TEST TUBE BREEDING OF RICE BY TISSUE CULTURE

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Introduction

It is urgently needed to increase the agricultural productivity for securing food as a counter-measure of population growth. Breeding should contribute to the expansion of yield potential and to the improvement of the quality of products. The efficiency of breeding depends on the efficient processes of broadening the genetic variability, selection, fixation and rapid propagation.

Conventional breeding in which each process is performed at population and individual plant level has yielded excellent results. However, recent progress in biological sciences reveals that some stages of breeding procedures can be performed more efficiently by adopting tissue, cell culture and molecular manipulation *i.e.* „test tube breeding". Table 1. shows the methods of test tube breeding, which are currently or potentially applicable in plant breeding.

This paper describes characteristics of test tube breeding, in particular, at the tissue and cell level or plant tissue culture, and its application to and efficiency for rice breeding and discusses technical prospects for the future.

Plant tissue culture

Plant tissue culture has progressed since 1930's by the intensive efforts of pioneer researchers, P. R. White, R. J. Gautheret and many other investigators. Plant tissue culture has been utilized in various disciplines not only in agricultural fields, but also in morphogenesis, physiology, pathology, pharmacology, etc.

This technique depends on *in vitro* control of plants. Some organs and tissues in culture can grow in keeping their own forms (root culture in rice, Kawata and Ishihara 1967) and their organic functions (test tube fertilization). It is also possible for excised stem tissues to develop *in vitro* buds and flowers (Nitsch and Nitsch 1967). A state of dedifferentiation is substantially induced from any organ of plants, and from any plant species. As the plant cells have totipotency, intact plants are able to regenerate from single cells. This ability of plant cells in culture which connects manipulation *in vitro* to field cultivation is an important technical factor in practical application. However, under the present situation regarding techniques, the merits of test tube breeding are only applicable to a limited number of crops. The methods for regeneration from cells and callus tissues were not established in all crops, although the phenomena of dedifferentiation and redifferentiation are completely regulated in some crops by changing the cultural media. This is because problems related to the regulation of genes *i.e.*, gene control of differentiation including cell division, meiosis and biosynthesis, etc. are not yet solved. Still it is possible to apply the test tube breeding method to many crops, by the accumulated knowledge of tissue culture. This promising technique has several characteristics which can be applied to each process of test tube breeding.

1 Variability in culture

Several methods in tissue culture can be considered for broadening the genetic variability such as mutation, somatic hybridization by using protoplasts and transformation by uptake of exogeneous DNA. Variant cells induced through these methods are usually cultured in a dedifferentiated state and under aseptic culture conditions. Therefore the manifestations of genes on protection systems against microorganism infections or environmental pressures are

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Table 1. Methods in test tube breeding.

		Breeding process				
		Variation	Fixation	Selection	Propagation	Gene preservation
Intact plant		Mutation Hybridization Recombination Ploidy	Pedigree Bulk	Genotype Phenotype	Seed Vegetative propagation	Low temparature storage Conservative cultivation
Test tube	Organ	Embryo culture Test-tube fertilization		Meristem culture (virus free)	Clonal multiplication	
	Tissue and Cell	Mutation Somatic hybridization Transformation	Anther and pollen culture	Cultural environment Genotype Phenotype	Clonal multiplication	Freeze storage in super-low temperature
	Organelle	Mutation Recombination (Uptaking)			Organelle culture	
	Molecule	Recombinant DNA molecule Hybrid plasmid	Gene cloning	Colony hybridization Hybridization	Gene amplification	Freezing Lyophilizing bacteria

not required for their survival in culture. For instance, in rice varieties there are several different genes against the rice blast disease, but the blast resistance in cultured tissues was not demonstrated as they all were susceptible to the fungus (Ishii and Sakamoto 1975). And, callus tissues of monocotyledone, such as rice, wheat, rye, etc. cultured with 2, 4-D (2, 4-dichlorophenoxy acetic acid) did not synthesize chlorophyll, and there was no difference of growth between cultured tissues induced from albino and normal plants.

In natural environment, those individuals with incomplete or unbalanced genetic information, such as mutants, aneuploids and intergeneric hybrids etc. hardly grow as intact plants. But in culture conditions, those genetically unbalanced cells, even inter-family somatic hybrids and interkingdom plant-animal somatic hybrids, can survive as heterogeneous populations with or without competition with normal cells. Chromosome numbers are variable in many cultured tissues, and in some cases aneuploid cells became dominant in culture. These kinds of weak selection pressures in culture are a primary factor for broadening the genetic variation.

