this may be the non-uniformity of seeds harvested from one panicle. The topmost spikelet of any branch fills up first, after which the sequence of grain filling is acropetal[33]. Furthermore, dominating spikelets seem to produce larger, heavier grains with less chance of sterility and abortion than the dominated ones. Non-uniformity of harvested seeds is further aggravated by the practice of combining the seeds from two or more panicles during harvest. Variability in response during tissue culture may thus be due not only to treatments but to variability in grain filling of the seeds used as explants.

The seeds of two cultivars, IR28 and Nona Bokra, which were used as explants in this experiment, were therefore classified according to their specific gravity. The response of the graded seeds to callus induction and plant regeneration was compared with the ungraded (mixed ones) as control.

Nona Bokra had higher callus production than IR28, mainly as a result of varietal differences (Table 8). The main point here, however, is that all the graded grains gave a lower standard deviation of the mean than the ungraded grains in callus induction efficiency. The same trend was observed in plant regeneration[37]. In both parameters, grains with a specific gravity higher than 1.2 responded the best, as these may have the optimal kernel development required for callus production and plant regeneration. These results underscore the importance of grading the seeds first, before using them as explants in somatic cultures. Grading based on specific gravity narrowed the range of distribution of the true values of percent callus production and green plant regeneration.

Screening of Plants Regenerated from NaCl-Stressed Calli

Salinity is one of the major soil stresses in rice-growing countries. In South and Southeast Asia alone, saline soils constitute 58.8 million ha (64%) of the total problem rice lands[11]. The problem of salinity is so serious that it is one of the most widely studied fields in tissue culture, in terms of generating variants adapted to this condition.

The usual procedure in generating variants

Table 8. Percent callus production in LS medium[z]

Grain classification		2,4-D concentration (μM) 0	2.5	5.0	10.0
Nona Bokra					
Ungraded		36.11 ± 13.17	52.78 ± 10.00	66.67 ± 11.38	63.89 ± 9.04
Graded	1.1>SG>1.0	5.56 ± 2.87	19.45 ± 3.69	22.22 ± 3.51	19.45 ± 2.78
	1.2>SG>1.1	63.89 ± 2.78	72.22 ± 3.51	75.00 ± 3.72	72.22 ± 3.51
	SG>1.2	72.22 ± 3.51	83.33 ± 4.30	88.80 ± 3.51	77.78 ± 3.51
IR28					
Ungraded		5.56 ± 2.87	16.67 ± 8.61	19.44 ± 14.72	25.00 ± 10.97
Graded	1.1>SG>1.0	0	2.78 ± 2.54	8.34 ± 2.63	8.34 ± 4.81
	1.2>SG>1.1	5.56 ± 2.87	11.11 ± 4.30	16.67 ± 3.04	13.89 ± 3.66
	SG>1.2	11.11 ± 2.03	16.67 ± 3.04	19.45 ± 3.69	25.00 ± 5.69

[z] $\% \text{ Callus production} = \frac{\text{Number of callus-producing seeds}}{\text{Total number of seeds inoculated}} \times 100 \pm \text{standard deviation of the means}$

tolerant to soil and environmental stresses is to expose *in vitro* cultured cells to toxic concentrations of a particular stress, in this case NaCl, on the assumption that the cells surviving the stress will regenerate tolerant plants.

Eighteen calli of Reiho exposed to 15 g NaCl/l for 32 passages (4 weeks/passage) were able to regenerate 35 plants[35]. Although the calli tolerated exposure to 1.5% NaCl, the plants did not survive when grown in culture solution containing 5, 10 or 15 g NaCl/l. Plants kept on a culture solution without NaCl flowered, but were 100% sterile. The regenerated plants therefore did not carry the tolerance present in the selected calli.

For confirmation, some of the selected calli were subcultured in different NaCl concentrations. Calli growth was inversely proportional to the concentration of NaCl in the medium (Table 9), suggesting that the stressed cells have only physiologically adjusted to high NaCl concentrations. If the selected cell lines were true mutants, they would have required NaCl for growth.

Somatic Embryogenesis in Cultivated Rices

Somatic embryogenesis is a way of producing plants from *in vitro* culture, to regenerate true-to-type plants or conserve the genetic integrity of germplasm. Successful somatic embryogenesis in rice is important in protoplast culture and fusion, and DNA-transfer techniques. From a practical point of view, this technique could also be used for the production of artificial hybrid seeds.

Table 9. Response of calli (variety Reiho) to different concentrations of NaCl

Treatment (g NaCl/liter)	Initial weight[z] (g)	Final weight (g)	Relative increase in weight[y] (%)
0	0.0498	0.6882	1,281.93
5	0.0513	0.6446	1,156.53
10	0.0512	0.4551	788.87
15	0.0497	0.2762	455.73
20	0.0521	0.1607	208.44

[z] Average of 10 calli

[y] $\frac{\text{Final weight-initial weight}}{\text{Initial weight}} \times 100$

Rice hybrids provide a significant yield advantage (14-34%) compared with the best check varieties. The yield advantage of the hybrid can be attributed to an increase in dry matter production (source size) and/or a higher harvest index, due to an increased number of spikelets per unit area and 1,000-grain weight (sink size)[27].

Although a hybrid rice program has been carried out in Mainland China for several years, its feasibility is still in question in most countries. The main bottleneck in hybrid rice technology is seed production. Farmers need to buy hybrid seeds every cropping season. Since production of such seed is labor-intensive, the cost is very much higher than when non-hybrid seed is used. If hybrid seeds could be cloned in large quantities via somatic

embryogenesis, with hybrid vigor retained, this might be a practical and economical method of hybrid seed production.

Somatic embryogenesis has been observed in several cultivated rice species: IR28, IR40, IR43, IR54 and Tetep[12]. The ontogeny of embryoids from scutellar tissues of mature seeds was studied, using histological and scanning electron microscope techniques. It was established that the scutellar tissues, especially the sub-epidermal portion, was the site of active cell division which eventually gave rise to callus. The scanning electron microscope showed the development of globular and organized structures into cup-shaped, bottle-shaped and embryoids with scutellum, coleorhiza and developing coleoptile, structures similar to the zygotic embryos.

Somatic Embryogenesis in *Oryza Perennis* and *Echinochloa Glabrescens*

Wild species of rice and their relatives are excellent sources of resistance to diseases and pests. However, in conventional breeding there is difficulty in obtaining successful interspecific and intergeneric hybridization. Parasexual methods of hybridization, such as protoplast fusion, are then the only option left. Successful protoplast fusion and eventual plant regeneration greatly depend on the ease with which callus can be induced and plants regenerated from the protoplasts involved in fusion. High culturability of fusion protoplasts, though no guarantee that somatic hybrids can be regenerated, is a helpful parameter in using this technique.

One of the more important wild rice species is *Oryza perennis* Moench, which is tolerant to stagnant flooding and acid sulfate soils, and has desirable floral characteristics for out-crossing. *Echinochloa glabrescens*, on the other hand, is one of the gramineae which can be adapted for cultivation as a forage and grain crop in arid and semiarid regions, since a single watering suffices from germination to harvest.

Before attempting protoplast fusion between cultivated and wild rices or other related species, it is worthwhile to assess the callus production and plant regeneration abilities of these species.

Plant regeneration through somatic embryogenesis from mature seed and young inflorescence of the wild rice *Oryza perennis* was observed[12]. The frequency of callus induction was higher when young inflorescences were used as the explant (62%) than when mature seeds were used (42%). Green plant regeneration frequency from both types of explant decreased with increasing duration of culture (Table 10). The regeneration capacity of the calli from the two types of explant did not differ significantly. However, mature seeds are a more practical source of explants, since these can be stored and are readily available any time of the year.

Formation of embryogenic calli and plant regeneration was likewise observed in somatic cultures of *Echinochloa glabrescens*, using young inflorescences and leaf segments as explants[31]. The callus induction frequency was 91% in young inflorescences and 41% in leaf segments. The green plant regeneration efficiency decreased from the 2nd to 6th passages. The young inflorescence was a better explant than young leaf segments, in terms of plant regeneration.

Table 10. Plant regeneration in cultures derived from young inflorescences and seeds of *O. perennis*

Passage/ no. months in culture	Young inflorescence-derived callus				Seed-derived callus			
	Calli inoculated (no.)	Calli responding (no.) (%)	Total plants produced (no.)	Av. green plant production[y] (no.)	Calli inoculated (no.)	Calli responding (no.) (%)	Total plants produced (no.)	Av. green plant production (no.)
6/8	25	20 (80)	117	5.85	20	15 (75.0)	97	6.5
7/9	20	13 (65)	78	6.0	20	10 (50.0)	58	5.8
8/10	20	12 (60)	62	5.2	15	10 (66.7)	60	6.0
9/11	25	15 (60)	64	4.3	12	7 (58.3)	35	5.0
10/12	20	12 (60)	42	3.5	43	30 (69.8)	132	4.4

[x] Data were computed after 4 weeks in culture on MS18-1 medium

$\frac{\text{Total no. of calli responding}}{\text{Total no. of calli inoculated for regeneration}} \times 100$

[y] Plants produced/calli producing plant

PROTOPLAST CULTURE

Non-sexual methods of hybridization, such as protoplast fusion and gene transfer techniques, are possible only through the establishment of efficient plant regeneration using protoplasts. Isolation of viable protoplasts and plant regeneration has been reported from cell suspension cultures from various explants, including leaf segments and scutella[1], anther-derived calli[28, 29], embryo calli[24], immature embryo calli[4], intact seeds[2, 34] and root tips[39].

While there have been some common features in the procedures described by different authors, the detailed steps or conditions suggested for each plant or plant part have been sufficiently different to suggest that perhaps specific conditions are required of each plant or plant material being processed. Therefore, we recently attempted to assess the significant factors in the isolation, culture, cell division, callus induction and plant regeneration in rice protoplasts. We thus established cell suspension cultures of genotypes of Japonica, Indica and wild species of rice, to secure a source of protoplasts which readily yield $5 \times 10^6 - 1 \times 10^7$ protoplasts per gram of cell FW, depending on the variety. To date, a number of protoplast derived calli, and sustained cell division, have been obtained from two genotypes of Japonica and Indica, respectively. This research is now working towards plant regeneration.

FUTURE GOALS

Strengthening of Collaborative Projects

The results of our collaborative projects with the national programs of Peru and Korea and in-house projects have been very encouraging. Preliminary results have shown that anther culture-derived lines for cold and salt tolerance were equal in performance with, if not better than, the most tolerant checks used in screening such stresses. Some promising lines have been forwarded to the International Rice Testing Program, and are now being screened in different parts of the world.

Lately, we have established collaborative projects with Colombia, India, Pakistan and Indonesia. Anther and somatic culture-derived lines have been sent to these countries' national programs for screening and selection under local conditions.

Increasing the Culturability of Indica Genotypes

Although some Indica varieties, such as IR43, have been found to be moderately amenable to anther and somatic culture, these are still far below the capacity of model Japonica varieties. Culturability of the Indicas is needed, not only for anther culture, but also for somatic cell culture for possible application in artificial seed production through embrogenesis, and in protoplast culture and fusion.

Identification of Indica genotypes with good callus production and plant regeneration capacity, and manipulation of conditions likely to enhance the response of these genotypes, is in progress.

Genetic Techniques to Transfer the Culturability of Japonicas into Indicas

Since Japonicas are highly culturable, one of the possible channels to transfer this capacity into the recalcitrant Indicas is through conventional hybridization, followed by anther culture. The homozygous lines derived will then be screened, so that isogenic Indica lines (i.e., all characteristics of a given line) are the same as the Indica parent, with only the genes controlling culturability from the Japonica parent incorporated. These isogenic lines may be useful for practical purposes, such as the generation of somaclonal and gametoclonal variants, for protoplast culture and fusion, or as parents in hybridization.

Isolated Pollen Culture

One of the doubts raised in anther culture is whether the plants originate from the pollen grains or from the anther somatic tissues. Although previous studies have shown that anther culture lines attain immediate homozygosity (i.e. the plants came from pollen grains), some cases have shown that segregation may also occur, indicating that the development of somatic cells of the anther is not a remote possibility. The best way of resolving these doubts would be to regenerate plants from isolated pollen. Several other advantages can be derived from this technique, such as the development of true-to-type plants through pollen embryogenesis, instead of passing through the callus stage where mutation could occur, and production of pollen protoplasts for gene transfer techniques.

Protoplast Culture and Fusion

Success in regenerating plants from wild and related species of rice, progress made in protoplast culture of Japonicas and Indicas, and the vast collection of rice germplasm at IRRI, offer us a great opportunity to develop protoplast culture and fusion techniques. As mentioned above, suspension cultures have been established in Indica, Japonica and wild rice species, and protoplast-derived calli have been produced from Indica and Japonica species.

In particular, this technique will be utilized to incorporate the resistances found in wild species into cultivated ones, especially in crosses where sexual incompatibility exist. Moreover, successful protoplast culture is important in gene transfer techniques.

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DISCUSSION

Q. (M. Vajrabhaya)
You have experience of selecting for salt tolerance *in vitro*. Over how many generations should you test the plants, to make sure they are stable?

A. Sequentially, over the generations, the longer cells are kept in cell cultures, the less likely are the plants to be normal. Some of the plants I showed in my slides appear to be normal, but have abnormal chromosomes. Salt tolerance is difficult to transfer. There are also other problems, such as phosphorus deficiency in the field, which affect the behavior of rice plants.

Q. (D.A. Somers)
You mention work on improving the lysine content of rice. How did you do this?

A. A few years ago, we were very interested in increasing the lysine content. However, we only achieved 15% free lysine, and the level needs to be 50% to be valid. The results were only achieved very slowly, so that research began to move into different fields. We are now more interested in trying to transfer the yellow plasma band, which may indicate a higher lysine content.

The level of lysine in the plant is also very difficult to measure, in that a high level of nitrogen in the soil, drought stress, or a combination of the two, tend to increase the lysine content. Conditions need to be very highly controlled.

Q. (K. Hayashi)
Under the CGIAR system, applied research on practical problems is encouraged. In the IRRI rice breeding program, the problem of sterility in wide crosses, as between Japonica and Indica, is not a very serious problem, unlike in Japan. After you have grown plants from the anther, you do not seem to have much of a problem in obtaining F_1 seed. Is this correct? Is this the reason why IRRI is not concerned with genetic engineering?

A. We must do research for which the results have a practical application. Many national programs will be unable to use the technology of genetic engineering in the near future For this reason, we are more interested in building up the technology of rice anther culture. At IRRI, we have been able to overcome problems of sterility in the varieties we have worked with, but this is not necessarily a general rule. IRRI feels that, although genetic engineering is developing very fast, until the methods can be used for practical purposes in national programs, it would be better for us not to use it. To begin genetic engineering at IRRI will also need new personnel, and a lot of investment into new equipment such as centrifuges.

Q. (M.T. Senawi)
There appears to be a bias at IRRI in favor of Japonica rices. However, a lot of successful work has already been done on these. Is there any chance that IRRI will start to concentrate on Indica rices?

A. In fact, we are working on many Indica crosses at IRRI – most of the rice shown in my slides were Indica varieties. Which varieties we work with at IRRI depends on national programs.

Comment: (D.A. Somers)
Apparently Indica rices are a problem in rice anther culture. Some recent publications have shown that it is possible to make very wide crosses in the graminae that result in elimination of a parental set of chromosomes from the hybrid embryo. The resultant crosses are usually haploid embryos, and in some cases the haploid embryos have been rescued for plant formation. It is very difficult to anther culture oats, but Dr. Rines has had some success with crosses of corn and oats, and

has isolated haploid oat plants. If it is possible to make very wide crosses between rice and another cereal species, this may be an alternative to anther culture for Indica rice.

Comment: (F.J. Zapata)

The system for anther culture of Japonica was developed fifteen years ago. I feel it is now time for countries using Indica rice to use this method to produce new Indica rice lines. As far as wide crosses are concerned, in Mainland China they claim to have achieved crosses of rice and sorghum, with rice type plants growing in very arid soils. This may mean that it is possible to transfer the drought resistance of sorghum to rice.

RICE (*ORYZA SATIVA* L.) ANTHER CULTURE

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ABSTRACT

Recent advances in rice anther culture emphasized, not only time- or labor-saving techniques and standardization, but also practical application for rice improvement. Gradual progress in our knowledge of the factors affecting culturability in rice anther culture, such as the optimal pollen stage, callus age, media, cold pretreatment and genotypic differences, have reduced the difficulty of obtaining pollen-derived plants over the last two decades. Suggestions on the practical application of anther culture techniques as a valuable breeding method for rice improvement are presented, together with a clarification of the genetic variability, segregation and recombination of desirable characters in pollen-derived plants.

INTRODUCTION

The greatest advantage of anther culture techniques are that they make possible the improvement of new homozygous lines and shorten the breeding cycle by haploid breeding. Since haploid plants of rice through anther culture were first successfully obtained by Nitzcki and Oono in 1968[23], many scientists throughout the world have worked to increase the efficiency of anther culture and apply it to rice improvement.

Although rice is recognized to be comparatively suitable for anther culture the application of this technique for rice improvement has been hampered by the many factors affecting the production of pollen-derived plants. Research on these factors in terms of methodology should therefore be carried out simultaneously with the development of efficient diploidization methods, and studies of the genetic variability of pollen-derived plants, in order to put these techniques to practical use.

This paper summarizes recent research on rice anther culture conducted at the Yeongnam Crop Experiment Station (YCES), Rural Development Administration (RDA), Korea.

FACTORS AFFECTING THE CULTURABILITY OF RICE ANTHER CULTURE

Pollen Stage and Callus Age

Comparative studies indicate that the frequency of callus induction is high when anthers containing pollen at the uninucleate stage are inoculated. Furthermore, differences in the frequency of callus induction within the uninucleate stage have also been recognized[2]. There are many methods by which to determine the stage of pollen development under a microscope: nucleus division, the position of the pollen pore relative to the nucleus[26], and the diameter of the microspore at different developmental stages[25]. However, the status of pollen development as determined by microscopic analysis differs according to varietal group, cultural conditions and the position of the spikelet within the panicle. From a practical point of view, the optimum inoculation stage of the anther is generally determined by the distance between the upper two leaf auricles, and the degree of anther development in the spikelet. However, even though the panicles may be at an appropriate distance between the upper two leaf auricles, pollen stage may be very variable within the same panicle. The

degree of anther development in the spikelet is the best method to determine the appropriate time for anther inoculation. Although Nagdongbyeo (a Japonica rice variety) had a little longer optimum period for anther inoculation than Milyang 23 (Tongil type), the best results were obtained when anther development reached up to 1/2–1/3 of spikelet length (Table 1). However, if the panicles were pretreated at a low temperature before inoculation, the optimum period was when the anthers reached 1/3 of the spikelet length. When anthers were inoculated at the late uninucleate stage, the frequency of green plant regeneration decreased and that of albino plants increased[3].

About 90% of total calli were induced 20-40 days after anther inoculation, while a few calli were induced after 50 days[40]. Callus age, the number of days from callus induction to transplanting, affected the rate of plant regeneration. The frequency of green plant regeneration was high when callus was 11-20 days old, but the frequency of albino plants increased when callus was more than 30 days old (Table 2). The most effective callus age for green plant regeneration, was about 20 days after callus induction, when the callus had a diameter of 2-3 mm.

Table 1. Effect of anther development on callus induction in rice anther culture[11]

Anther stage	Anther[z] development	Variety[y]	No. of anthers inoculated	No. of calli induced (%)
Early	1/3	Nagdongbyeo (J.)	180	22 (12.2)
		Milyang 23 (T.)	460	53 (11.5)
Middle	1/3–1/2	Nagdongbyeo	280	42 (15.0)
		Milyang 23	440	31 (7.0)
Late	1/2–2/3	Nagdongbyeo	190	10 (5.3)
		Milyang 23	310	1 (0.3)

[z] Anther position in spikelet (1/3: one third from bottom)
[y] T: Tongil type variety
J: Japonica type variety

Table 2. Effect of callus age on plant regeneration in rice anther culture

Callus age (No. days)	Variety[z]	No. of calli transplanted	Plant regeneration (%) Green	Plant regeneration (%) Albino
0–10	Milyang 23 (T.)	65	12 (24.6)	12 (18.5)
	Nagdongbyeo (J.)	80	29 (36.3)	8 (10.0)
11–20	Milyang 23	78	37 (47.4)	16 (20.5)
	Nagdongbyeo	95	42 (44.2)	8 (8.4)
21–30	Milyang 23	80	29 (36.3)	28 (35.0)
	Nagdongbyeo	80	31 (38.8)	18 (22.5)
31–40	Milyang 23	50	7 (14.0)	17 (34.0)
	Nagdongbyeo	63	10 (15.9)	17 (27.0)
41–50	Milyang 23	20	1 (5.0)	2 (10.0)
	Nagdongbyeo	41	3 (7.3)	5 (12.2)

[z] T: Tongil type variety
J: Japonica type variety

Media

In the early studies, MS and Miller basic media were mainly used for rice anther culture, but recently N_6 basic media with a modified in ammonium content has been widely adopted[8]. However there is some difficulty in using N_6 basic media for all genotypes, especially when the rice is an Indica type. It is known that the medium used for callus induction influences the rate of plant regeneration after callus transplanting. The source and amount of total nitrogen in the basic media in particular are critical for callus induction[31]. In an evaluation of basic media for rice anther culture, the N_6-Y_1 basic medium gave better results than MS, Miller, or N_6 media (Table 3). The total nitrogen content of N_6-Y_1 basic medium was the same as that of N_6, but in N_6-Y_1 medium it took the form of 28 mM KNO_3, 1.75 mM $(NH_4)_2SO_4$, and 1.75 mM L-Glutamine, while in N_6 the nitrogen was present as 28 mM KNO_3, 3.5 mM $(NH_4)_2SO_4$ (Table 4).

Table 3. Effect of media on callus induction and plant regeneration in rice[z] anther culture

Culture medium	No. anthers inoculated	Callus induction		Plant regeneration	
		No.	%	No.	%
M S	600	58	9.7	15	2.5
Miller	600	148	24.7	3	0.5
N_6	600	208	34.7	23	3.8
N_6–Y_1	600	272	45.3	37	12.3

[z] Rice variety: Nagdongbyeo
Cold treatment: 15 days at 10°C
Growth regulators: Callus induction → NAA 2 mg/l + Kinetin 1 mg/l
Plant regeneration → IAA 0.2 mg/l + Kinetin 1 mg/l
Source: Chung and Sohn 1986[1]

Table 4. Composition of N_6-Y_1 medium for rice anther culture[11]

Components	Content (mg/l)
Macronutrients salt	
$(NH_4)_2SO_4$	231.5
KNO_3	2,830
KH_2PO_4	400
$CaCl_2 \cdot 2H_2O$	166
$MgSO_4 \cdot 7H_2O$	185
Micronutrients salt	
$MnSO_4 \cdot 4H2O$	4.4
$ZnSO_4 \cdot 7H_2O$	1.5
H_3BO_3	1.6
KI	0.8
$FeSO_4 \cdot 7H_2O$	27.85
Na_2 EDTA	37.25
Organic supplements	
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine Hcl	0.5
Thiamine Hcl	1.0
L - Glutamine	265

Under line: Difference between N_6-Y_1 and N_6 medium

Exogeneous growth regulators are also required for callus induction and plant regeneration in rice anther culture. A combination of 2 mg/l NAA (1-naphthalenacetic acid) and 1 mg/l kinetin showed better results than the other growth regulators used, alone or in combination, for callus induction[38]. The effect of ABA (abscisic acid) on plant regeneration has recently been recognized in tobacco anther culture[16]. When rice anthers were pretreated at 10°C for 15 days, a combination of NAA, kinetin and ABA gave a better response, in terms of both callus induction and green plant regeneration, than either NAA alone or a combination of NAA and kinetin[10]. The interaction between exogenous growth regulators, especially with ABA, and cold treatment should be given further study[11]. Most of the callus derived from rice anther culture lost its totipotency rapidly when the callus was more than 20 days old, but the addition of ABA was effective in maintaining the regeneration ability of callus derived from anther for a longer period of time (Fig. 1). These results should contribute to the practical application of this technique on a large scale.

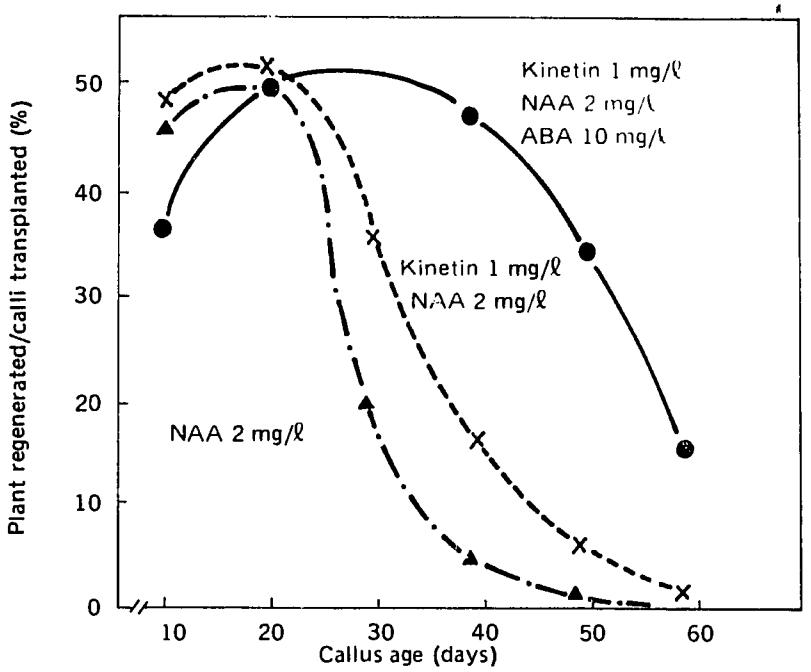

Fig. 1. Relationships between callus age and plant regeneration under various plant growth regulators treatment
Cold treatment: 15 days at 10°C
Source: Chung 1985[10]

In order to increase the frequency of green plant regeneration and save time and labor, a liquid culture method has been developed. In this method, Japonica varieties showed a better response than Indica ones[7]. Callus could be also obtained from panicle culture in a liquid medium[36]. However, normal callus growth was observed only when the anthers were cultured in liquid medium; when panicles and spikelets were cultured, a limited amount of abnormal callus growth was observed[34]. The frequency of callus induction in the liquid media was much higher than in the solid media, but the frequency of plant regeneration in liquid media was much lower compared with that in solid media. Poor rates of regeneration may be due to the insufficient aeration of callus precipitated to the bottom of the liquid media[35]. To improve aeration, potato extract can be added to the medium, or a paper bridge used for liquid culture[19]. Callus induced from a combination of NAA, kinetin and ABA in liquid media showed higher frequency of plant regeneration than when 2.4-D was used (Table 5).

Table 5. Comparison of regeneration efficiency of liquid medium and solid medium used for rice[z] anther culture[34]

Medium	Concentration of growth regulators (mg/l) Callus induction	Plant regeneration	No. anthers inoculated	Calli induced No. (%)	No. calli transplanted	Plant regeneration No. (%)
Solid	2.4-D (2)	Kinetin (1) IAA (0.2)	360	122 (34)	112	23 (21)
	NAA (2) Kinetin (0.2) ABA (4)	"	700	196 (28)	214	82 (38)
Liquid	2.4-D (2)	"	250	455 (182)	455	10 (2)
	NAA (2) Kinetin (0.2) ABA (4)	"	300	612 (204)	612	44 (7)

[z] Rice variety: Nagdongbyeo Basic medium: N_6-Y_1 Cold treatment: 15 days at 12°C

The practical application of liquid culture techniques needs more study. When N_6 medium supplemented with NAA (0.5 ppm) and kinetin (4 ppm) was used for callus induction in one-step culture, the frequency of plant regeneration was higher than when two-step culture was used[18].

Cold Pretreatment

One important factor which can increase culturability in anther culture is the use of various pretreatments before inoculation. Cold pretreatment has become an essential step in rice anther culture since it was first described by Chaleff *et al.* in 1975[1]. Researchers have reported various optimum times and temperatures needed for cold pretreatment[1, 2, 14, 43]. When panicles of Milyang 23 (a Tongil type rice variety) were pretreated at either 6°C or 12°C for 5-25 days, cold pretreatment at 12°C for 15-20 days proved to be most effective in increasing green plant regeneration. Cold pretreatment at 12°C for more than 20 days increased the frequency of albino plants (Table 6). Callus induction from anthers pretreated at 12°C for 20 days occurred earlier compared to the control, although most calli were still induced between 20 and 30 days after anther inoculation (Fig. 2). The effect of cold pretreatment in increasing culturability has been recognized regardless of rice variety, or even in F_1 hybrids[38]. When a lot of anthers are cultured at the same time in a breeding program, cold pretreatment can have an important practical benefit, in terms of saving time and labor. To carry out cold pretreatment, the excised panicle at an appropriate pollen stage should be kept in a jar filled with up to 5 cm water, and stored in a polyethylene bag in the dark to maintain the freshness of the panicles[32]. In tobacco[15] and barley[12], a high proportion of S pollens enhanced the formation of embryoids[13]. It is interesting that rice, the proportion of S pollens in Japonica-type rice varieties is much higher than in Indica. When cold pretreatment was applied in rice anther culture, there was also a correlation between the proportion of S pollen and callus induction frequency.

Table 6. Effect of low temperature treatment on callus induction and plant regeneration in rice[z] anther culture

Low temperature treatment		No. anthers	Callus induced		No. of calli	Green plants regenerated		Albino regenerated		Green plants/ 100 anthers
°C	days	inoculated	No.	%	transplanted	No.	%	No.	%	inoculated (%)
6	5	310	27	8.7	17	2	11.8	6	35.3	0.6
	10	270	4	1.5	2	0	0	1	50.0	0
	15	300	1	0.3	–	–	–	–	–	0
	20	300	0	–	–	–	–	–	–	0
	25	300	0	–	–	–	–	–	–	0
12	5	110	6	5.5	6	4	66.7	1	16.7	3.6
	10	290	47	16.2	41	21	51.2	13	31.7	7.2
	15	270	58	21.5	54	24	44.2	10	18.5	8.9
	20	250	79	31.6	62	34	54.8	20	32.3	13.6
	25	200	58	27.0	54	4	7.4	31	57.4	2.0
Control		300	24	8.0	19	9	47.4	2	10.5	3.0

[z] Rice variety: Milyang 23
Source: Chung and Sohn 1986[11]

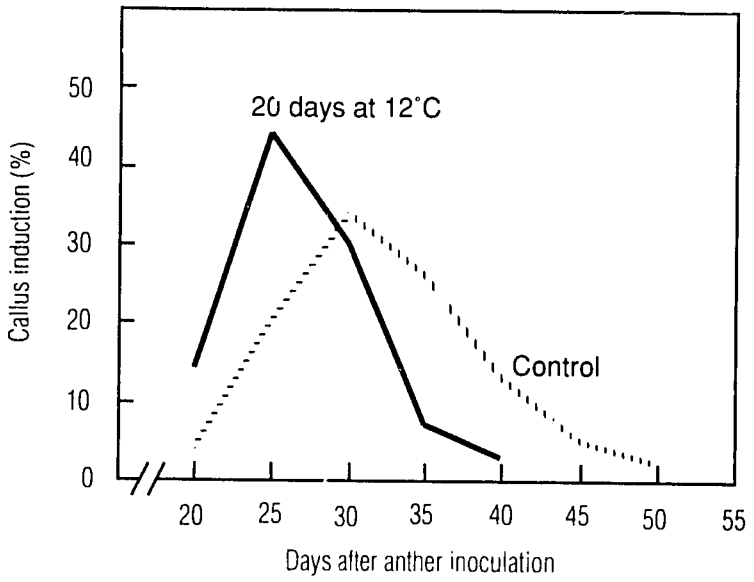

Fig. 2. Percent distribution of callus induction from anther treated for 20 days at 12°C[11]

Genotypic Difference

The success of rice anther culture is also affected by the genotype of the materials inoculated[9, 10, 14]. There are distinct differences in the efficiency of rice anther culture in different species, subspecies or even varieties. Most Japonica type rice varieties have a better response in terms of callus induction than other Indica or Tongil (Indica/Japonica) type rice varieties. There is also considerable variability within each varietal group[10, 11]. The frequency of callus induction and plant regeneration in F_1 hybrids varied according to the parents of the crosses. The culturability of F_1 hybrids was very low when crosses were made between Indica- and Tongil-type rice varieties. If one parent had good culturability, The F_1 hybrids showed a comparatively high level of response (Table 7). Miah *et al.*[22] considered callus formation ability in anther culture of rice to be a heritable character. The success of rice anther culture may greatly increase if selection is made of genotypes with a good response.

Table 7. Comparison of F1 rice plants with parental variety in terms of callus induction and plant regeneration[2]

Material	No. anthers inoculated	No. callus induced		No. plants regenerated			
				Green		Albino	
		No.	(%)	No.	(%)	No.	(%)
Milyang 74/Dashukei 2	6,330	2,155	(34.0)	681	(10.8)	234	(3.7)
Milyang 74	1,550	497	(32.1)	65	(4.2)	55	(3.5)
Dashukei 2	4,370	1,802	(41.2)	304	(7.0)	19	(0.4)
Milyang 23/Sinseonchalbyeo	600	135	(22.5)	8	(1.3)	9	(1.5)
Sinseonchalbyeo	600	141	(23.5)	28	(4.7)	6	(1.0)
Milyang 23	1,870	361	(19.3)	31	(1.7)	9	(0.5)
IR50/Milyang 23	1,710	103	(6.0)	5	(0.3)	8	(0.5)
IR50	1,110	30	(2.7)	0	–	0	–

[2] Cold treatment: 15 days at 10°C
Source: Chung and Sohn 1986[11]

PLOIDY LEVEL OF POLLEN-DERIVED PLANTS

Even if the main objective of anther culture is to get haploid plants, in practice plants with various levels of ploidy are regenerated. The proportion of haploid to diploid plants is much the same in various reports concerned with the ploidy level of pollen-derived rice plants. Variation in the ploidy level ranges from n to 5 n[24]. Many factors, such as the developmental stage of the inoculated anthers, the use of cold pretreatment[37, 39], the type of medium[11, 17], and genotype[34, 38], as well as callus age, affect the ploidy level of plants derived from anther culture. The proportion of haploid plants increases significantly if cold pretreatment is used before anther inoculation (Table 8). When cold pretreatment is used, the inclusion of 2,4-D, kinetin and NAA, or a combination of kinetin and NAA, increased the proportion of haploids, but a combination of ABA, kinetin and NAA increased the proportion of diploids (Table 9). The origin of some diploids, polyploids and aneuploids is known to be the result of division of the microspore itself, or of callus cells *in vitro*, but the process is still unclear[21]. To apply rice anther culture techniques to varietal improvement, haploid plants must be diploidized by various chromosome doubling methods. These are essentially spontaneous diploidization by tiller separation, leaf sheath injection, or submergence in colchicine[20, 27, 42]. The frequency of spontaneous diploidization by tiller separation is 2.7%, while treatment with 0.2% colchicine for 12 hours produced 23% diploidization, even though some tillers were killed by toxicity (Table 10). More effective diploidization methods should be developed as soon as possible, in order to shorten the breeding cycle.

Table 8. Effect of low-temperature treatment on the ploidy level of anther-derived rice plants

Treatment	Variety	No. plants	Ploidy level (%)		
			Haploid	Diploid	Polyploid
Cold treatment (15 days at 10°C)	Nagdongbyeo	213	70.4	24.4	5.2
	Milyang 74	110	68.2	30.0	1.8
	Total	323	69.7	26.3	4.0
Control	Nagdongbyeo	128	46.9	43.0	10.1

Source: Chung and Sohn 1986[9]

Table 9. Effect of plant growth regulators on the ploidy level of anther-derived plants of rice[z]

Growth regulators (mg/l)	No. plants	Ploidy level (%)		
		Haploid	Diploid	Polyploid
2,4-D (2)	32	68.7	21.9	9.4
Kinetin (1)	89	64.1	29.2	6.7
NAA (2)	38	79.0	10.5	10.5
Kinetin (1) + NAA (2)	72	72.2	22.2	5.6
Kinetin (1) + NAA (2) + ABA (10)	50	38.0	54.0	8.0

[z] Variety: Nagdongbyeo
Cold treatment: 15 days at 10°C
Source: Chung and Sohn 1986[9]

Table 10. Diploidization of haploid rice plants induced from anther culture

Treatment	No. haploid plants treated (A)	No. diploid plants Total (B)	From tillers (C)	From spikelets (D)	(%) B/A	C/B	D/B
Colchicine							
Injection (0.1%)	128	24	8	16	18.8	33.3	66.7
Submerged for 12 hours (0.2%)	784	180	68	112	23.0	37.8	62.2
Tiller							
Separation	600	16	0	16	2.7	0	100

Source: Chung and Sohn 1986[11]

VARIABILITY OF POLLEN-DERIVED PLANTS

Many scientists working in the field of rice anther culture have pointed out that further work is needed on genetic variability of plants derived in this way, before the segregation and recombination of desirable characters can be used in rice improvement[44]. The uniformity of pollen-derived plants is reflected by the fact that roughly 90% of regenerated plants showed genetical homozygosity in the subsequent population. Some variants gave important information on plant genetics and breeding[6, 28]. In most A_2 lines, more than 90% of subsequent generations of pollen-derived plants were much the same as the donor variety in terms of phenotypic appearance, while 6-8% of A_2 lines showed a change in phenotypic pattern without segregation, while segregation within the line occurred in a small proportion of lines (Table 11)[38]. Schaeffer[29] has recognized the occurrence of variants with a shorter culm than the donor variety, Calrose 76.

Table 11. Phenotypic patterns of the A_2 generation derived from two varieties by rice anther culture

Variety	No. A_2 lines	A_2 lines in given phenotypic pattern: No segregation, Similar No.	(%)	No segregation, Dissimilar No.	(%)	With segregation No	(%)
Milyang 74	49	46	(93.9)	3	(6.1)	0	–
Dashukei 2	98	89	(90.8)	8	(8.2)	1	(1.0)

Discussions on the advantages or disadvantages of haploid breeding through anther culture are also concerned with the haploids that can be obtained from a heterozygous F_1. Generally, it is well known that haploid breeding gives faster progress in varietal improvement because pollen-derived plants originate from a haploid microspore, so that phenotypes coincide with genotypes in diploidized pollen-derived plants, without any interference from recessiveness covered by dominance. The segregation mode of major agronomic characters such as days to heading, and panicle length in both pollen-derived A_2 lines and the F_2 population derived from Milyang 74/ Dashukei 2 F_1 hybrids was similar to a normal distribution curve (Fig. 3). The distribution frequency of culm length in A_2 lines and the F_2 population derived from hybrids of variety Milyang 74, with a short culm, and Dashukei 2, with a tall culm, could be divided into two parental groups.