2 Fixation with the culture of haploid cells

Haploid, diploid and triploid plants were induced by tissue culture of pollen, protoplast, cultured single cell and endosperm cell. Haploid breeding by anther culture depends on the efficient process of pollen to plant regeneration and diploidization. Since it is possible to consider that a single cell is an intact plant with full genetic diversity, pollen culture of hybrid plants with different genotypes using 1 ml medium, may be the equivalent for the cultivation of thousands of plants. If the selection systems from a large quantity of cultured cells become available with correspondence to specific genes in culture and desirable characters in intact plants, efficiency of the selection by tissue culture will remarkably increase.

3 Selection with the control of cultural environment

Generally, it is easy to control the cultural environment. The physical aspects against cultured cells are temperature, day length, intensity and quality of light, osmotic pressure and gaseous conditions, etc. Culturing on agar media or in liquid media *per se* is somewhat different

from the cultivation of intact plants in natural environment. Chemical aspects of environment are pH, buffer action of media, medium constituents, such as nutrients, metabolic precursors, plant growth regulators, growth inhibitors, mutagens, etc. Biological aspects consist of uptake and release of substances by cultured cells, cell densities in culture, cell to cell contact, competition between cultured cells and symbiosis with other organisms, etc. These factors might play an important role in selection for test tube breeding.

4 Propagation

Propagation of plant with the same genetic background is possible by the rapid growth of cultured cells or organs and regeneration from them, although, genetically aberrant plants, especially polyploid plants, are frequently observed in tissue culture. Substantial number of genetic variants with various chromosome numbers were also induced from shoot apex calluses in sugar cane (Liu and Chen 1976). However, the frequency of genetically aberrant plants can be reduced considerably, even avoided entirely, if the plant multiplication can be achieved through an enhancement of axillary shoot formation, *e.g. Asparagus officinalis* and *Gerbera jamesoni* (Murashige 1974).

Presently, for avoiding genetically aberrant plants it might be important to obtain plants from the culture which keeps a higher level of organization, and not from dedifferentiated tissue or cells. At any rate, vegetative propagation by tissue culture has been practically established as an effective technique in orchids and several garden crops.

5 Preservation and genetic stability

It is a well known phenomenon that cultured tissues often include cells with diverse chromosome number, with some exceptions. As endopolyploidy occurs *in vivo,* it is difficult to say that the variations of chromosome number are induced only by culture conditions *in vitro.* However, most of the established media in tissue culture have been developed along criteria of growth rate of cultured tissues, and as described later, the high frequencies of gene mutations were induced in rice tissue culture. The variations of chromosome number or mutations might be easily inducible in cultured tissues in any other crops. The genetic instability of cultured cells can be applied to increase genetic variability.

On the other hand, the instability of cultured cells will cause some difficulties in applying them to the preservation of genetic resources by using tissue culture. The preservation of gene resources, not only of seed propagating crops, but also of vegetatively propagating crops is important. The preservation methods of seed propagating crops are practically established, but in vegetatively propagating crops many difficulties persist. An advantage of using tissue culture in preservation is that single cells are resistant to low temperature. Combination of tissue culture with strorage at very low temperature which preserves the genetic stability of cultured cell by freezing, will help the storage of genetic resources especially in vegetatively propagating crops. At any rate, it is necessary to develop a method enabling the cells in culture to be genetically stable.

Tissue culture of rice

Tissue culture of rice started with embryo culture (Amemiya, Akemine and Toriyama 1956), and by using this technique hybrid plants between tetraploid of *Oryza sativa* and *Oryza minuta* or *Oryza sp.* (Paraguay) were obtained (Nakajima and Morishima 1958). Meanwhile, callus induction and its subculture, which had been considered to be difficult to achieve in rice compared with dicotyledone, was successfully obtained by culturing explant of stem node with 2, 4-D media (Furuhashi and Yatazawa 1964). Subsequently, regeneration of the plants could be obtained by using the callus induced from seed (Tamura 1968, Maeda 1968, Nishi, Yamada and Takahashi 1968), from root (Kawata and Ishihara 1968) and from pollen (Niizeki and Oono 1968). Plant regeneration rate from seed callus was *ca.* 80% using media with low concentration of auxin and high concentration of sucrose, yeast extract or benzyladenine (Oono 1975).