The ratio of tall plants to short ones was 3:1 in the F_2 population, and 1:1 in the A_2 lines. The short culm of Milyang 74 was controlled by a single recessive gene (Table 12). This shows that the selection efficiency of recessive genes can be increased by haploid breeding through anther culture.

The segregation mode of the culm length of a remote cross, Singwangbyeo/Eunhabyeo, appeared to follow a normal distribution curve, although the average culm length in A_2 lines was shorter than that of the F_2 population[38]. A comparison was made of major agronomic characters controlled by multiple genes in A_2 lines and F_2 populations[5,6]. It would appear that the selection efficiency of quantitative characters, will be equal in both populations, if sufficient pollen-derived plants are evaluated[6].

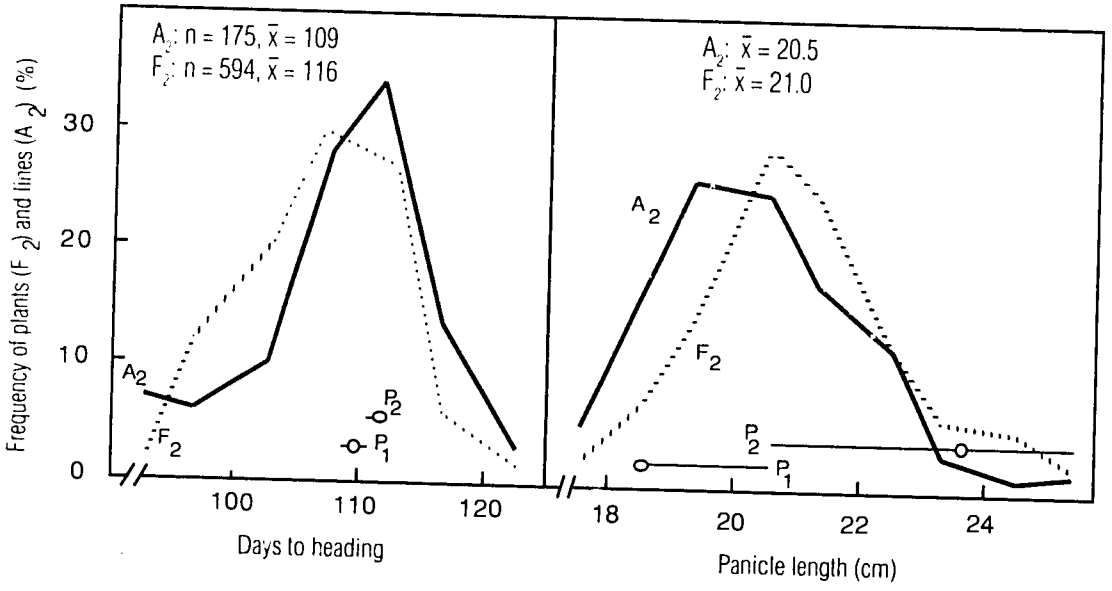

Fig. 3. Distribution of days to heading, and panicle length of F_2 and A_2 derived from Milyang 74/Dashukei 2

Table 12. Segregation of culm length in A_2 and F_2 populations derived from Milyang 74/Dashukei 2

Generation	Culm length		Total	Ratio	X^2	P
	Tall	Short				
A_2	98	77	175	1:1	2.520	.10–.25
F_2	466	128	594	3:1	3.773	.05–.10

In rice improvement, the most deleterious barrier to making remote crosses is sterility. The fertility of pollen-derived A_2 lines derived from F_1 hybrids of a remote cross was quite different to that of an F_2 population. The fertility of the A_2 lines had a narrower range, but was higher than that of the F_2 population (Fig. 4). These A_2 lines which demonstrated higher fertility will supply important material for remote cross breeding[38].

There are no variants with leaf blade pubescence in A_2 lines derived from the original variety, which had a glabrous leaf blade[38]. When an F_1 hybrid was bred from Milyang 74, with a pubescent leaf blade, and Dashukei 2, with a glabrous one, the ratio of pubescent to glabrous was 3:1 in the F_2 population, and 1:1 in A_2 lines. The glabrousness of Dashukei 2 is controlled by a single recessive gene (Table 13). There are no variants in terms of reaction to bacterial leaf blight (pathotype K_1), in A_2 lines derived from the original variety resistant to this pathogen. When a F_1 hybrid was bred from resistant and susceptible parents, the ratio of resistant plants to susceptible ones was 3:1 in the F_2 population, and 1:1 in A_2 lines. Resistance to bacterial leaf blight (pathotype K_1) in Milyang 74 was controlled by a single dominant gene (Table 14). These results on the type of gene coincide with the results of Chen *et al.*[4] in their segregation of lines with a narrow and/or liguleless leaf.

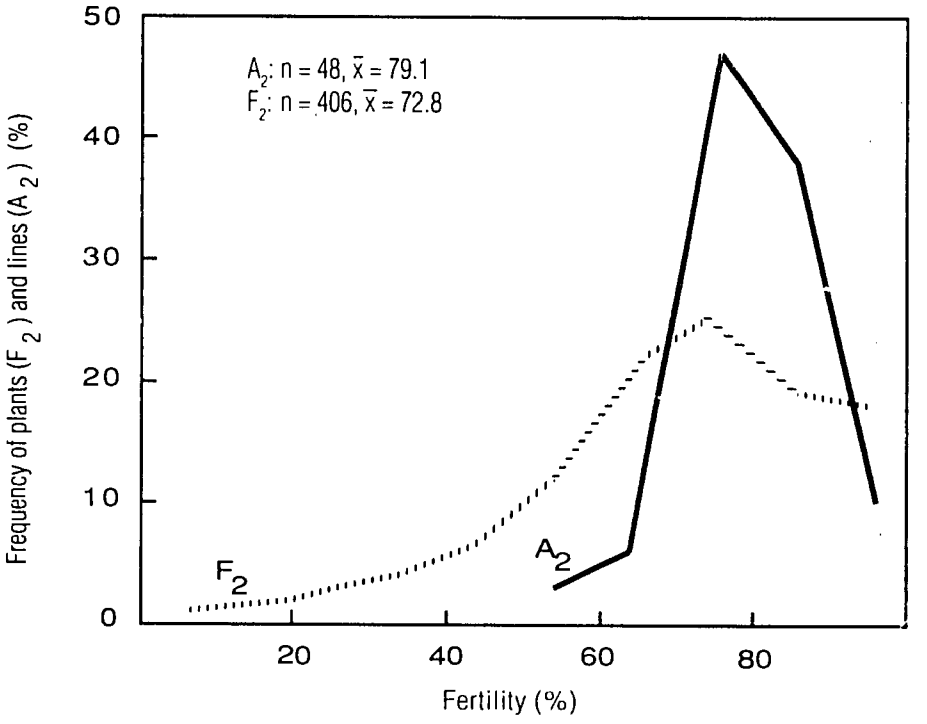

Fig. 4. Distribution of fertility of F_2 and A_2 lines derived from a Singwangbyeo/Eunhabyeo cross

Table 13. Segregation of leaf blade pubescence in A_2 and F_2 derived from Milyang 74/Dashukei 2

Material	Generation	Leaf blade		Total	Ratio	X^2	P
		Pubescence	Glabrous				
Milyang 74		*					
Dashukei 2			*				
Milyang 74/Dashukei 2	A_2	96	83	179	1:1	.9441	.25–.50
	F_2	439	155	594	3:1	.3793	.50–.75

* Characteristic of parental variety

Table 14. Segregation of resistance to bacterial leaf blight, pathotype K_1 in A_2 and F_2 derived from Milyang 74/Dashukei 2

Material	Generation	Reaction to BLB (K_1)		Total	Ratio	X^2	P
		Resistant	Susceptible				
Milyang 74		*					
Dashukei 2			*				
Milyang 74/Dashukei 2	A_2	97	82	179	1:1	1.2570	.25–.50
	F_2	457	137	594	3:1	1.1874	.25–.50

* Reaction of parental variety

APPLICATION OF ANTHER CULTURE IN RICE IMPROVEMENT

Rice breeders attempt to develop high-yielding rice varieties with resistance to diseases and insects, and with stress tolerance. However, the accumulation of various desired characteristics into any one variety is almost impossible to achieve within short breeding cycles. However, anther culture has made possible the development of desired characteristics within short breeding cycles[30], and the selection of new lines from interspecies F_1 or F_2[41], so that it is now used for breeding commercial rice varieties[44]. Recent progress in rice anther culture at the Yeongnam Crop Experiment Station (YCES), Korea, has increased the frequency of plant regeneration from 0.1% in 1982 to 5.6% in 1986 (Table 15), and already two promising elite lines are ready for field testing, to determine their suitability for local conditions. More than 2,000 pollen-derived lines will be tested in the next year in observational yield trials at YCES. Milyang 90, a Japonica-type rice variety derived from anther culture, has shown resistance to brown planthopper (Bph) and stripe virus disease (SV), and has good grain quality. In producing these desired characteristics,the breeding cycle of Milyang 90 took only four years, at which point it was ready for field testing (Fig. 5).

Rice improvement through anther culture needs further detailed study on the artificial control of ploidy level, and of albino plants occurring *in vitro*, as well as improvement of the culturability of Indica type rice varieties.

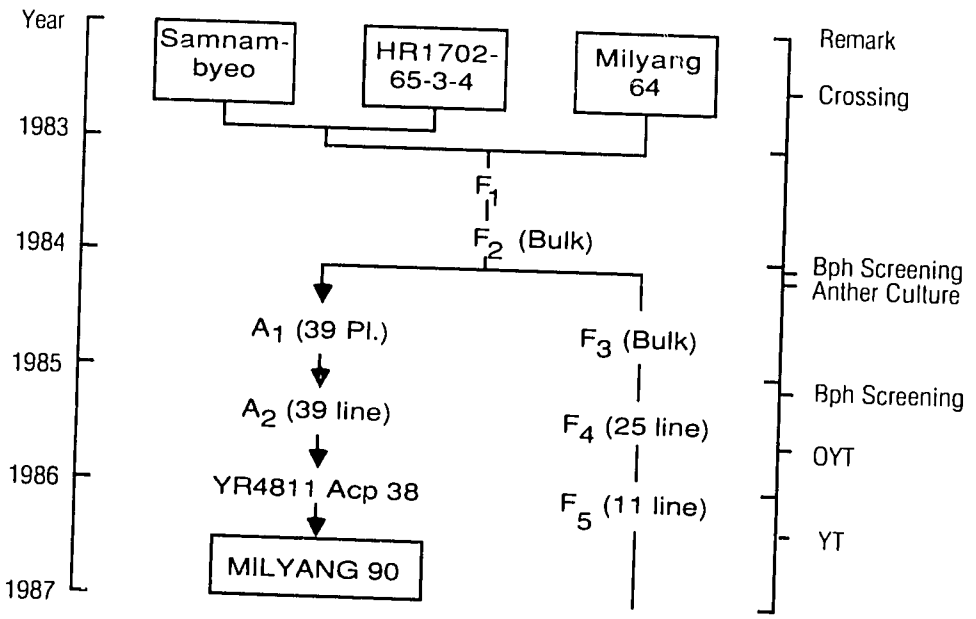

Fig. 5. Breeding of promising new rice line, Milyang 90, through anther culture

Table 15. Recent progress of rice anther culture at YCES*

Year	Culturability and performance	
	Green plant/100 anthers (%)	No. of anther-derived lines
1982	0.1	83
1983	2.5	296
1984	3.1	606
1985	5.0	1,950
1986	5.6	2,693

* YCES: Yeongnam Crop Experiment Station, RDA, Milyang, 628-800 Korea

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DISCUSSION

Q. (D.A. Somers)
How many varieties of rice have been developed through anther culture so far?

A. In Korea, two varieties and three good lines are available at present.

Q. (M. Vajrabhaya)
When you get the haploid plant, how do you double the chromosomes to achieve the normal diploid plant?

A. Haploid plants are usually treated with colchicine. Sometimes we separate the material and plant it directly in the field. We then obtain about 2.7% diploid plants by spontaneous diploidization.

Q. (F.J. Zapata)
For the selection of salt tolerant lines by means of tissue culture, after you have obtained salt tolerant lines *in vitro*, how many generations do you think we should select to make sure that the selected line has true resistance?

A. As you know, it is possible to select salt tolerance lines *in vitro*. However, it is impossible to obtain salt tolerant mutants without any changes in other morphological characters.

Q. When you select anthers, you ascertain whether it is at an optimum inoculation stage by analyzing the position of the flower. I understand you look for one third chrome, at the bottom of the spikelet?

A. We first look at the flag leaf and second leaf, when we select and cut the material. However, I must emphasize that there are many variables, which differ according to the variety, whether the variety is Japonica or Indica, etc.

SOMATIC MUTATION IN RICE TISSUE CULTURE

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ABSTRACT

Various tissue culture techniques have been developed in rice tissue culture over the past ten years. The development of pollen culture, protoplast culture and somatic embryogenesis is the most effective approach for improving the breeding methods for rice by means of anther culture, in vitro selection, cell fusion, propagation and transformation.

However, genetic unstability in tissue culture soon became a well known phenomenon for many plant species. The use of this somatic mutation is a promising technique to increase the range of genetic variability in rice breeding. Somatic mutation is frequently induced in cultured rice tissues. Among these somatic mutants, useful mutants are often observed. Furthermore, cultured rice tissue is a good material for somatic cell genetics. It is possible to carry out genetic analysis of regenerated plants and their offspring, as well as molecular studies. Rice mutants with unusual genetic behavior have been obtained by means of tissue culture.

INTRODUCTION

Plant tissue culture techniques for various plant species have developed rapidly during the past ten years. These techniques, from a single cell level to an organ level, are useful not only for applied purposes, but also for studying basic sciences in the plant. One potential application of particular interest is plant cell genetics based on single cells. The control of totipotency of plant cells is a great advantage in this respect.

Cultured plant cells are often habituated to cultural conditions, and show alterations in their nutrient or auxin requirements and lose totipotency. Furthermore, an increasing number of experiments indicate that the chromosome number and structure are highly variable. Phenotypic variations in regenerated plants from cultured cells have been known to occur since the late 1960's. In rice, the presence of abnormal or variant regenerated plants has been documented since the first success in plant regeneration, e.g., morphological variants from seed callus[4], and albinos[2] and polyploids[3] from pollen callus. Albinos, and morphological variations in plant height and panicle length, were also observed in anther culture studies. Variations in plant height and panicle length were also observed in regenerated haploid plants obtained from a single pollen callus[5].

We therefore planned to clarify the nature and causes of variations in cultured rice cells, as well as in regenerated plants, in order to develop means of both broadening genetic variability and establishing genetically stable cell lines.

GENOME MUTATION

Rice callus tissues are unstable at a chromosomal level. For instance, calli of anther culture from the F_1 plants of a cross Arborio x Loctjan were examined. These calli were induced on 2,4-D or NAA (5×10^{-6}M each) media. The calli weighed 1,000 mg each, and showed very wide variation in chromosome number. The frequencies of haploid cells were 34.5% in the calli induced on 2,4-D medium, and 31.1% on the NAA medium. The frequencies of diploid cells were 42.5% and 25.9% in the two different media, respectively. The highest chromosome number observed was 114.

In one callus, 8 different chromosome numbers between 10 and 36 appeared, and in another callus 15 between 12 and 144 were found. These

results suggest that cell divisions in pollen calluses are not always normal, and that polyploid and aneuploidy are common. It appears that the culture conditions do not exert strong negative selection pressures on abnormal cells or chromosomal mutants[5].

Chromosomal observations of regenerated plants from rice anther culture were also made. 40% were found to be in a haploid state, and 48.5% in a diploid. A group working in Mainland China obtained the same responses: 60% of about 1,000 pollen plants obtained from F_1 anther culture were found to be diploid, 35% haploid and 5% triploid or aneuploid[5]. In addition, pentaploid rice plants were also obtained from anther culture[3].

Chlorophyll deficient plants, especially albinos, were frequently regenerated from pollen callus. Albinos appeared not only as haploid but also as diploid and triploid plants.

In wild rice species, *O. perennis*, some triploids have been obtained[5].

GENE MUTATION

One thousand, one hundred and twenty-one D_1 plants were regenerated from 75 homozygous seed calluses. Eighty-three D_1 plants were studied, and their plant height, flowering date, morphological features and seed fertility at maturity were recorded. Plant height of the D_1 plants ranged between 77 and 124 cm. Heading date of the D_1 plants ranged between Aug. 9 and Sep. 1.

Seed fertility of the randomly investigated 489 D_1 plants varied widely. Seed fertility was less than 80% in 58.7% of the D_1 plants, and less than 40% in 18.2% of the D_2 plants. Morphological changes of some characters, e.g., grain density and long awn, were also observed.

Chimerism of D_1 plants in seed fertility (difference in seed fertility of more than 20% between panicles on the tallest two culms on one D_1 plant) and in chlorophyll deficiency was also observed, with a frequency of 23.7% and 29.3%, respectively. One of the twelve tetraploids was a 2x – 4x chimera, bearing one 4x panicle among 10 panicles.

The agronomic characters of successive generations (D_2, D_3 . . .) of these D_1 plants were further investigated. Heading date of the 6,382 D_2 plants fluctuated between Aug. 19 and Oct. 7. Early- and late-heading variants were observed, including D_2 plants which had a heading date 20 days later than the control. Plant height of the D_2 plants ranged between 29 and 135 cm. There was a distinct shift to low plant height in the D_2 population, and 4.1% of D_2 plants had a plant height of less than 90 cm. Plants less than 50 cm tall were mostly malformed and/or completely sterile. The difference in the mean heading date and plant height between the D_2 and the control was significant at a 1% level. Seed fertility of the D_2 varied from 99.2% to 0%. The frequency of D_2 plants with seed fertility of less than 80% or 40% was 19.5% and 5.0%, respectively. Completely or partially sterile plants showed various types of pollen abortion, such as completely shrivelled pollen, incomplete accumulation of starch, or combinations of these while others had normal pollen, and pollen of normal and small size.

D_1 panicle progenies were examined for the presence of segregation of chlorophyll-deficient mutants in the D_2 generation. Chlorophyll mutants segregated in 34 out of the 438 tested panicle progenies. This mutation frequency, 8.4%, was comparable to the mutation frequencies in M_2 following X-ray or γ-ray irradiation of rice seed. Segregation frequency of chlorophyll mutants in the D_2 lines (D_1 panicle progenies) was higher than 20% in 22 (64.7%) of the 34 mutated D_2 lines.

Among 6,328 D_2 plants at maturity, 1,491 plants were recorded as mutants in some agronomic characters. The frequency of mutation, excluding the lethal chlorophyll mutants, was 23.4%. Table 1 shows the number of D_2 lines segregating such mutants, in addition to those of polyploids, mutants showing drastic morphological changes, and viable chlorophyll mutants. As the Table indicates, many D_2 lines did not segregate any mutants. The average number of mutant characters per D_1 panicle (D_2 line) was 1.08 (826/762) when five characters were examined. The results suggest the frequent induction of mutations in the process of callus induction up to D_1 plant regeneration.

Mutants selected for chlorophyll deficiency, plant height, heading date, etc., were true-breeding in the D_3. The breeding behavior of eight mutants showing drastic changes was investigated in crosses between the mutants and the original varieties. F_2 segregation ratio patterns of six of the mutants fitted the 3:1 ratio, indicating that the mutations were controlled by single recessive genes[7].

HOMOZYGOUS MUTATIONS

Ten seed calli from a panicle of the same plant were obtained after 22 days of culture on Miller's medium supplemented with 10^{-5}M 2,4-D. These calli were subcultured on medium of the

Table 1. Changes of characters of mutants in D_2 lines derived from panicles of D_1 plants regenerated from rice calli

Mutated characters	No. of lines	%
Normal	214	28.1
Ploidy (4x)	12	1.6
Fer	273	35.8
Ht	19	2.5
Hd	3	0.4
Mor	1	0.1
Ch	27	3.5
Fer and Ht	74	9.7
Fer and Hd	14	1.8
Fer and Mor	7	0.9
Fer and Ch	59	7.7
Ht and Ch	3	0.4
Hd and Ch	1	0.1
Mor and Ch	1	0.1
Fer, Ht, and Hd	11	1.4
Fer, Ht, and Mor	2	0.3
Fer, Ht, and Ch	27	3.5
Fer, Hd, and Ch	2	0.3
Fer, Mor, and Ch	1	0.1
Fer, Ht, Hd, and Mor	1	0.1
Fer, Ht, Hd, and Ch	9	1.2
Fer, Ht, Mor, and Ch	1	0.1
Total	762	100

Abbreviations for the mutated characters are as follows: Fer, seed fertility; Ht, plant height; Hd, heading date; Mor, morphological traits; Ch, chlorophyll deficiency

same composition, but containing 10 g/l NaCl. Two calli (NA-2, NA-3) were selected after three subcultures (123 days of culture in total, including 101 days on media containing NaCl). Sixty-nine D_1 plants regenerated from three calli (NA-1, NA-2, NA-3) were grown to maturity. Of 37 plants regenerated from callus line NA-3, 33 plants showed a dwarf trait. A genetic analysis of progeny of putative homozygous dwarf mutants derived from NA-3 callus has been carried out. There were no significant differences in heading date, pollen fertility or seed fertility between putative homozygous mutants and plants of normal height regenerated from NA-3 callus. However, the culm length and panicle length of the putative homozygous mutants decreased, and the number of grains produced by these plants was smaller than normal. The dwarf trait was expressed from the seedling stage to maturity, and all internode and panicle lengths were therefore shorter than those of normal plants.

In the second generation, D_2 progenies of 36 D_1 plants examined showed significant differences in height. The frequency distribution of plant height in 744 D_2 plants was examined. All 1,143 D_2 plants in four D_2 lines from plants of normal height were also of normal height. However, all of the 24 D_2 lines, including 441 D_2 plants produced by dwarf D_1 plants, were also short, as if the trait resulted from a cytoplasmic mutation or a nuclear mutation for which the D_1 plants were homozygous. Seven D_2 lines segregated 1 or 2 normal individuals, to yield 8 normal and 152 dwarf plants in total. One D_2 line segregated 7 normal and 22 dwarf plants. In the third generation, all 11 D_3 lines derived from normal D_2 lines or from normal segregants showed normal traits. However, all 24 D_3 lines (938 D_3 plants) produced by dwarf D_2 plants were dwarf. Six D_3 lines from dwarf D_2 plants segregated only one to three normal plants. The overall frequency of normal plants was 4.1% (10 normal/235 dwarf). One D_3 line included 41 plants of medium plant height (90.0 ± 3.6 cm, range 84-100 cm). In the fourth generation, 57 D_4 lines were examined: a 31 D_4 lines from normal or revertant D_3 plants were normal. In 21 D_4 lines derived from short D_3 plants, the dwarf trait was fixed. Four D_4 lines from dwarf D_3 plants segregated normal plants (12.8%). Transmission of the dwarf trait was stable, with a similar low frequency of segregation of normal revertants in the subsequent four generations through to D_8.

Putative homozygous mutant D_3 plants were analyzed by crossing. The dwarf trait was expressed by F_1 plants produced by crosses between two dwarf plants e.g. 9Na57-1 x 9Na56-1. The dwarf trait was transmitted to the F_2, F_3, and F_4 generations of these crosses. The frequency of normal plants in the F_2 was 3.8% (14N/351S). The normal stature trait was transmitted to the F_3 and F_4 without segregation.

The dwarf trait was not expressed at all by the F_1, F_2, F_3, and F_4 progenies derived from reciprocal crosses of dwarf plants with the control (N8P5) or with the progeny of the normal regenerated plants, such as 9Na107-13 (normal D_3 plant), derived from the same callus. Although a few short plants were observed in the F_2 generation, this distribution was within the normal range for normal plants. Moreover all subsequent progeny of these plants were normal. Plant height is a quantitative trait. However, the F_3 data did not indicate that the plant height of the F_2 had any

influence on the plant height of the F_3 lines. These results indicate that reversion from the putative homozygous mutation occurred in the F_1 generation[6].

DNA AMPLIFICATION AND DIMINUTION

Changes in the differentiation and dedifferentiation processes at a DNA level were analyzed. To obtain information about DNA changes during callus induction, rice nuclear DNAs from embryo and 30-day-seed callus were digested with several restriction enzymes. Of these enzymes, BamHI showed a clear difference between embryo and callus DNA. Callus DNA showed a pattern of many discrete bands on a smeared background. These bands suggest that some DNA sequences were amplified during callus induction. To elucidate the mechanism of the DNA amplification, we have tried to identify those DNA fragments that were amplified. DNA fragments corresponding to banks in the region between 3.0kb and 1.0kb were extracted from embryo DNA, and cloned into bacterial plasmid pBR325. Recombinant plasmids containing rice nuclear DNA sequence were named pRB. Fifteen high copy number clones were selected and purified. Southern blot hybridizations of BamHI digested embryo and callus DNAs were performed, with the ^{32}P-labelled cloned DNAs as probes. The fragments contained in plasmids pRB301 and pRB401 showed very interesting patterns. The bands in embryo and callus DNA that hybridized with pRB301 were the same size those of the cloned fragment (1.7kbp), but their intensities differed. That is, hybridization of callus DNA with pRB301 was ten-fold stronger than that of embryo DNA with this fragment. Even more dramatic differences were discovered between hybridized sequence (1.4kbp) in embryo DNA, which was estimated to be ten thousand per haploid genome, while no hybridization signal was detected in callus DNA. The limit of detection of the experimental methods employed is between one and five copies per genome. By using a probe of higher specific activity, a weak hybridization signal could be detected even in callus DNA. Copy number changes were estimated quantitatively by the co-hybridization experiment.

The fragments cloned in pRB301 and pRB401 originated from embryo nuclear high copy number sequences that change in copy number between embryo and callus DNA.

Because plants can be regenerated from rice callus by changing the medium conditions, copy numbers of the DNA fragments cloned in pRB301 and pRB401 were studied during redifferentiation. Several seed calli were transferred to a regeneration medium, and 30 days later shoots and roots were regenerated. Nuclear DNA was extracted from shoots approximately 5 cm in length. For the preparation of nuclear DNA from regenerated shoots, embryos and callus were digested with BamHI and hybridized with the ^{32}P-labelled fragments cloned in pRB301 and pRB401. pRB301 hybridized with regenerated shoot DNA and embryo DNA with almost same intensities, which were lower than the intensity of hybridization with callus DNA. Furthermore, pRB401 hybridized to DNA prepared from regenerated shoot and embryo with the same intensity, but also hybridized only weakly with callus DNA. These results suggest that the copy numbers of both clones also change during redifferentiation, and that in regenerated plants the copy numbers of these cloned fragments return to the level present in the embryo. Therefore, the copy number changes of the fragments cloned in pRB301 and pRB401 were reversible during dedifferentiation[1].

Table 2. Somaclonal variation in tissue culture

Type of variation	Observed in		Heritability
	Callus	Regenerated plant	
Physiological variation	Yes	Yes	No
Genome mutation	Yes	Yes	Yes
Chromosomal mutation	Yes	Yes	Yes
Gene mutation	Yes	Yes	Yes (Mendelian)
Plasmon mutation	Yes	Yes	Yes (Maternal)
"Homio" mutation	not Det	Yes	Yes (non-Mendelian)
DNA amplification and diminution	Yes	Yes	not Det

CONCLUSION

Somaclonal variation in tissue culture is summarized in Table 2. Some of these phenomena were first discovered and studied in rice and therefore cannot be assumed to apply generally to other plant species. However, it is reasonable to expect that similar phenomena will soon be observed in other plant species in the process of dedifferentiation and redifferentiation. We are rapidly improving tissue culture techniques for many plant species. These improvements include the manipulation of protoplasts, pollen culture, transformation, etc., which support the rapid development of somatic cell genetics in plants.

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DISCUSSION

Q. (G.S. Chung)
Some mutations, such as short stature in plants, may be due to some other genetic factor, and bring changes in other factors besides small size. Do you think that more than one character may be involved in short size of plants?

A. With regard to short stature in plants, only in the case of homozygous mutation is a single character involved. If it is not homozygous, other factors may be involved.

Q. (H. Uchimiya)
Dr. Oono, you are getting good results from your work with protoplast culture of rice. How does your system compare with that for corn?

A. Formerly we had considerable problems, but recently we have found it possible to regenerate plants from protoplasts. I am beginning to feel that the culture period may not be a major problem. Some ten years ago, I was convinced it was impossible to regenerate plants when cultures were repeatedly subcultured. I have now changed my mind, and feel that plants can be regenerated after subculturing.

We have also tried to establish an embryogenic system. We have recently published a study of 500 varieties, which found that the ability to form the embryogenic state is highly variable between varieties.

Section III.

Tissue Culture of Woody Plants

COTYLEDON TISSUE CULTURE AND SHOOT BUDS ON ROOTS IN *CITRUS*

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ABSTRACT

Tissue culture has proved to be a useful method for Citrus crop improvement and propagation. However, very little work has been done on the indigenous species of S.E. Asia. Cotyledonary tissues and the smaller embryos of Citrus microcarpa, C. limon, C. sinensis and Fortunella polyandra were cultivated on MS basal medium with NAA and kinetin. The development of both callus and root was common, although the cultures responded variably to different concentrations and combinations of growth substances. Whole embryos of C. microcarpa could germinate on basal medium alone, but NAA was helpful in callus induction. Shoot buds developed on roots in 3 months old cultures of C. grandis, C. limon and C. microcarpa. Nucellus and leaf segments did not respond well in vitro. The usefulness and the feasibility of the results are discussed, with reference to clonal improvement and the mass production of plantlets. The present position of Citrus in S.E. Asia and the potential and practical applications of tissue culture to improve the Citrus industry are briefly discussed.

INTRODUCTION

Considerable research data has accumulated on the *in vitro* culture of *Citrus* species, and most of the important findings have been reviewed[7, 9, 11]. *Citrus* is a major fruit crop, and the usefulness of tissue culture methods for crop improvement and the elimination of pathogens in certain species are well documented[3, 9]. In view of the vast number of species and cultivars involved in the *Citrus* industry, and considering the commercial possibilities and possible economic gains, much more research needs to be done. Very little research has been done on species indigenous to Southeast Asia. The species available in this region are good genepool materials, that can be used for studies on hybridization, protoplast fusion and somaclonal variation. This paper reports on *in vitro* regeneration potential of the cotyledons and root tissues of four *Citrus* species and *Fortunella polyandra* – a *Citrus* ally.

MATERIALS AND METHODS

Plant materials used in the experimental studies included *Citrus limon* (Linn.) Burm. f., *C. microcarpa* Bunge, *C. sinensis* Linn. Osbeck var. Liu Ching and *Fortunella polyandra* Ridl. Tanaka (Fig. 1a-d). All plant materials were obtained from a local fruit farm in Singapore. Both vegetative and reproductive structures were used as explants. Intact seeds were surface sterilized in 20% v/v commercial Chlorox solution for 15 minutes. In nucellus tissue culture experiments, mature fruits were surface sterilized with 25% Chlorox for 25 minutes. In leaf tissue culture experiments, leaves were washed, rinsed and soaked in 20% v/v Chlorox for 15 minutes. All the materials were then rinsed five or six times in sterile water. After the seed coats were removed, the embryos were separated. The whole embryo, whether small or large, or part of the cotyledon (0.3 cm x 0.3 cm) was used as the explant, and cultured separately to determine the relative growth potential of the tissues from different sized embryos. The nucellus was isolated by removing the integuments, exposing a translucent white, puffy tissue. This was cut for inoculation. Of the vegetative tissue, only the lamina was used. 0.5 cm x 0.5 cm segments were inoculated lying

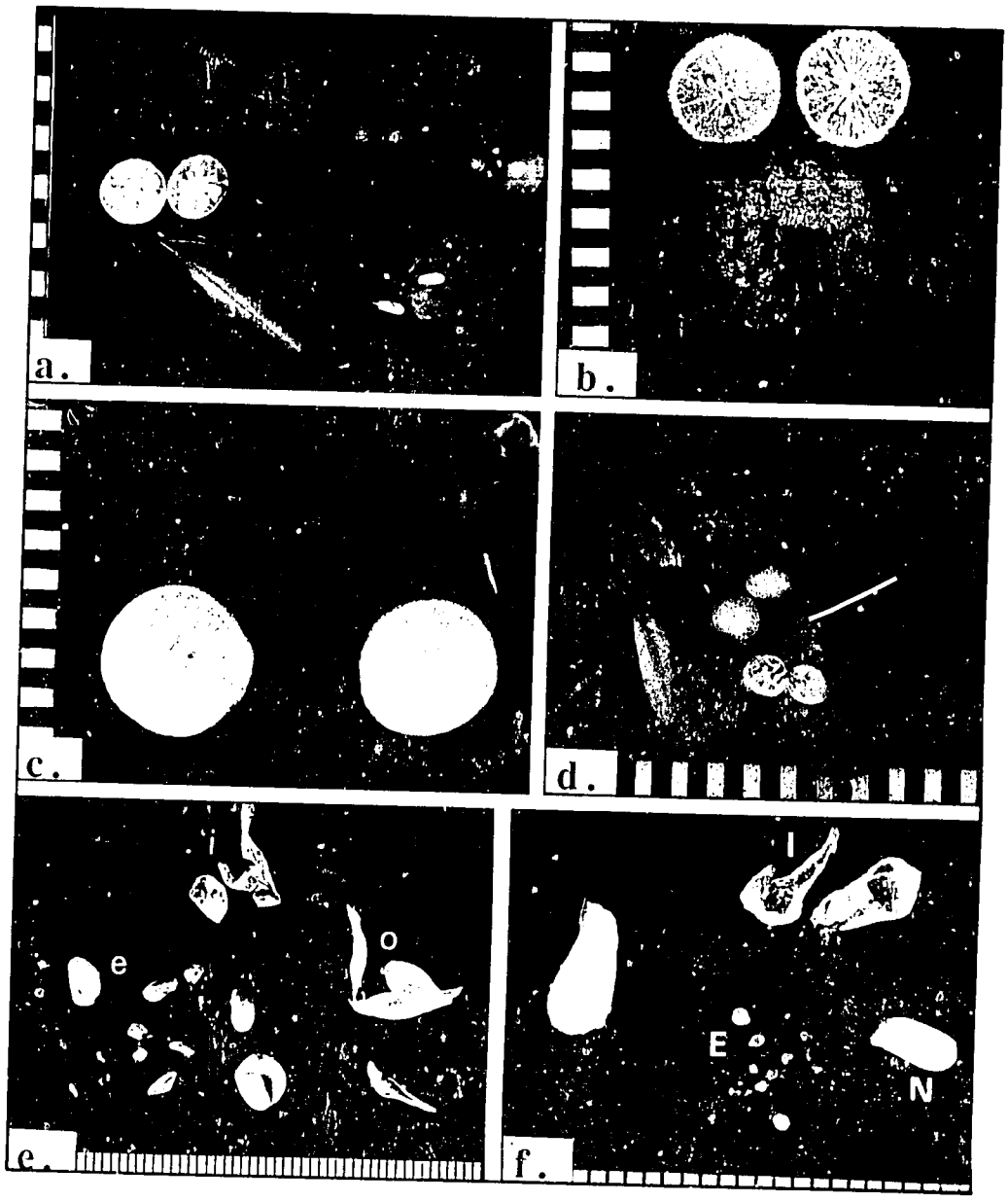

Fig. 1 **a-d** Plant materials of *Citrus microcarpa*, *C. limon*, *C. sinensis* and *Fortunella polyandra* (cm scale)
e,f *C. microcarpa*. Mature and immature seeds dissected to remove embryos (e), outer (o) and inner (i) seed coats. In **f** whole immature seed is on left, integuments (I), fleshy nucellus (or) and embryos (E) (mm scale)

flat, with either the lower or the upper epidermis in direct contact with the medium. Nodal and internodal segments were also cultured.

The basal medium used was that of Murashige and Skoog[7] (MS), with 2-4% sucrose; casein hydrolysate (400 mg/l) and coconut water (10% w/v) were added separately. The growth regulators used included α-Naphthalene acetic acid (NAA), Kinetin (K), 6-Benzylaminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Indole-3-acetic acid (IAA). These were added either individually or in combination, in varying concentrations ranging from 0-10 mg/l. The pH of the medium was adjusted to 5.8 before the addition of agar, and the medium was gelled with 0.8% Difco Bacto agar. All cultures were maintained in the culture room at a temperature of 24°C ± 2°C, and with a light intensity of 45 $\mu Em^{-2}s^{-1}$ for 18 hours followed by six hours of darkness. Standard micro-technique methods were followed for microtoming the tissues and staining the sections[9].

OBSERVATIONS AND RESULTS

The size and shape of the seeds of some of the species investigated are illustrated in Fig. 2. Details regarding the average seed length, number of embryos per seed, approximate size of the largest embryo in each seed, and the color of the chalazal end of the inner seed coat and cotyledon are presented in Table 1 (Fig. 1e-f). In all the experiments, two types of cotyledon tissue explants were cultured separately. The first comprised segments excised from one of the largest embryos, and the second, segments excised from one of the smaller embryos, about two sizes larger than the smallest available embryo (Fig. 3a-d).

A general morphological description of growth and differentiation of cotyledon tissue in culture is given first, followed by histological details of the tissue, and finally the effects of growth substances on tissue or organ growth.