Fixation by anther culture

It usually takes many years to obtain the homozygous plants with conventional breeding methods. But diploidization of haploids will easily establish fixed lines. Haploids have been induced by interspecific and intergeneric hybridization, pollinators, delayed pollination, substitution of nucleus, multigerm plants, irradiation, etc. However, haploids were not sufficiently induced from desired materials by these methods, and their application for breeding was practically difficult. Haploidy is induced by meiosis or somatic reduction and the culture of those tissues will induce haploid calluses and plants. Haploid calluses were first induced from pollen culture of *Ginkgo biloba* (Tulecke 1953), and from female gametophyte of *G. biloba* (Tulecke 1964) Subsequently, embryoids induced from pollen were obtained by anther culture of *Dature innoxia* (Guha and Maheshwari 1964), plants through embryoids induced from pollen in *Nicotiana tabacum* (Bourgin and Nitsch 1967, Nakata and Tanaka 1968) and plants through callus induced from pollen in *Oryza sativa* (Niizeki and Oono 1968). These results revealed the possibility of achieving haploid breeding crops by anther culture. At present the induction of haploid plants by anther culture has already been successfully realized in more than 30 species of crops. Haploid plants were obtained through embryoid or callus induced from pollen at uninucleate stage which was adequate for anther culture in most of the crops. Haploid rice plants (2n = 12) were obtained from pollen callus and induction rate of callus was improved from 0.6% (Niizeki and Oono 1968) to 50% (Yin *et al.* 1976) although the rate differed depending on varieties. Induced calluses from pollen were different in their morphology and function of biosynthesis even in early days of culture (Fig. 1). Also the calluses

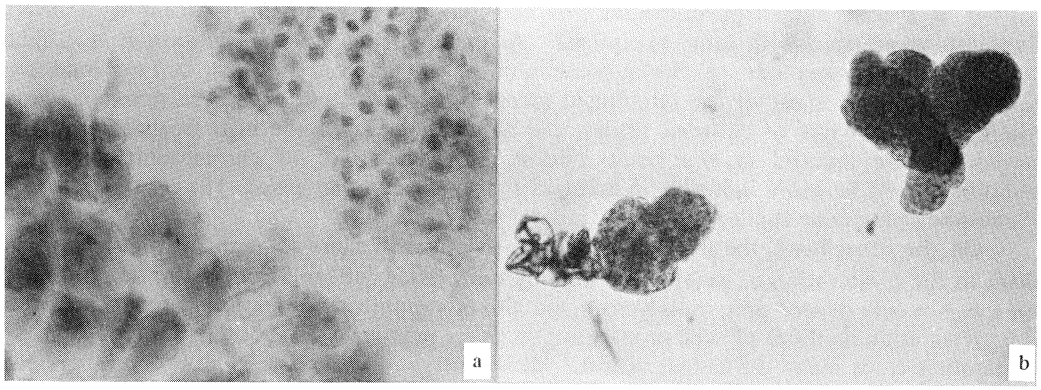

Fig. 1. Differentiation of calluses.
a: 50 day-old pollen callus (callus derived from a single pollen), cell size and nuclear size were different in a callus.
b: Two calluses in the same anther, showing different responses to iodine potassium (left side, callus with accumulated starch grains).

consisted of cells with different nuclear sizes and different chromosome number. Fig. 2 shows the variation of chromosome number of calluses obtained after 50 to 55 days incubation of anthers. Calluses were induced on media with 2, 4-D or NAA (α-naphthaleneacetic acid), and the fresh weight of calluses ranged from less than 10 mg to 1000 mg. Although the tendency for chromosome variation of calluses was similar in both media, significant differences were observed in each callus. The proportion of haploid cells was 34.5% (69/200 cells) in calluses induced by 2, 4-D and 31.5% (45/143 cells) by NAA. The highest number of chromosomes observed was 114 on NAA medium. The reason for the variation in chromosome number in rice anther culture is considered to be due to repeated endomitosis and abnormal multipolar nuclear divisions. These results show that the callus tissues mixed with cells of different chromosome number may easily vary. These phenomena could be used for inducing

homozygous diploid plants directly from pollen calluses. Fifty to eighty per cent of inoculated pollen calluses regenerated plants in early culture stages. But the regeneration rates rapidly decreased by prolonged culture and disappeared completely after 120 days of culture (Wang *et al.* 1974) or by subculture (Oono 1975). Attempts to prevent the decrease by changing the medium constituents are not successful yet. The loss of the ability of regeneration was also reported in carrot (Syono 1965) and tobacco (Murashige and Nakano 1965). These phenomena were generally attributed to "habituation", as both physiological and genetical changes bring about the loss of regeneration ability of cultured cells. The induction of haploids in rice anther culture through calluses has several advantages. Direct regeneration of plants from inoculated anthers aimed at improving the efficiency should be studied further. Direct regeneration of plant by using medium with NAA instead of 2, 4-D (Niizeki and Oono 1971), or embryoid formation like anther culture of *N. tabacum* (Guha-Mukherjee 1973) were observed in rice anther culture.

The regenerated plants by anther culture were not only haploid but diploid, triploid, tetraploid, pentaploid and aneuploid. It should be emphasized that a high frequency of diploids was obtained in rice anther culture. The range of diploids in regenerated plants was 50 to 60% against 30 to 40% in haploids (Oono 1975, Mok and Woo 1976, Academia Sinica 1976, Chen and Lin 1976). The frequencies of haploid (2n = 12) and diploid cells (2n = 24) were almost the same in pollen calluses (Fig. 2). Nevertheless the high frequency of the regeneration of diploid plants indicated the existence of some superiority of diploid plant to haploids or a selection mechanism for diploid. These diploid plants were confirmed to have a pollen callus origin through the progeny tests. High frequency of diploid regeneration is desirable for application to breeding. The higher the frequency of diploid, the greater the efficiency.

Chlorophyll deficient plants, especially albino were frequently regenerated from pollen callus. Sun *et al.* (1974) observed rice albino plants by electron microscopy and found that proplastids with primary lamellae existed in the leaf cells. However, the proplastids of the

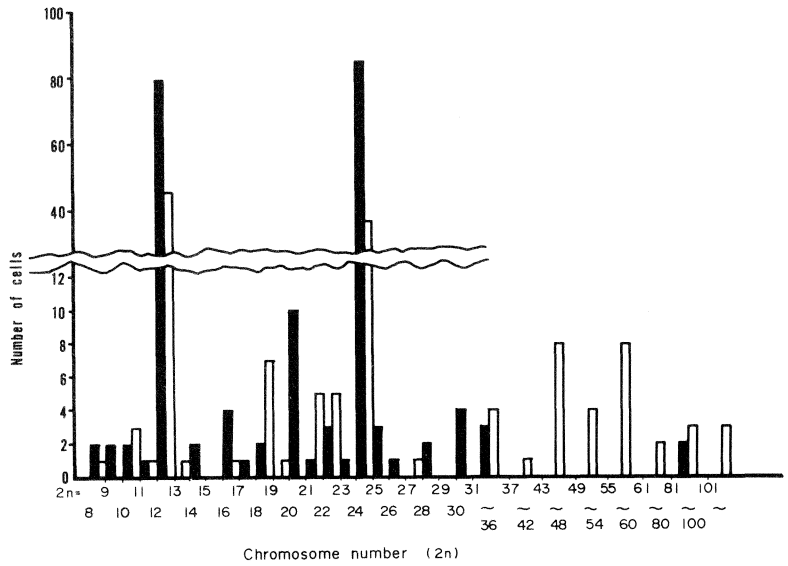

Fig. 2. Chromosome number of cells of pollen calluses.
Materials: Calluses obtained after 50 – 55 days incubation of anthers of F_1 plant in Arborio x Loctjan. Total number of observed calluses = 22. Total number of observed cells = 343.
■ : Calluses obtained on medium of Miller + 2,4-D(5×10^{-6} mol)
□ : Calluses obtained on medium of Miller + NAA(5×10^{-6} mol)

albino could not develop into normal chloroplasts with granum lamellae. In the albino plastids DNA-like fibrils were present, but ribosomes were absent. They considered that the absence of ribosomes was directly responsible for albino formation. Offsprings of albino have not been obtained yet, but further studies on them will provide information on the cause of albinism. However the chlorophyll mutation of green plants regenerated with albino in the

Table 2. Comparison of some characters among progenies of diploid pollen plants and parental variety.