After about four days in culture, the cotyledon explants appeared to be greener and slightly swollen. The callus tissue developed between five and seven days after inoculation, mostly on the cut surface of the explants and on the regions embedded in the culture medium. Depending on the composition of the medium, the callus tissue so formed grew three or four times the size of the original explant after seven to eight weeks in culture. The root primordia appeared as pale yellow or light green outgrowths on the tissue. In certain cases, there was no callus formation and the roots developed

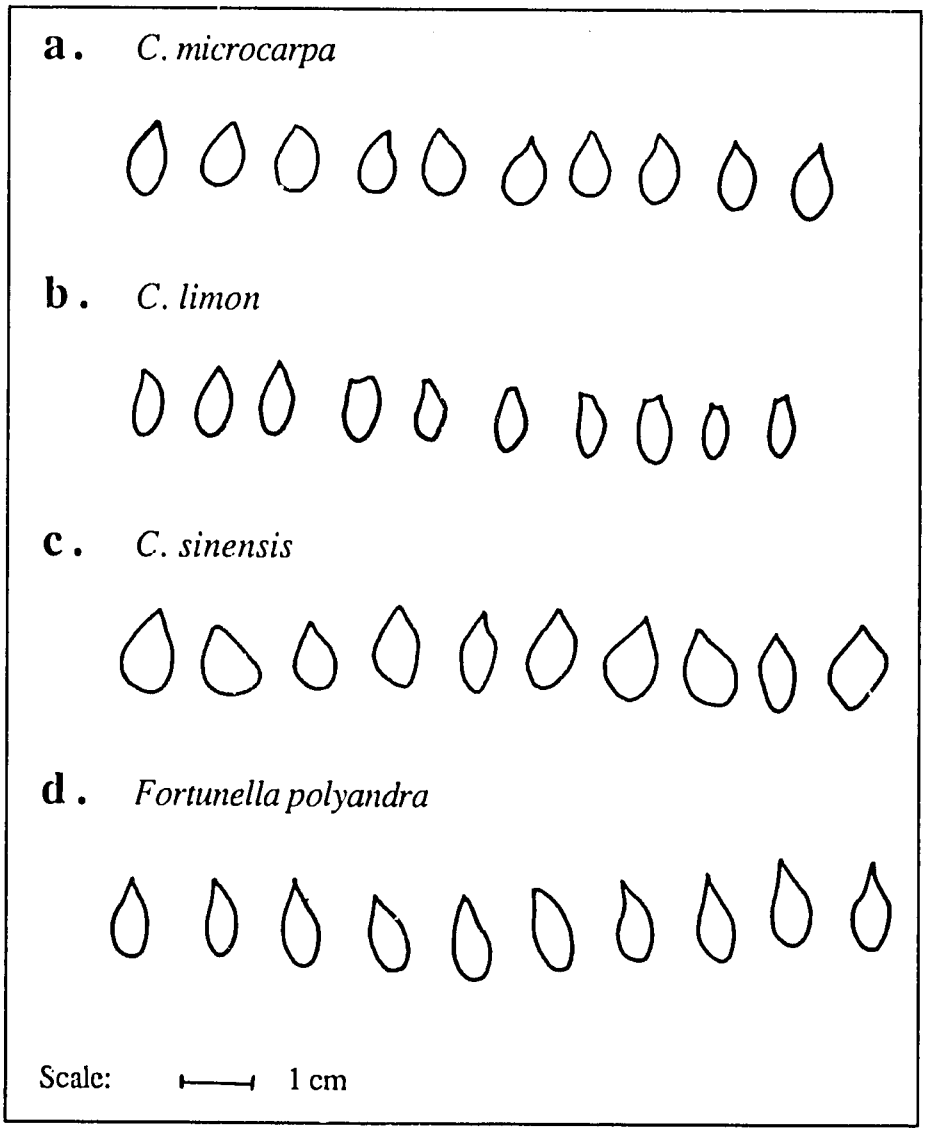

Fig. 2. Relative variations in seed size and shape in four *Citrus* species

Table 1. Seed[z] characteristics of *C. microcarpa*; *C. limon*; *C. sinensis* and *Fortunella polyandra*

Plant material	Average seed length[y] (cm)	Average no. embryos/ seed[x]	Approximate size of largest embryo (cm)	Texture and color of outer seed coat	Color of	
					Chalazal end of inner seed coat	Cotyledon
C. microcarpa	9.4 ±0.6	9.2 ±0.9	(8-6) x (7-5)	smooth, pale yellow	brown	green
C. limon	8.8 ±0.4	5.6 ±0.7	(8-6) x (5.5-4)	ridged, cream	maroon	pale yellow-green
C. sinensis	11.1 ±0.9	5.2 ±0.4	(10-8) x (7-5)	ridged, cream	reddish brown	off-white
F. polyandra	11.1 ±0.6	3.2 ±0.6	(9-7) x (7-5)	smooth, pale	brown	green

[z] All of these were polyembryonate seeds
[y] Mean calculated from sample of 10 seeds
[x] Size of smallest embryo not recorded, because the irregular shapes of embryos made designation of the horizontal and vertical axes very difficult

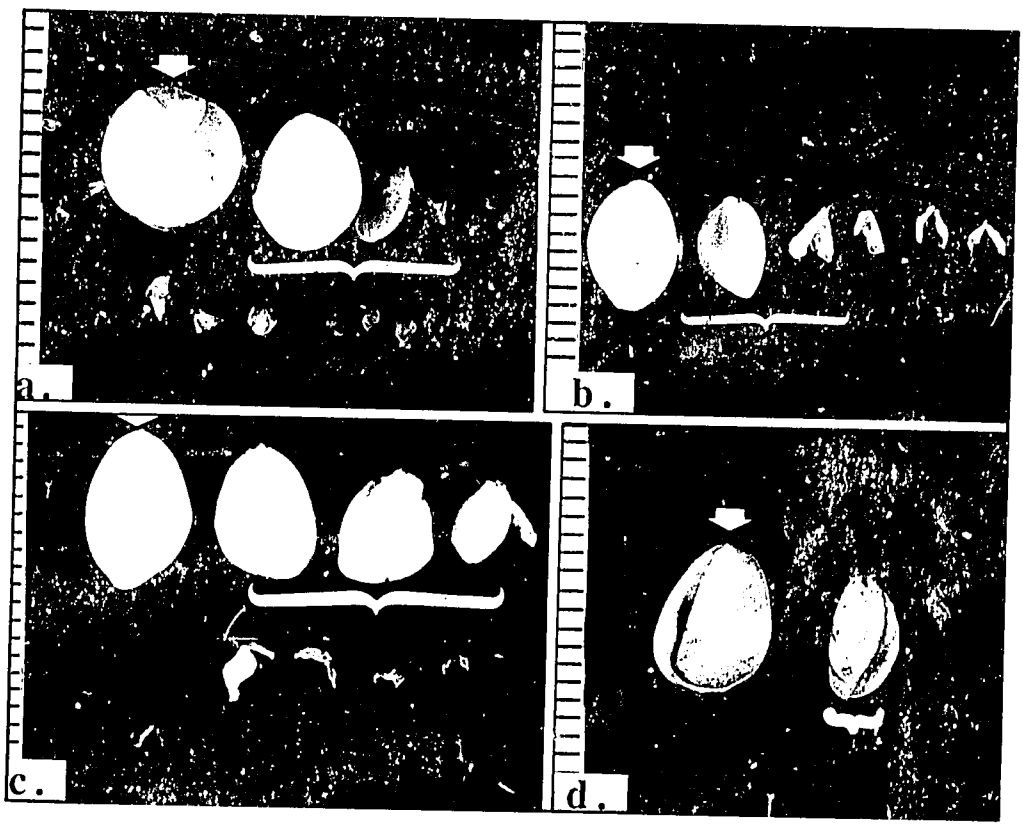

Fig. 3. Embryos isolated from a single polyembryonate seed of **a.** C. microcarpa; **b.** C. limon; **c.** C. sinensis; **d.** F. polyandra. Cotyledon segments were excised from the largest embryo (see arrows); the smaller embryos used were at least 2 mm long

directly from the explants. The surface of the roots appeared flaky initially, and was sometimes covered densely with root hairs. The roots were positively geotropic, and grew into the medium as they elongated. They also turned green and no longer had any root hairs once in the culture medium. The color, texture and pattern of callus and root development were similar for the cotyledon explants excised from both the largest and the smaller embryos. The general pattern of growth and organogenesis was similar in the explants of all the species, and unless there were distinct labels it was difficult to distinguish the cotyledon cultures of the different species from one another.

The cotyledon explant tissue was mainly composed of storage parenchyma, with some vascular strands and oil cavities (Fig. 4a-b). The surface cell layers in the excised regions served as healing tissues, and there was some suberin deposition on the cut surface (Fig. 4b).

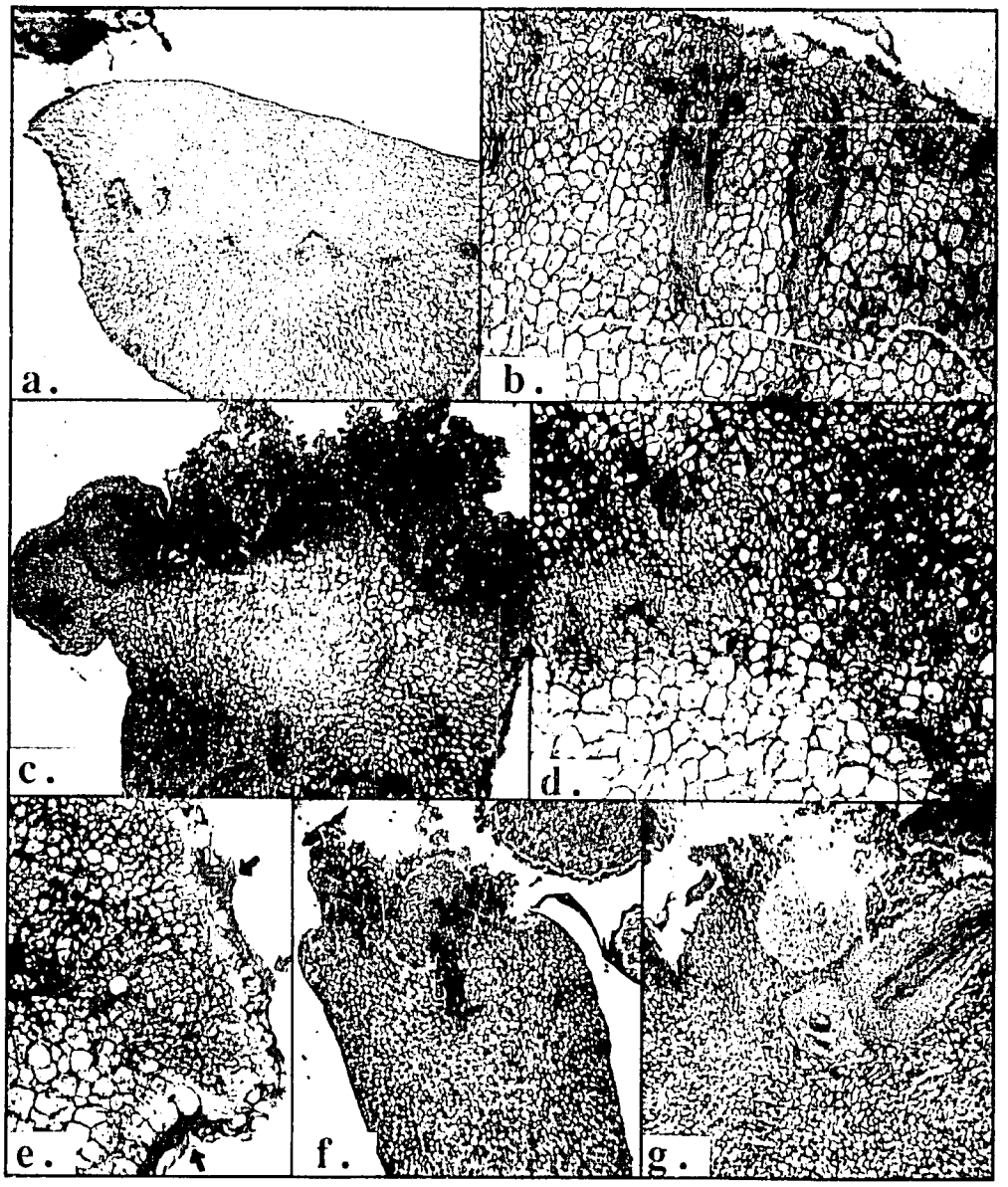

Fig. 4. **a-e.** *F. polyandra*

a. L.S. cotyledon segment, mostly parenchyma cells of fairly uniform size with vascular strands

b. Part of a segment enlarged to show subsurface cambium, with more intensive activity in and near the vascular strands

c. L.S. of the segment, showing profuse heterogenous callus with uneven outline surface; on right is a thick suberin deposition (50 days)

d. Part of **c** enlarged to show the callus tissue (darker region), thick cambium above the original tissue of the explants (lighter region)

e. Peripheral part of the growing explant, showing suberin deposition (arrows), active cambium and necrosis of some cells (50 days)

f-g. *C. microcarpa*

f. Cotyledonary segment with peripheral callus and root primordia (30 days)

g. Part of segment showing well developed roots, T.S. and L.S. (60 days)

The cell layers immediately below the healing tissue divided to form the subsurface or peripheral cambium (Fig. 4b). The cells adjacent to the cambial zone lost their contents and resumed meristematic activity, thus widening the cambial zone. Tissues nearer the vascular strands divided more vigorously, and so also did the parenchyma of the vascular strands. Depending on the relative intensity of the meristematic activity, several growth regions were formed which resulted in an uneven surface outline (Fig. 4c) and tissue protuberances of various sizes. The new tissue formed was darker brown in color, with rich tannin and other inclusions (Fig. 4d), while on the surface suberin deposition was prominent (Fig. 4e). Primarily both the callus and root development were of subsurface origin, and there was no correlation between the position of the original vascular strands in the tissue and the origin of new root primordia (Fig. 4c, f, g).

Effects of Growth Substances

Cotyledon explants of *C. microcarpa* were used to test the effects of NAA, and there was no tissue growth in the control. The concentration effects of NAA were obvious: at a lower concentration (0.2 mg/*l*) only roots developed; both callus and roots developed with 2 mg/*l*; and profuse callus developed with 10 mg/*l*, with few or no roots (Fig. 5a). The roots developed at lower concentrations were larger (Fig. 5a-b). The results were similar in the explants from both the largest and smaller embryos (Fig. 5a-b).

All the explants of *C. microcarpa* cultured in MS medium supplemented with only K (0-10 mg/*l*) failed to produce any growth response for the entire culture period of 12-15 weeks. Subculturing the tissues onto the fresh medium did not help. In subsequent experiments, combinations of both NAA and K were used, and effective concentrations for callus and root development varied (Fig. 5c-d). When the explants showed a greater degree of callus formation, the roots developed were shorter than those observed in other media with low NAA (Fig. 5c-d). The growth response of explants of *C. microcarpa* and *C. limon* were similar. The explants of *C. sinensis* failed to form any callus, but a few of them produced roots. In *F. polyandra*, the growth response of the explants was similar to that found in *C. limon* and *C. sinensis*, and again there was no significant difference between the explants of larger or smaller embryos (Fig. 5e). The best tissue growth was obtained in medium with NAA (10 mg/*l*) + K (0.2 mg/*l*) media, which for optimum root development a lower concentration of NAA (2 mg/*l*) + K (0.2 mg/*l*) was very helpful. An increase in the concentration of K (2 mg/*l*) helped root growth, but there was no bud development (Fig. 5f-h). From the above results, it was clear that both callus and root induction were possible when growth substances were used at particular concentrations.

In certain cases, full embryos were used as explants. These developed into normal seedlings bearing roots, hypocotyl and leaves (Fig. 6c). The size of the seedling was proportionate to the size of the embryo used. When the embryonal axis was separated from the cotyledons and cultured, this did not grow on the control medium but in media with NAA and K, the tissue growth was profuse (Fig. 6d-e). Initially the callus tissue appeared pale yellow, and took the form of a loose to fairly compact, granular mass. With further tissue proliferation, the explant became covered with callus. Whenever there was profuse callus, shoot development was arrested (Fig. 6d-e).

SHOOT BUDS ON ROOTS

Some of the cultures were left on the same medium for an extended period of 12-14 weeks, to study the growth response under undisturbed conditions. The roots continued to elongate, especially when petri dishes were used instead of test tubes. In cultures of *C. limon*, shoot buds developed on the roots, and eventually each of these developed into a leafy shoot (Fig. 6a-b).

Further experiments were then conducted on *Citrus grandis* and *C. microcarpa*. It was obvious from the above studies that under the influence of NAA and/or K very few buds developed. Therefore, for further work K was replaced with benzyl adenine (BA). The procedure for setting up the cultures was the same as described earlier.

Fresh seeds of *C. grandis* and *C. microcarpa* were used. They germinated readily on MS medium, and seedlings 4-5 weeks old had two to four roots and two pairs of leaves. These seedlings were transferred to fresh nutrient medium with BA (5 mg/*l*). About eight weeks after transfer, bud primordia appeared on the middle portion of the roots. They were green in color and could easily be seen against the pale white color of the roots (Fig. 7a-c). Almost 80% of the seedlings of both the species had buds on their roots.