Variety and progeny	A_2 - (1970) No. of plants	Heading date	Plant height (cm)	Panicle length (cm)	No. of panicles per plant	Seed fertility (%)
Minehikari	39	Aug. 26	94.5	20.5	15.0	94.5
Y-861	23	Aug. 25	96.5	21.3	21.6	95.1
Y-864	20	Aug. 25	60.3**	14.8**	44.0**	86.7*
Y-1052	26	Aug. 25	66.3**	14.5**	35.4**	86.2*
Y-1054	13	Aug. 25	67.8**	15.2**	35.2**	93.1
Y-1060	24	Aug. 26	97.3	20.9	17.2	95.8
Y-1063-1	13	Aug. 26	98.9	21.3	15.7	94.0
Y-1063-2	26	Aug. 25	98.2	20.6	16.3	93.7
Y-1063-3	25	Aug. 26	97.6	21.2	14.2	89.3
Y-1069	25	Aug. 25	95.4	20.1	19.8	96.7
Y-1071	13	Aug. 24	90.4	21.6	19.0	91.6
Y-1329	12	Aug. 24	60.1**	13.8**	34.5**	91.1

Variety and progeny	A_3 - (1971) No. of plants	Heading date	Plant height (cm)	Panicle length (cm)	No. of panicles per plant	Seed fertility (%)
Minehikari	39	Aug. 25	100.1	19.6	12.3	93.3
Y-861	39	Aug. 25	97.7	19.7	16.1	91.9
Y-864	37	Aug. 25	61.8**	13.3**	39.6**(13.1)	91.5
Y-1052	38	Aug. 25	68.9**	13.5**	29.2**(11.3)	86.4
Y-1054	38	Aug. 25	73.1**	14.8**	29.3**(12.7)	94.8
Y-1060	39	Aug. 25	99.7	19.6	15.0	93.0
Y-1063-1	39	Aug. 25	98.1	20.4	13.7	93.7
Y-1063-2	39	Aug. 25	100.2	20.8	13.4	93.9
Y-1063-3	38	Aug. 25	95.5	20.3	13.1	93.0
Y-1069	39	Aug. 25	91.5	19.0	16.0	95.0
Y-1071	39	Aug. 25	87.1	20.2	12.7	91.6
Y-1329	39	Aug. 25	60.3**	13.6**	39.8**(15.7)	90.5

Material: Minehikari
Seeding: May 22, 1970 Transplanting: June 23, 1970
: June 1, 1971 : June 23, 1971
* Significant at 5% level
** Significant at 1% level

Table 3. Comparison of some characters among progenies of pollen plants, two parental varieties and A_2 and A_3 strains.

Parent and progeny	Generation	No. of plants	Heading date	Plant height (cm)	Panicle length (cm)	No. of effective tillers per plant	Seed fertility (%)
Muyozetsuto (female parent)		39	Sep. 13 ± 1	127.4 ± 5.2	22.8 ± 1.4	14.5 ± 3.6	83.4
B_1F_2 (F_2 of male parent)		39	Oct. 8 ± 13	110.3 ± 15.3	24.4 ± 2.3	15.5 ± 4.3	28.8
F_2 of hybrid		38	Sep. 20 ± 6	129.1 ± 16.7	20.3 ± 4.0	14.9 ± 4.0	79.2
AG-1-2 (*maculata*)	A_2 (1970)	2	Sep. 10	73.0 ± 0	15.0 ± 0	11.0 ± 0	–
AG-1-15		16	Sep. 3 ± 1	106.9 ± 6.2	22.2 ± 1.5	14.7 ± 5.7	87.3
AG-1-22 (normal)		27	Sep. 2 ± 1	108.6 ± 4.3	22.4 ± 1.6	13.0 ± 3.5	88.3
AG-1-22 (short culm)		10	Sep. 2 ± 1	72.0 ± 4.1	17.6 ± 2.4	9.2 ± 3.9	68.8
AG-1-24		39	Sep. 4 ± 1	113.1 ± 6.4	22.9 ± 1.5	13.2 ± 4.4	86.1
AG-1-2 (*maculata*)	A_3 (1971)	50	Sep. 8	58.3 ± 4.7	16.7 ± 1.0	15.3 ± 6.2	–
AG-1-22 (normal)		56	Sep. 8	94.4 ± 6.3	22.6 ± 1.7	13.1 ± 4.6	–
AG-1-22 (short culm)		82	Sep. 8	67.2 ± 4.9	20.2 ± 2.0	10.1 ± 2.8	–
AG-1-24		54	Sep. 8	98.9 ± 3.9	23.5 ± 1.3	11.3 ± 2.7	–

Material: Muyozetsuto x [Kinmaze x Morak Sepilai) x Kinmaze B_1F_1]
Seeding: May 22, 1970 Transplanting: June 23, 1970
: June 1, 1971 : June 23, 1971

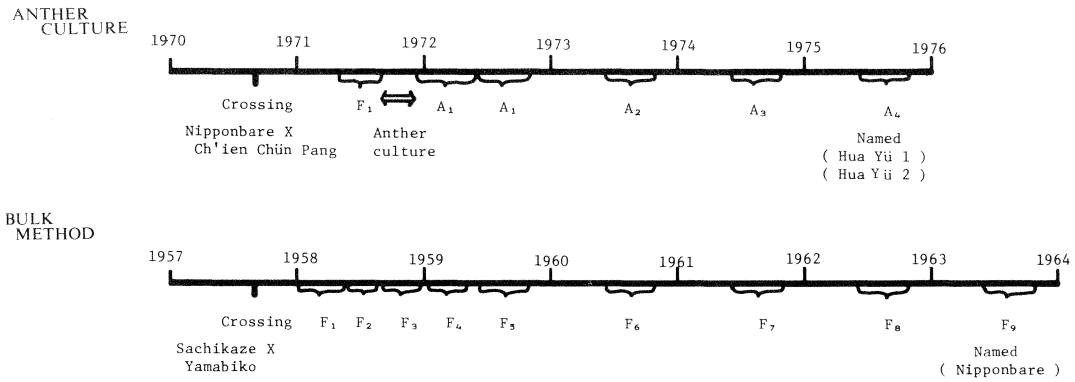

Fig. 3. Breeding of new varieties by anther culture and bulk method.

same test tube from the same seed calluses was investigated in the offsprings. Relatively high frequencies of chlorophyll mutants were observed, but there were not restricted to albino in M_2 generation (unpublished data). These results suggest that some of the albino were chromosomal mutants, but that others resulted from incompleteness of chloroplasts in the callus culture or at the time of redifferentiation. In any case, prevention of albino regeneration is one of the problems to be solved for efficient application for breeding.

Besides albino or high ploidy plants, a number of homozygous lines from haploids and

spontaneous diploids were obtained. Their progenies were homozygous and inbreeding depression was not observed (Table 2). Fixed lines were also obtained from F_1 of an intersubspecies hybrid, *Indica* x *Japonica*, by anther culture (Table 3), proving that anther culture is really an efficient method for establishing pure lines. Several improved varieties have already been bred by anther culture. Fig. 3 compares the breeding process of the first varieties, Hua Yü 1 and 2, which were bred by anther culture (Rice Research Laboratory *et al.* 1976) and those of Nipponbare obtained by bulk methods. Also results from the first successful application of anther culture on rice in 1968 and successful breeding of new varieties by anther culture confirm the efficiency of this method.

Induction of variability

The basic source of genetic variations is represented by gene mutation, and change of chromosomal structures and chromosome numbers. Therefore, many investigations on mutation have been studied by irradiation and chemical mutagen treatment. Induction of genetic variants in tissue culture has also been investigated by irradiation (Howland and Hart 1976) and chemical mutagen (see: Table 5) as the use of tissue culture offers great advantage in enabling the treatment of millions of cells. However, the merits of *in vitro* cell culture cannot be fully exploited owing to insufficient screening of variant cells and regeneration from cultured cells. On the basis of recent progress in anther culture, it was reported that many chlorophyll deficient plants and polyploids regenerated from pollen calluses, and Oono (1975) suggested the occurrence of gene mutations during culture. If high frequencies of gene mutations were induced in culture, it would be possible to increase the efficiency of test tube breeding combined with haploid breeding by anther culture and to perform an important part in breeding.

It was confirmed through the analysis of regenerated plants from diploid rice callus that mutations were easily induced in cultured cells. Calluses were induced from the seeds of

Table 4. Chlorophyll mutation in immediate progenies (D_2) of regenerated plants from rice culluses.

No. of panicles examined	No. of panicles mutated	Type of mutation						Mutation %
		Albina	*Xantha*	*Viridis*	*Striata*	Others	Total	
438	34	11	9	7	4	6	37	8.4
(Relative frequency %)		29.7	24.3	18.9	10.8	16.2	100	

$$\text{Mutation frequency (\%)} = \frac{\text{Total No. of mutation in panicles}}{\text{No. of panicles examined}} \times 100$$

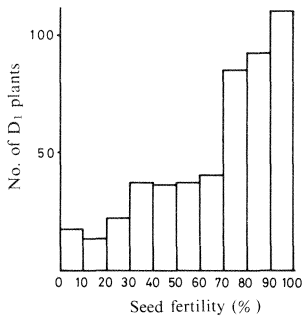

Fig. 4. Variation of seed fertility of D_1 plants regenerated from rice seed calluses.