At first, the shoot bud primordium was globular and button shaped (Fig. 7a). Soon after emergence, the two lobes of the primordia were

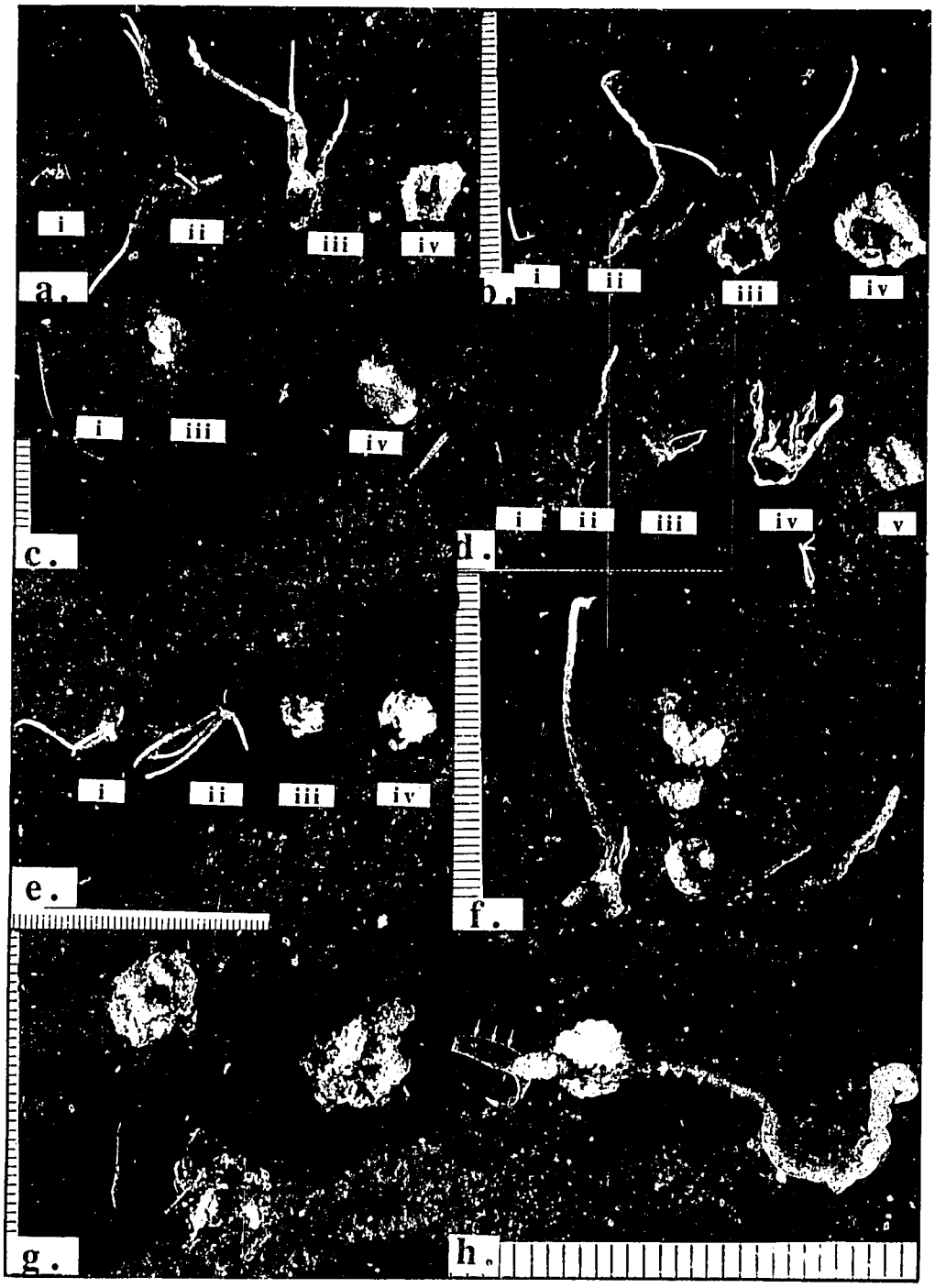

Fig. 5. **a-b.** *C. microcarpa*. Explants of cotyledons from largest **a.** and smaller **b.** embryos. MS full strength. i-iv 0, 0.2, 2 and 10 mg/l NAA respectively. Note that lower concentrations encourage root development, and the higher concentration callus development (42 days)

c. *C. limon*. Cotyledon explant from largest embryo. i. 0.2 mg/l NAA; iii. 2 mg/l NAA + 0.2 mg/l K; iv. 10 mg/l NAA + 0.2 mg/l K. Note the variations in the size, and the absence of roots (40 days)

d. *C. microcarpa*. Explants from the largest embryo. i. no growth regulator; ii. 0.2 mg/l NAA; iii. 0.2 mg/l NAA + 0.2 mg/l K, very little callus mostly roots; iv. 2 mg/l NAA + 0.2 mg/l K, callus and roots formed; iv. 10 mg/l NAA + 0.2 mg/l K, mostly callus formed

e. *F. polyandra*. Explant from smaller embryo. i. 0.2 mg/l NAA; ii. 0.2 mg/l NAA + 0.2 mg/l K, mainly root formation; iii. 2 mg/l NAA + 0.2 mg/l K; iv. 10 mg/l NAA + 0.2 mg/l K, mostly callus in iii and iv. 28 days old

f-h. *C. microcarpa* (50 days)

f. Callus and roots formed in medium with 2 mg/l NAA and K

g. Mostly callus, compact and friable, 10 mg/l NAA + 2 mg/l K

h. Explant enlarged to show callus development on root. Arrows indicate small outgrowths on the cut surface of the cotyledons

Fig 6. **a-b.** *C. Limon*

a. Spontaneous bud formation (90 days)

b. Enlargement to show development of leafy shoot with root (120 days) (0.2 mg/l NAA)

c-e. *C. microcarpa*

c. Seedlings developed on MS medium without any growth substances (42 days). Variations in size reflect original embryo size

d. Profuse callus around embryonal axis. 2 mg/l NAA + K each (42 days)

e. Whole embryo culture on the same medium as **d.** Note profuse callus from cotyledons and arrested shoot development (42 days)

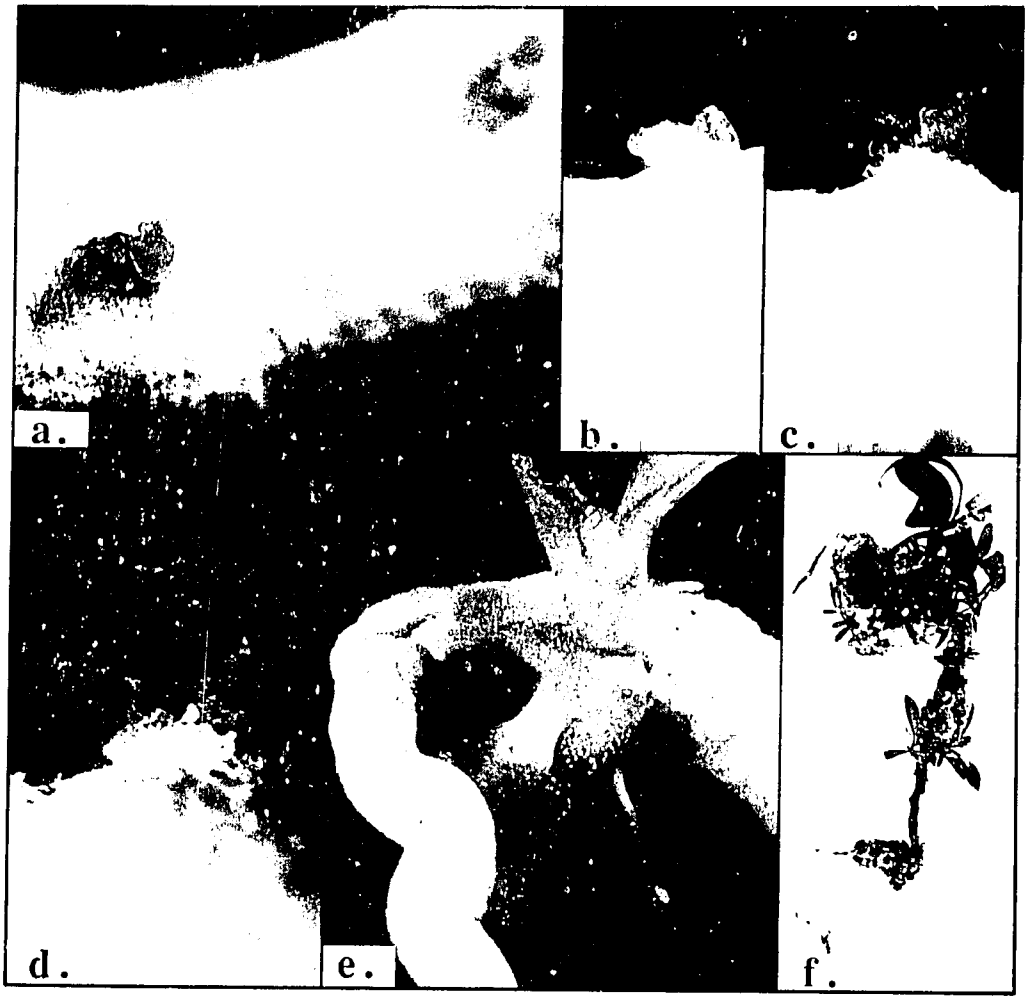

Fig. 7. **a-e.** *Citrus grandia*
a. Enlargement of root showing shoot bud primordia, button-shaped structure (out of focus) (80 days)
b. Primordium developed further, showing erect axis and leaf primordia (left) (80 days)
c-d. Primordia enlarged, showing rupture of root tissue and emergence of axis and the first leaf (left)
e. Enlargement of shoot bud, showing four leaves and two roots; it is now ready for excision to obtain a new plantlet
f. *C. microcarpa.* 1 month old seedling in NAA (10 mg/l) medium (with callus) transferred to BA (5 mg/l) medium. Note that some of the callus is present almost at the end of the root, and the development of numerous plantlets on the upper part of the root and hypocotyl. One of the seedling leaves is intact and producing callus; 20-30 shoots developed on each root; each of these could be separated into individual plantlets (60 days old)

clearly visible, and developed into the first two leaves. The axis of the bud was round and swollen, and in 6-8 weeks old cultures the axis emerged prominently. In cultures 10-12 weeks old, the buds also developed individual roots and thus many plantlets developed. These could be separated into plantlets (Fig. 7d-f).

Some of the seedlings, 2-3 weeks old, were transferred to NAA (0.5 mg/l) medium, and the roots produced profuse callus in three or four weeks. When the seedlings were transferred to BA (5 mg/l) medium, many shoot buds developed from the callus region. New shoot buds also developed directly from other parts of the root that were not producing callus. The shoot buds so developed were isolated and transferred to fresh medium, sometimes with NAA (0.5 mg/l) and sometimes without. In two to three weeks, they developed roots and full plantlets were obtained (Fig. 7f).

CITRUS IN S.E. ASIA

Vast number of *Citrus* species, natural hybrids and cultivars are present in S.E. Asia, and our knowledge of them is meager. Both basic research and experimental studies urgently need to be undertaken. Some of the points discussed below are very important to those working on tissue culture, for both plant improvement and genepool conservation.

The taxonomy of *Citrus* species is confused. It prompted Tanaka[24] to write on "Species problems of *Citrus*" and Dodds[7] to state that "... with the exception of the limes, these fruits represent on the whole natural groups of horticultural varieties and to apply the concept of formal taxonomy is more confusing than helpful."[2, 15]. These complications in the taxonomy are due to the ease with which *Citrus* species hybridize, by polyembryony, by mutations, and by the spontaneous production of autotetraploids with thicker leaves and rinds[2, 15].

Many species of *Citrus* are indigenous to Southeast Asia. Some are cultivated, while others exist as wild or primitive species that are less commonly grown. Table 2 lists the various species and cultivars commonly found and used in the region. It includes most of the commonly cultivated species described earlier, as well as some wild indigenous species such as *C. celebica*, *C. halimii*, *C. ichangensis*, *C. latipes*, *C. micrantha* and *C. microptera*. Furthermore, the region is also rich in species of closely related genera. Among these are *Fortunella polyandra*, *Feronia limonia* and *Feroniella lucida*[6]. In spite of the richness of *Citrus* germplasm, citriculture has not been as extensively developed in Southeast Asia as in other areas in the warm temperate zone, subtropics and drier parts of some tropical areas. Citrus fruits have not yet been exploited commercially as a large scale plantation crop in tropical areas, but are widely grown as a backdoor and orchard crop for local consumption[1, 6]. Relatively limited use has been made of the native genetic diversity in upgrading *Citrus* in Southeast Asia. Furthermore, cultivation in each of the Southeast Asian countries is plagued by problems ranging from pests and diseases to poor farm management. Nevertheless, efforts are being made to overcome these problems[6].

The International Board for Plant and Genetic Resources (IBPGR) is trying to develop an awareness of the need for preservation, evaluation and utilization of *Citrus* in Southeast Asia. This is recognized as a difficult task in view of the many constraints. It is widely believed that native crops are of low quality, and that there is no export demand for them. Low production yields are another problem.

Germplasm preservation efforts are further hindered by the problems of cost and space involved in the maintenance of *Citrus* germplasm collections. At the present time, almost all (if not all) the *Citrus* germplasm collections in Southeast Asia are maintained vegetatively[11]. This means that relatively large areas of land are occupied, and their maintenance can be expensive. The lack of users i.e. breeders and horticulturists in Indonesia[21], population pressure, the need for higher incomes for rural people and competition from other crops in Thailand[6] also threaten the future of germplasm collections in these countries. Nevertheless, there have been recent reports of *Citrus* collecting missions working all over Southeast Asia. This is because a dedicated group of people are aware of the need to continually acquire more germplasm to broaden the resource base. This is essential for the improvement, not only of *Citrus* in the Southeast Asian region, but also in all the *Citrus* growing regions of the world[21].

Both nucellar embryos and shoot buds on roots provide a convenient means of propagating select or elite plants on a massive scale. The plantlets so obtained are true clones of the mother plant, since no sexual cycle or meiotic changes are involved. Tissue culture thus offers an excellent opportunity of producing the good quality plantlets that are required to establish *Citrus* plantations in various developing countries. Tissue culture methods will also help to save the precious germplasm which is disappearing very fast along with the humid rain forests of the tropical zone. *In vivo* methods alone may not be adequate to conserve all ecotypes and clones[4, 12, 19, 23].

DISCUSSION

Citrus seeds are well known for their polyembryonate nature. Whether fertilization is necessary for the development of nucellar embryos is still not proven, for the whole genus or for other genera of different angiospermous families[5, 17]. Earlier, it was shown that in *C. microcarpa* and *C. grandis* both shoot and root development could be induced by changing the composition of the medium and light/dark regime[18]. Some of the recent observations made are discussed in the following section.

Embryos in mature seeds are crowded, and

Table 2. Citrus commonly used in Southeast Asia

Scientific name	English name
Citrus umbilycarpa (Hassk.) Oshse	
C. aurantifolia (Christm. & Panz) Swing.	lime
C. aurantifolia var. *italicus* Hort.	Italian lime
C. aurantium Linn.	sour orange, bitter orange, Seville orange, bigarade
C. celebica Koord.	Celebes papeda
C. grandis (Linn.) Osbeck	pomelo, pummelo, shaddock
(*C. maxima* Merr.)	
C. grandis var. *racemosa* (Roem.) Stone	grapefruit
(*C. paradisi* Macf.)	
C. halimii Stone	
C. hystrix DC.	leech lime, porcupine orange, Mauritius papeda
C. ichangensis Swing.	Ichang papeda
C. jambhiri Lush.	rough lemon
C. latifolia Tanaka	Persian lime, Tahitian lime
C. latipes (Swing.) Tanaka	Khasi papeda
C. limetta Risso	sweet lime
(*C. limetlioides* Tanaka)	
C. limon (Linn.) Burn. f.	lemon
(*C. medica* var. *limon* Linn.)	
C. limonia Osbeck	Rangpur lime
C. macroptera Montr.	false shaddock, Melanesian papeda
C. macroptera var. *kerrii*	Kar's Thailand papeda
C. medica Linn.	citron
C. medica var. *sarcodactylis* Swing.	Buddha's fingers, fingered citron
C. micrantha Wester	small-flowered papeda
C. micrantha var. *microcarpa*	small-fruited papeda
C. mitis Blanco	calamondin, musk lime
(*C. microcarpa* Bunge)	
(*C. madurensis* Lour.)	
C. reticulata Blanco	Tangerine, mandarin orange
C. reaculans x *C. sinensis*	king mandarin, tangor
(*C. mobilis* Lour.)	
C. reticulata var. *austera* Swing.	sour mandarin
C. semperflorens Lush.	
C. sinensis (Linn.) Osbeck	sweet orange
Fortunella polyandra (Ridl.) Tanaka	Malayan kumquat
F. punctata Tanaka	
F. crassifolia Swing.	Meiwa (sweet) kumquat
F. japonica (Thumb.) Swing.	kumquat, Marumi (round) kumquat
F. obovata Tanaka	Changshon kumquat
Poncirus trifoliata (Linn.) Raf.	trifoliate orange

closely appressed. Those studied in these experiments were of varying sizes. As many as 22 were isolated from the seed of *C. microcarpa*, as against 40 embryos/seed in *C. unshiu*[7]. Nucellar embryos are generally recognized by their lack of suspensors, by their smaller size, or by their lateral location within the seed. Both large and small embryos have good growth potential.

The nucellus is an unique tissue without any vascular strands. In certain species, adventive embryogenesis was induced from *in vitro* culture of nucellus tissues excised from unpollinated ovules, proving that the stimulus of pollination and/or fertilization was essential[10, 11].

As stated above, many studies have been made to exploit the full regenerative potential of both vegetative and reproductive tissues of *Citrus* species. Nucellus, which is the mother tissue for adventive embryo development *in vivo*, also regenerates well *in vitro*, giving rise to embryos. The effect of various growth substances on tissues of various species in culture has also been studied[13, 16]. Also of interest are the studies concerned with the regenerative tissues derived from stem and leaf segments, leading to full plantlet development[9]. Shoot tips of mature trees have also been used to induce multiple shoots, and some of these could be rooted to obtain full plantlets. Obviously, this procedure can be used as an effective method of eliminating viruses[5, 14]. There is thus a great deal of technical expertise available for the mass propagation of certain *Citrus* species and their cultivars. However, whether any one method is equally well suited to all species is not certain, since each study has been concerned only with one or a few species. There is thus a need for further studies which include a wide range of species or cultivars with commercial potential. It is possible that such studies have already been completed, and suitable methods already developed, by large commercial companies in the citrus producing countries of the developed world such as Australia or USA. However, only very limited work has been done on the species available in Southeast Asia.

Some interesting observations have been made on direct shoot bud development on roots[22]. In previous years, liquid medium was used to establish root meristem cultures of orange, lemon, *Poncirus trifoliata* and Citrange. After initial growth, larger pieces of roots were transferred to agar medium where they grew further, giving rise to shoots, either directly or through the callus phase. Eventually plantlets were obtained. In the present study liquid medium was not used, and shoot buds developed directly on the roots without any callus. Root callus tissues, when subcultured, gave rise to a large number of shoots that could be separated and rooted to obtain plantlets. Thus, the possibility now exists of obtaining plantlets, with or without a callus phase. It may prove very interesting to extend this method of study for important commercial varieties of *Citrus*, using roots developed both *in vivo* and *in vitro*. Besides maintaining the desirable genetic qualities of mother or elite plants, this method also offers a good system of eliminating viruses. With further improvements in these methods, mass propagation of valuable *Citrus* plants can thus be achieved, to satisfy the huge demands of *Citrus* plantations in developing countries. The natural hazards and variations that vegetative propagation methods carry can thus be avoided.

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DISCUSSION

Q. (N.M. Bhattacharya)
I am very interested in the culture of tea. What medium are you using with tea tissue culture in Singapore, and what complications do you have in obtaining embryogenesis?

A. I think a scientist from Taiwan, a Dr.Wu, was the first to succeed with tea tissue culture at the Taiwan Tea Research Institute in 1982. He used a BS media initiator, later transferring the culture to a series of other media. I do not think his work has been published yet.

Q. (D.A. Somers)
Have you been able to induce embryogenesis in nucellar tissue?

A. Yes, we were able to do this as early as 1962.

MICROPROPAGATION: THE PROBLEMS WITH WOODY SPECIES

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ABSTRACT

The potential of micropropagation in crop plants has been well expressed, particularly with regard to the herbaceous plants. However, success with woody plants has to date been limited to a handful of species. This appears to be related to the greater difficulty of establishing cultures of woody plant tissues in vitro. The following paper reviews the various problems encountered when working with tissues of woody plants for the purpose of micropropagation.

INTRODUCTION

Micropropagation can be described as *in vitro* multiplication of a plant. It normally involves the hormonal release of dormancy of the axillary buds, and their subsequent outgrowth. Micropropagation has two advantages. Firstly, no treatments for the neoformation of shoot-buds are required. When a bud is used as the explant, the total shoot is *a priori* present. Secondly, the risk of genetic variation is minimized. The procedure was pioneered by Morel and Martin, who used it to recover virus-free plants through shoot-tip cultures from infected plants[80]. It was subsequently extended for clonal multiplication of orchids[49], and has since become the standard procedure for *in vitro* propagation in the orchid industry. The current wave of related activity with other crops probably owes much to its success with orchids.

The potential application of micropropagation for woody plants, particularly tree species, has been well reviewed[5, 28, 40, 87]. However, the application of micropropagation techniques to woody plants has had a rather late beginning, and many problems remain to be solved. This paper reviews the various problems which are found to be commonly associated with the micropropagation of woody plants.

THE PHYSICAL ASPECTS

Availability of Explant Material

Woody plants differ from other plant species in several important respects. They are generally long-lived, and can take a long time to reach the mature stage for evaluation. There is thus a pressing need to propagate material of proven field performance. The use of tissues from mature plants can, however, be very difficult, since success in culturing their tissues is known to be inversely related to their age[13]. Generally, one has to resort to the few juvenile growths that can be found in the mature plant. Reversion from adult status to juvenility is, however, not a common phenomenon, though in some plants it may be induced artificially by applications of growth regulators, grafting, etc. In species where the phenomenon of topohysis is found, the availability of material that can be used as the explant is further limited[9, 29, 68].

In *Theobroma cacao*, for example, only a very small number of the preferred orthotropic shoots can be found, and then only the top 30-50 cm (accounting for about 8-12 meristematic centres) of the shoot can be utilized as the source of the material[46]. This situation has placed a severe constraint on the number of explant cultures that can be made available for experimental purposes.

This is particularly the case when the choice of the mother plant is based on a single stand selection.

Disinfestation of Explant Material

During the long growing period in the field, plants are continually exposed to numerous field contaminants. They also harbour various insects, the activities of which further increase microorganism contamination in the host-plant tissues. Thus, getting adequate disinfestation of the explant material from these plants can be very difficult. Debergh and Maene[21] have recently published an excellent review of this problem. While several options, such as pretreatment of material in the field[22], or the incorporation of antibiotics into the culture medium[43, 74, 76, 82, 83], are available to complement the usual surface sterilization procedure, their effectiveness may vary according to the species and field situation[6, 35, 36, 72].

One possible solution which has only recently been given serious consideration is to isolate the mother stock plant from the usual field contaminants. This preparatory stage for the stock mother plant material has been termed stage 0[21], and it can complement the stages I, II and III of propagation scheme proposed by Murashige[53, 54, 55, 56]. Mother plants prepared under this condition produced healthier explants with a uniform response[7, 36].

Tissue Browning

Another serious problem known to affect the initiation of cultures of woody plants is the phenomenon of explant browning, which eventually causes the culture to die. The relationship of tissue browning with plant phenol production is fairly well documented[30, 70, 77]. Such a phenomenon can be an indication of tissue sensitivity to physical stress (e.g. the dissection process) and/or to chemical stress (e.g. the surface sterilization process). Kostuge[30] has suggested that it can also be a stress response to persistent fungal or bacterial infection, and data from other published work seems to support this view[34, 35, 36]. Several solutions to this problem have been proposed. These include the incorporation in the culture medium of antioxidants[4, 36, 44, 46, 69, 74, 77, 87] or adsorbents[4, 27, 46, 65, 66, 80], manipulating the salt concentration in the medium[2, 5, 26, 78, 81], avoiding the use of N^6-substituted adeninine cytokin in the first culture[71, 79], or imposing a culture environment which may not be conducive to oxidative reaction[15, 90, 91]. Pretreatment of the explants by soaking them in distilled water may also reduce this problem[25].

Emission of Excudates

With some woody species, the emission of exudates from the cut surfaces can pose a problem in the handling of the explant material, and can even interfere with the surface sterilization process. In certain species such as *Theobroma cacao*, the situation can become unmanageable as the exudate may become quite viscous on contact with water[46]. Blake and Maxwell[7] even postulated that the limitation of tissue growth *in vitro* of *Theobroma cacao* may be due to blockage of vessels by the excessive mucilage. In certain genera of *Ribes*, inhibitory exudates are secreted only after the explants have developed complete shoots[37, 84].

BIOLOGICAL ASPECTS

Nutrient Requirements

Apart from the explant origin, the other important factor that governs the success of plant cultures is the culture medium. The description of a defined culture medium is, in fact, the first step in the development of a plant tissue culture system. The nutrient formulation utilized for a particular plant culture system is generally based on an empirical approach, with a general preference for the established formulations. One such medium that has been widely used is that of Murashige and Skoog[52], the high salt content of which appears to be suitable for the faster growing herbaceous plants. With the woody species, data from recently published work appears to indicate otherwise. The general preference of these species appears to be for a nutrient formulation of lower salt concentration[7, 42, 46, 63, 71, 84]. In light of this information, any assumption that established media which work well with a broad range of non-woody species may also work with the woody species can lead to an inherent serious flaw in the work. The sensitivity of the tissues of woody species to small imbalances is not well documented, but in *Theobroma cacao*, for example, the tolerance range has been found to be very restricted[48, 57].

Correlative Inhibition

In micropropagation, the axillary or terminal buds are induced to develop *in vitro*. A single

bud may produce only a single shoot, or, depending on the species and the medium used, may produce multiple shoots. As each of the shoots in turn develop buds along their axis, the procedure may be continued indefinitely[45]. With many of the woody species, there appears to be a general difficulty in getting good development of buds into shoots *in vitro*[46]. The underlying cause may be the correlative inhibitions or innate (bud) dormancy which are fundamental to the species. Seasonal variation in the field may affect the morphogenetic response of the explant, and this phenomenon has been reported in a number of species[21, 45, 67]. Morphogenetic ability appears to be limited to the period of vegetative growth. This area has not been well studied, but a better understanding of correlative inhibitive effects on the explant may help us understand how to exploit the morphogenetic effects of dormancy. This particular problem has been a major constraint in establishing a culture system for many woody plant species.

In certain species, the recalcitrant nature of the explant tissues can be overcome through a process that can be appropriately termed 'conditioning'[46]. The change in the tissues can possibly be attributed to adaptation of the tissues to the new environment, or leaching of substances which are inhibitory to the development of the explant. This 'conditioning' process may be achieved either by soaking the explants for some period in sterile distilled water prior to inoculation[15], or by inoculating the explant onto an initial medium devoid of any growth regulators[14, 37, 43, 46, 63, 75].

Loss of Morphogenetic Potential

The loss of morphogenetic potential has been reported to occur with callus or cell suspension cultures of a number of herbaceous species, and there is increasing evidence to show that this occurs generally with extended subculturing[32, 60, 64, 79]. The few published works on the micropropagation of woody species have highlighted this problem[7, 46, 55, 60, 63, 85], and it may well be a common feature in cultures of woody species. It is very serious, in view of the fact that tissues of woody species are generally slow growing, so that initiating the necessary morphogenetic responses may require a passage through several subcultures. Various reasons have been forwarded, including the reduction of endogenous levels of growth regulators[16, 55], systematic contamination of the cultures with microorganisms[6, 59], and the presence of gasses which are inhibitory to the tissues[55].

Tissue Vitrification

The development of shoots or plantlets *in vitro* with succulent, translucent leaves has been reported in cultures of a good number of woody species[8, 10, 23, 24, 31, 33, 35, 46, 47, 62, 63, 71, 85, 86]. This phenomenon, termed vitrification, is considered to be a physiological disorder frequently affecting plants in the course of their vegetative multiplication *in vitro*[18, 19, 20, 21]. It is hypothesized that when the tissues are under stress, ethylene is produced through the peroxidase-IAA-oxidase systems which in turn produces such a reaction[38]. Vitrification generally results in a low survival rate in plantlets regenerated *in vitro*, particularly during the transfer of plants from *in vitro* to *in vivo* for establishing in the soil. Debergh and Maene[21] have made an excellent review of this problem, indicating that it can be overcome or minimized by using a high agar concentration, a low incubation temperature, culture vessels with better aeration, a short culture duration and low levels of cytokinins.

FUTURE CONSIDERATIONS

Clearly, there are a large number of factors, both physical and biological, that can influence the successful application of micropropagation techniques to woody plant species. It may be that woody plants are inherently more difficult to manipulate *in vitro* than the herbaceous plants, but it is also true that not much research has been done in this field, and further systematic research is urgently needed.

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DISCUSSION

Q. (A.N. Rao)
I was interested in your statement that vitrification could be controlled by raising the agar concentration.

A. An increase in the agar concentraion does in some cases control vitrification, but the multiplication rate is low. A balance has to be struck between control of vitrification and an acceptable rate of multiplication. In our experience, beginning the culture using a medium with a low agar concentration which may vitrify the plants, and then subculturing them into a second medium with a higher agar concentration, does not solve the problem. It is better to begin the procedure with a high agar concentration medium.

Q. (M. Vajrabhaya)
When you propagated plants using tissue culture, did you encounter any problems from somaclonal variation?

A. In micropropagation, we were using preformed somatic tissues, and didn't go through the culturing process. When callus is used, there is a possibility of variation, but not when preformed tissue is used, which should be true to type. To date I have not encountered such a problem.

Q. (J.D. Chung)
Could you give us more information on the browning of tissues, and also the exudation of cell sap, and perhaps tell us how they can be prevented?

A. The use of anti-oxidant, reducing and absorbent agents to control tissue browning *in vitro* is fairly well documented. In my experience with such recalcitrant tissues as that of coconut, a combination of an antioxidant (e.g. Cleland's reagent) and an absorbent (e.g. activated charcoal) incorporated in the culture medium has shown to be effective. To me, exudation is more of a nuisance than a problem, and in my experience, contact of the explants with water appears to make the situation worse. Minimize contact with water!

IN VITRO DEVELOPMENT OF SHOOT APICES FROM POLLEN CALLUS IN THE ANTHER CULTURES OF TEA (*CAMELLIA SINENSIS* L., O. KUNTZ)

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ABSTRACT

Since tea (Camellia sinensis L., O. Kuntz) is a perennial, cross pollinated and virtually self-incompatible tree, it is difficult to produce isogenic lines (inbreds) of distinctive varieties or clones through conventional methods of breeding. Attempts have been made to produce pollen derived haploid/homodiploid lines by anther culture, in order to improve this important plantation crop. This paper reports on our success in developing shoot apices from pollen calli from tea anthers cultured on B_5 medium with 2,4-D 0.1 mg/l, kinetin 0.1 mg/l, and sucrose 7%, supplemented with NAA 0.1 mg/l and glutamin 400 mg/l.

INTRODUCTION

Since Guha and Mahaswari first reported the successful production of haploid plants through anther culture (using *Datura innoxia*[10, 11, 12]), a great deal of attention has been paid to the production of haploids using this technique. Haploids have now been produced for 247 plant species[19]. However, many reports announce only limited success, and in most cases the efficiency of haploid plant production is low[3]. Current attempts to produce haploids *in vitro*, using a wide range of plant species, are nevertheless of great significance. Different crops vary markedly in their response to various *in vitro* techniques. Such attempts enable us to make a judicious choice of the proper crop species for experimentation.

The application of *in vitro* culture methods to produce pollen derived plants for utilization in crop improvement, advocated initially by Melchers[20, 21], has obvious benefits, and the subject has been dealt with at length in a recent review[8]. In terms of plant breeding, haploids are most useful for obtaining homozygous lines by chromosome doubling. Using conventional breeding methods, it takes about five to six crop generations to produce homozygous lines, whereas tissue culture can produce haploids in large numbers within months[3]. By doubling the haploid chromosomes, homozygous lines can be produced with ease.

This procedure has been widely used for field crop improvement, particularly by Chinese and Japanese scientists, who have published reports on the successful release of a number of commercial varieties of rice[13, 15] and tobacco[24] bred by anther/microspore culture techniques. These techniques also have great potential for haploid production in the breeding of perennial species such as tea.

ANTHER CULTURE OF TEA (*CAMELLIA SINENSIS*)

Tea (*Camellia sinensis*, 2n = 30) is an important plantation crop in many tropical and subtropical countries, including India. Since tea is a highly cross-pollinated, virtually self-incompatible perennial shrub, which takes 5-10 years to flower and fruit, the use of conventional breeding methods to produce inbreds or isolate mutants is extremely difficult, if not impossible. Hence, any attempt to produce androgenic haploids/homodiploid lines of tea *in vitro* is of immense importance.

This paper discusses our work to produce shoot apices from pollen callus in the anther cultures of tea.

Materials and Methods

Flower buds of different sizes were collected as required from healthy tea bushes of three different var./clones (TV-1, TV-23 and Tocklai generative clone-124/488). All were growing in the Mathura Tea Estate, Jalpaiguri, West Bengal, India, about 30 km from our Institute.

Procedures for Handling Buds and Removal of Anthers

Buds of varying sizes were initially collected in conical flasks filled with distilled water, and were brought to the laboratory for culturing. Surface sterilization was achieved by treatment with mercuric chloride solution (0.1% w/v) for 30 min., followed by five rinsings with sterile double distilled water. Surface sterilization, anther excision and anther planting were carried out in a sterile environment provided by a laminar air flow work bench (Klenzaids, India). The buds were opened with the help of two sterilised forceps, and anthers were carefully detached from the filaments.

Culture of Anthers and Choice of Media Composition

Initially, for selecting the media composition, anthers of different sizes from all three var./clones were planted aseptically on six different media, namely: SH[31], MS[23], modified MS[14], B_5[9], NN[26] and H[5], to judge their level of response. On each medium, 35-50 excised anthers per tube were planted in 10 culture tubes (150 x 25 mm) containing 10-12 *ml* of nutrient medium solidified with 0.8% agar (BDH, England), at a pH recommended in the original reports on the media. Cultures were incubated in the dark at 25 ± 1°C for three weeks, and were then transferred to beneath white fluorescent tubes (1500 lx).

Standardization of Bud and Pollen Size for Improved Callus Induction

35-50 excised anthers from Tocklai Generative clone (T.G.C.), representing 124/488 flower buds of varying sizes (ranging from 0.638 ± 0.008 to 1.039 ± 0.006 cm in diameter) were inoculated in each tube containing 10-12 *ml* of B_5 solid medium. For each bud size, 10 tubes were inoculated. Bud size (diameter) was based on the mean of 10 measurements taken from different angles with the help of lide callipers, as the flower buds of tea are almost round after removal of the stalk. Attempts were made to develop a method for determining responsive pollen size by comparing response with the diameter of the flower bud.

To determine pollen size, the diameters of twenty randomly selected pollen grains were measured from smear preparations made from five randomly selected anthers of each bud under a microscope, with the help of an ocular-micrometer. These measurements were then calibrated into microns (μ).

Medium for Organogenesis

Fresh excised anthers of T.G.C. - 124/488 (bud size ranging between 0.768 ± 0.006 to 0.827 ± 0.005 cm in diameter) were planted on B_5 solid medium with various modifications at pH 5.5, following the procedure described above. The cultures were incubated in the dark for three weeks, and then kept under continuous light (1500 lx) at 25 ± 1°C.

Cytological Method

To identify the developmental stage of the microspores responsive to callus induction, anthers were fixed (overnight in 3:1 alcohol and propionic acid), and were then smeared onto 2% propiono-carmin for observation.

In order to analyse the ploidal status of the shoot apices originating from anther callus, calli with white globular structures were fixed in a 1:3:6 mixture of propionic acid, chloroform and ethanol overnight. Each callus was then washed thoroughly in distilled water, and stained in a solution containing 1 part 1N HCl and 9 parts 2% propiono-orcein. It was then warmed for a few seconds, and pulped in a drop of 1% propiono-orcein solution.

Histological Study

Differentiating tissues (not larger than 5 mm^3) at different stages were fixed in FAA (Formaline 5 *ml*, Acetic acid 5 *ml* and 70% alcohol 90 *ml*) and stored at 4°C. Sections were cut either by hand or by microtome (10-15 μm thickness), stained in safranin and Light Green[14], and finally mounted in DPX. For microtomy, specimens were dehydrated in ethanol-chloroform in a series, following

30 hours fixation, and were embedded in paraffin with a low melting point (52-54°C, E. Merck).

STATISTICAL ANALYSIS

A completely randomized design with five replications (two tubes for each replication for each treatment) was followed for both series of experiments on the response effect of different media, and the response effect of different bud size, on callus induction from anthers. A analysis of variance was performed accordingly.

In order to study the effect of sucrose x glutamine and serine x abscisic acid on the induction of pollen callus, 3 x 4 and 2 x 3 factorials were performed, respectively, in randomized complete block design with 5 replications in each.

In order to ascertain whether treatment variations were significant or not, only percentage values were analysed by angular transformation. Wherever the response effects were found to be nil, the treatment(s) were excluded from the analysis.

RESULTS

Choice of Media Composition for Callus Induction and Proliferation from Anthers

The response of the excised anthers (from flower buds of varying sizes) taken from all the three var./clones to six different standard culture media was tested. Only the anthers from TGC-124/488 responded uniformly.

Table 1 depicts observations on the response of anthers from TGC-124/488, in terms of callus induction and proliferation, to six different standard media. It is evident from these data that the response of anthers on B_5 nutrient composition was significantly higher (26.33%), in terms of callus induction, whereas no response was observed on NN medium. The growth of callus was also prolific on SH and modified MS media. Response to B_5 medium was moderate.

The nature of the calli on all five media which produced a response was generally compact, except for the development of numerous minute globular structures on the surface of calli growing on B_5 medium (Table 1, Fig. 1d), the growth of these ceased, however, after 7-10 weeks of incubation. The color of the calli was initially creamy white on all five media at the time of induction, but varying pigment development was observed following 2 weeks' incubation in continuous light (i.e. about 5 weeks after inoculation).

Table 1. Relative response of tea (cv. T.G.C. – 124/488) anthers, in terms of induction and proliferation of callus, to different standard culture media

Media	No. cultured anthers	No. anthers showing response	% anthers showing response	Color of callus at induction	Color of callus 6 weeks after inoculation	Growth and nature of callus
SH	458	90	19.65	CW	PY	+++ C, Gr
MS	473	73	15.43	CW	LG	+ C
MS (modified)	367	58	15.80	CW	VP	+++ C, Gr
B_5	452	119	26.33	CW	LG-VP	++ C, Gl
NN	458	–	–	–	–	–
H	429	92	21.45	CW	P	+ C
LSD at 0.05		–	2.45			

–	no response	VP	variously pigmented
+	poor growth	LG-VP	light green to variously pigmented
++	moderate growth	P	deep pink
+++	prolific growth	C	compact
CW	creamy white	Gr	granular
PY	pinkish yellow	Gl	globular
LG	light green		

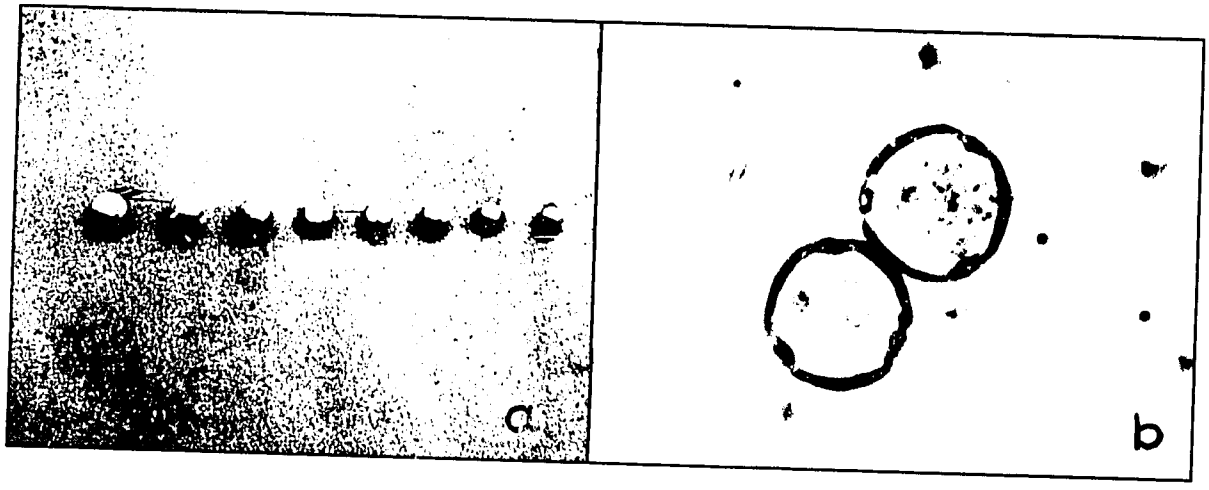

Fig. 1a-b. (a) Eight different sizes of tea buds, utilized to identify optimum size for anther callus induction
(b) Uninucleate microspores from responsive anthers at inoculation

Fig. 1c-d. (c) Induction of callus from anther (remnants of anther wall at the base)
(d) Numerous globular structures at the surface of the intervening callus

Fig. 1e-f (e) Numer of shoot apices developing on intervening calli
(f) Enlarged view of shoot apices covered with pubescent hairs at the initial stage

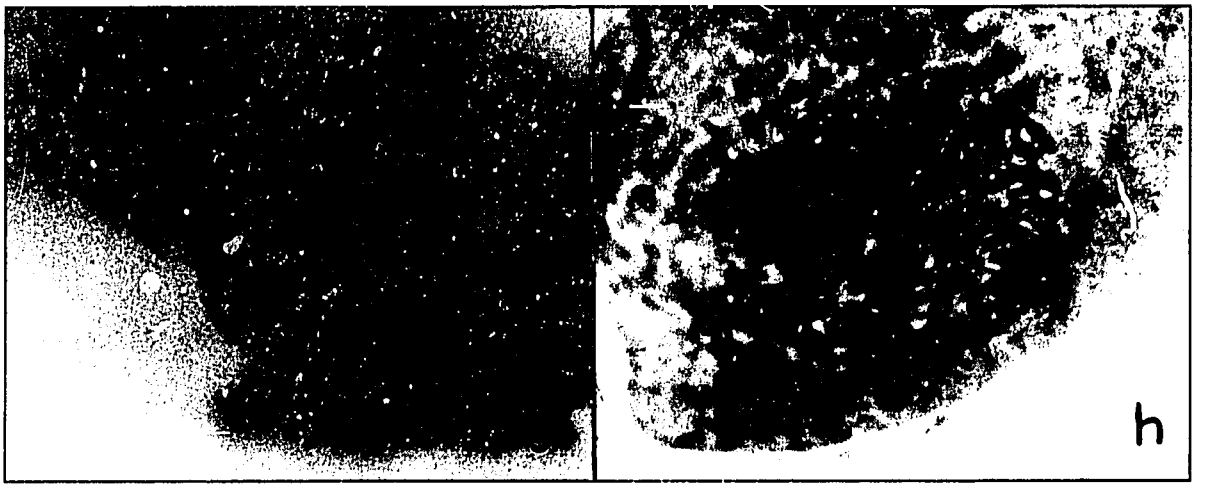

Fig. 1g-h (g) Deep green shoot apex at late stage of development
(h) Cytological preparation from globular structures, showing anaphase chromosomes in a haploid cell (n=15)

Standardization of Flower Bud and Pollen Size for Induction of Callus

The response of anthers excised from flower buds of varying sizes (Fig. 1a) was determined, in terms of the percentage of anthers inducing callus on B_5 medium (Table 2). It seems that anthers of three different bud sizes, ranging from 0.768 ± 0.006 to 0.827 ± 0.005 cm diameter (pollen size 40.66 ± 1.56 to 45.45 ± 0.53 μ in diameter), were responsive. However, no significant variation in response could be seen between these three different bud sizes. Later cytological study of the anthers from these three bud sizes revealed that the microspores of these anthers were at the uninucleate stage of development (Fig. 1b) at the time of inoculation.

Media for Organogenesis

The nutrient composition of the B_5 medium with varying amounts of auxins (2,4-Dichlorophenoxy acetic acid 0.1, 0.5, 1.0, 1.5 and 2.0 mg/l; Napthalene acetic acid 0.1, 0.5, 1.0 and 2.0 mg/l) and cytokinin (kinetin 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 mg/l) in various combinations, was tried initially (data not shown). None of these combinations contributed positively towards differentiation of calli, except that a marked increase in the number of globular structures was seen on medium containing the B_5 original nutrient composition with a lower range of 2,4-D and kinetin, supplemented with NAA 0.1 mg/l. These auxin and kinetin concentrations were thus used in all later studies of organogenesis.

Table 2. Response of various sizes of flower bud and pollen, in terms of callus induction in anther culture of tea (T.G.C. -- 124/488)

Bud size (diameter in cm)	Pollen size (diameter in μ)	No. cultured anthers	No. anthers showing response	% anthers showing response
0.638 ±0.008	Terad	410	–	–
0.694 ±0.003	34.82 ±0.92	430	–	–
0.768 ±0.006	40.66 ±1.56	386	86	22.28
0.732 ±0.004	43.16 ±0.55	429	138	32.17
0.827 ±0.005	45.45 ±0.53	445	90	20.22
0.852 ±0.008	47.12 ±1.12	412	–	–
0.958 ±0.007	56.08 ±0.60	429	–	–
1.039 ±0.006	56.61 ±0.53	400	–	–
F value	–	–	–	10.55 NS

NS indicates non-significant

Further modifications were introduced by supplementing the medium with higher levels of sucrose (4%, 7%, 10% and 12% w/v) and glutamine (400, 800 and 1200 mg/l) (Table 3a). Induction of callus was observed in all the treatment combinations, after 2-3 weeks of incubation in the dark. Observations revealed that callus induction was significantly higher (response in 42.08% of anthers) on medium containing low levels of sucrose (4%) and glutamine (400 mg/l). Induction was found to be lowest (response in 5% of anthers) with higher levels of sucrose (12%) and glutamine (1200 mg/l). The data presented in Table 3b show that different concentrations of both glutamine and sucrose have a significant effect on callus induction, but that they have no significant interaction.

Cultures were transferred to light after three weeks. After incubation in light for the next three weeks, numerous minute globular structures appeared (i.e. 6-8 weeks after inoculation) over the variously pigmented compact calli growing on the B_5 original medium with 2,4-D 0.1 mg/l, NAA 0.1 mg/l, kinetin 0.1 mg/l, and an increased level of sucrose (7%), supplemented by glutamine 400 mg/l. Initially, the globular structures were whitish in color (Fig. 1d), but gradually developed into pale yellow to light/deep green shoot apices (Fig. 1e-f), covered with white pubescent hairs along their whole length, except for the meristematic zone. Out of 143 anthers inducing callus, only 18 anthers

Table 3a. Effect of sucrose and glutamine on organogenesis in pollen callus of tea (cv. T.G.C. – 124/488)

Sucrose (% w/v)	Glutamine (mg/l)	No. cultured anthers	No. callus inducing anthers	% callus inducing anthers	No. pollen calli showing organogenesis	% pollen calli showing organogenesis
4 +	400	366	154	42.08	–	–
7 +	400	374	143	38.24	18	12.59
10 +	400	385	119	30.91	–	–
12 +	400	420	63	15.00	–	–
4 +	800	392	128	32.65	–	–
7 +	800	432	136	13.48	–	–
10 +	800	395	111	28.10	–	–
12 +	800	430	106	24.65	–	–
4 +	1200	398	85	21.36	–	–
7 +	1200	397	78	19.65	–	–
10 +	1200	412	46	11.16	–	–
12 +	1200	440	22	5.00	–	–

Table 3b. Effect of sucrose and glutamine on pollen callus induction in tea anther cultures

Glutamine (mg/l)	Sucrose (% w/v) 4	7	10	12	Means
400	40.54	38.40	33.89	22.85	33.92
800	34.93	33.93	31.85	29.65	32.59
1200	27.23	26.12	19.46	12.63	211.36
Means	34.23	32.82	28.40	21.71	

LSD at 0.05 for Glutamine means = 3.84; sucrose means = 4.44 and for Glutamine x sucrose means = NS
LSD at 0.01 for Glutamine means = 5.13, and for sucrose means = 5.93

(12.59%) showed organogenesis (Table 3a). These shoot apices enlarged in size (Fig. 1g), and sometimes even branched, but did not grow further, even after a further 2-3 weeks on a low sucrose medium.

To achieve complete plantlet development, further modification of the above medium composition by supplementation with serine (100 mg/l) and abscisic acid (0.1 and 1.0 mg/l) was tried in a later set of experiments (Table 4). Results indicated that anthers showed organogenesis in the presence of serine alone, as well as in a combination of serine with abscisic acid at a lower concentration level of 0.1 mg/l. However, no improvement could be achieved in the number of shoot apices, nor in their morphogenetic behavior.

Cytological Study of the Globular Structures

Cytological study of the globular initial structures confirmed their haploid (n = 15) genomic status (Fig. 1h).

Histological Study

Histological examinations showed that the globular structures originated from the surface of the intermediary callus mass. Tissue orientation, and the presence of multicellular epidermal hairs in the histological preparations, confirmed the development of a shoot meristem (Fig. 2a-d).

Table 4 Effect of serine and abscisic acid on organogenesis in pollen callus of tea (cv. T.G.C. – 124/488)

Serine (mg/l)	Abscisic acid (mg/l)	No. cultured anthers	No. callus inducing anthers	% callus inducing anthers	No. pollen calli responded for organogenesis	% pollen calli responded for organogenesis
0 +	0	244	79	32.38	8	10.17
100 +	0	250	46	18.40	3	6.52
0 +	0.1	240	80	33.33	–	–
100 +	0.1	249	75	30.12	5	6.67
0 +	1.0	230	50	21.74	–	–
100 +	1.0	241	59	24.48	–	–

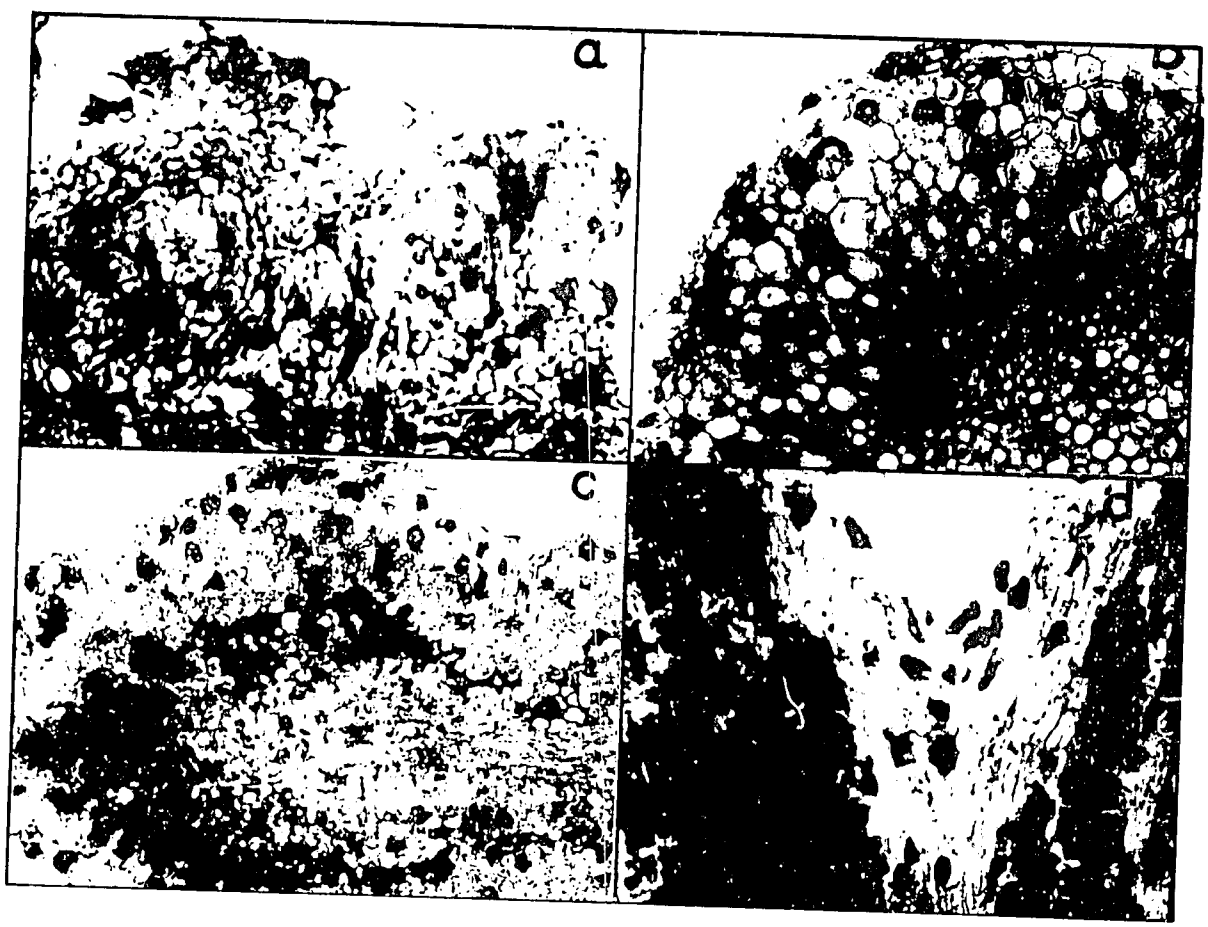

Fig. 2. (a) Microtome T.S. of globular structures
(b) Hand section showing T.S. of shoot apex
(c) Microtome T.S. of shoot apex at point of bifurcation
(d) Microtome L.S. of shoot apex

DISCUSSION

The *in vitro* development of plantlets from somatic callus of tea, utilizing a modified MS medium has already been reported[25, 11, 34]. Induction of callus from the excised anthers of tea on the same medium has also been reported, but the calli did not respond towards organogenesis[34].

At the beginning of our anther culture studies, it was therefore essential to test a wide range of media, including the one used by Wu *et al.*[34]. Our results demonstrated prolific callus growth, which took a compact granular form, on both modified MS and SH media (Table 1). Although callus growth was only moderate on B_5 medium, the suitability of this medium for organogenesis was indicated by the nature of the calli, following 2-3 weeks incubation in light. They differed from other calli, in that they showed numerous whitish globular structures (Fig. 1d), as are often observed at the inductive phase of organogenesis[29] and embryogenesis[5]. As the B_5 original nutrient composition was found to be inadequate in generating further growth in these globular structures, further modification of the medium is essential.

However, before trying any modification of the medium, excised anthers of varying sizes were tested in terms of their responsiveness, for both callus induction and formation of globular structures (Table 2), in an attempt to develop a method of determining optimum pollen size compared with bud size. This study indicated that bud size (0.768 ± 0.006 to 0.827 ± 0.005 cm diameter) could be used to indicate responsive anthers. This method is quite similar to that used by Keller *et al.*[17] for *B. campestris* anther culture.

The auxin – kinetin balance has been shown to play a critical role in the induction of somatic organogenesis in tea[25, 34]. This supports the hypothesis that the hormonal balance has an essential role in directing the course of differentiation[5]. However, our initial attempts towards manipulating the auxin:cytokinin balance in the B_5 medium, using various combinations of 2,4-D, NAA and kinetin (data not shown), did not contribute positively towards organogenesis or embryogenesis, except that they produced a marked increase in the number of globular structures on B_5 medium with 2,4-D 0.1 mg/*l*, NAA 0.1 mg/*l* and Kn 0.1 mg/*l*.

Further modifications of the medium with increased sucrose levels supplemented by varying concentrations of glutamin (Table 3a, 3b) were of interest, since these modifications contribute significantly towards organogenesis, as is evident from the development of shoot apices in the intervening pollen callus (Table 3a, Fig. 1e-g). Although callus induction occurred in all combinations, callus induction gradually decreased with an increase in the concentration of both sucrose and glutamin. It is beyond the scope of the present paper to identify their individual roles in promoting the callus induction at lower concentration levels. Numerous globular structures were found to develop from calli on media combinations modified by increasing the sucrose level to 7%, supplemented with glutamine at the two lower concentrations used. However, a combination of 7% sucrose with 400 mg/*l* glutamine was found to be conducive to organogenesis, in that the globular structures gradually enlarged and developed into shoot apices covered with multicellular shoot hairs. These observations clearly indicate the critical role of sucrose and glutamine in inducing organogenesis in anther derived callus of tea.

The effect of high sucrose concentrations in promoting callus formation have been observed in anther cultures of barley[7], tomato[42], sugarcane[22] and triticale[27]. Our findings with tea anthers indicate the opposite, in that there was a marked decrease in response with higher doses of sucrose. However, sucrose was shown to be an important factor influencing organogenesis in tea anther cultures. A positive response to high sucrose concentrations, in terms of organogenesis[6] and embryogenesis[16, 17, 15], has been reported in the past. These authors indicated that high concentrations of sucrose are necessary for the induction of embryogenesis, but that post-induction transfer helps in plantlet development. However, we failed to achieve plantlet regeneration, even after post-induction transfer to a 2% sucrose level.

Our results also indicated that not only sucrose, but also glutamine, play an essential role in organogenesis in tea. A similar role for glutamine for embryogenesis in *B. campestris* anthers has already been reported[17].

Abscisic acid[1, 30] and L-serine[16, 25] have been reported to have a beneficial effect on organogenesis/embryogenesis. Our attempts in this respect have not yielded any positive result.

Although complete plantlet regeneration from haploid shoot apices (identified as such in cytological analysis) developed from pollen callus is yet to be achieved, we hope that the data presented in this paper will provide useful information as a basis for further development.

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DISCUSSION

Q. (M.T. Senawi)
When you carried out anther culture, how did you ascertain whether the callus was in fact derived from pollen?

A. It is true that in many cases, embryos will develop if you culture the anther directly. The ideal situation is to culture the pollen isolated from the anther, but the anthers are very small in the case of tea, although there are many anthers in each flower. Removing the pollen is a very tricky procedure which we had hoped to be able to avoid, although we now think it may be necessary.

When the calli in our experiments developed, there was always a remnant of the anther wall at the bottom, never the calcified remains of the whole anther. Microscopy could identify the chromosome number, which was either haploid or close to haploid genomic status.

Q. (A.N. Rao)
Why did you start anther culture? Was it for the induction of haploidy?

A. The idea was to obtain haploid or homodiploid (i.e. inbred) lines. Tea is widely crossed, and in any breeding program it is very difficult to get inbred lines.

Comment: (A.N. Rao)
In some of the structures in cultured tissues, it was not quite clear whether these were shoots or roots. This often happens, particularly especially with a highly heterogeneous plant such as tea. If this happens, what you have achieved is undifferentiated hypocotyl. If you take that part, isolate it from your culture, cut it into pieces, and transfer it into a growing medium with auxins, you should soon get root or shoot differentiation.

Comment: (A. Komamine)
The whole problem of differentiation is very interesting one. The papers we have heard show that there are quite different methods of inducing differentiation for different species, which makes me wonder whether any general principle is involved. It is easier to induce differentiation in dicotyledons than in monocotyledons, for example, and there is a particular problem of regeneration in the culture of woody species. Does anyone have any comments on regeneration in woody plants?

A. (A.N. Rao)
The problem with woody plants is the loss in meristematic activity. The meristem of herbaceous plants can normally be kept alive in culture. In the case of woody plants, the nature of the material is quite different. If you take the shoot system of any woody plant, and count from axis 1 to axis 10, normally the buds will not develop in culture. Even if you remove the tip of the apex, apical dominance is lost. In the case of herbaceous plants, on the other hand, apical dominance is a very common phenomenon. Our understanding of woody plants is still very limited.

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