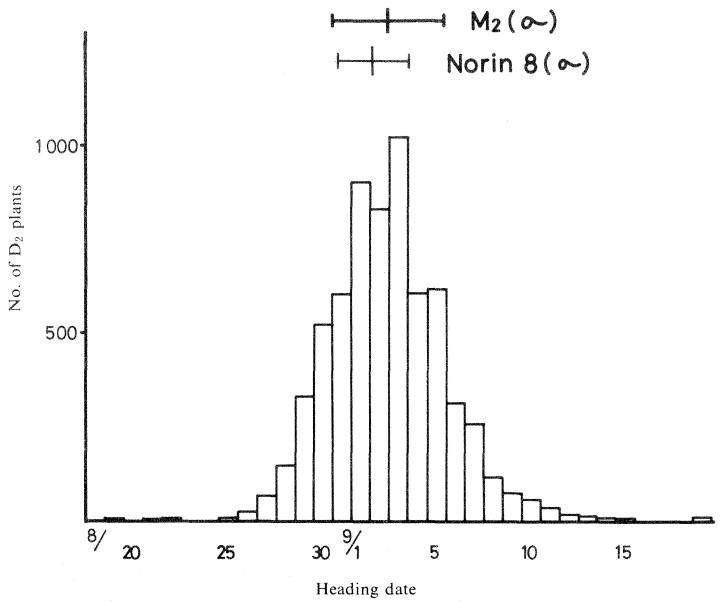

Fig. 5. Variation of heading date of D_2 plants regenerated from rice calluses.

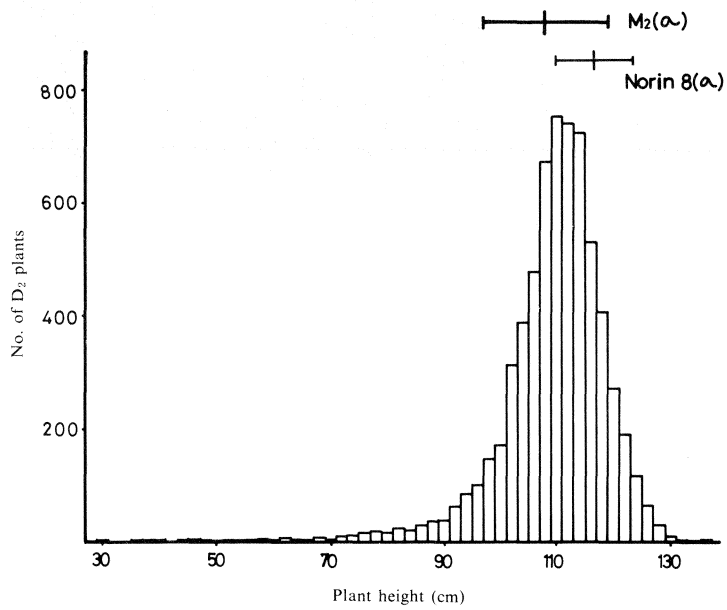

Fig. 6. Variation of plant height of D_2 plants regenerated from rice calluses.

spontaneous diploid from spontaneous haploid and plants were regenerated from them without subculture. Seventy five seeds were used and 1121 regenerated plants (D_1) were obtained with 83 albino plants (7.4%). Average number of plants obtained from 1 seed callus was 10.7

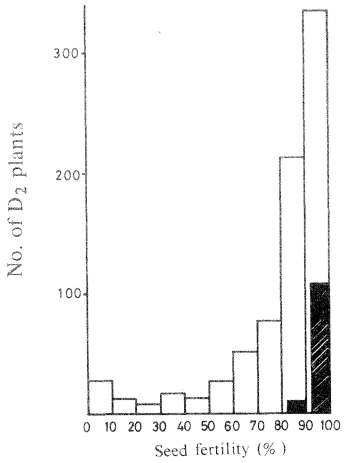

Fig. 7 **Variation of seed fertility of D_2 plants regenerated from rice calluses.**
D_2: □ , Control (Norin 8) : ▨

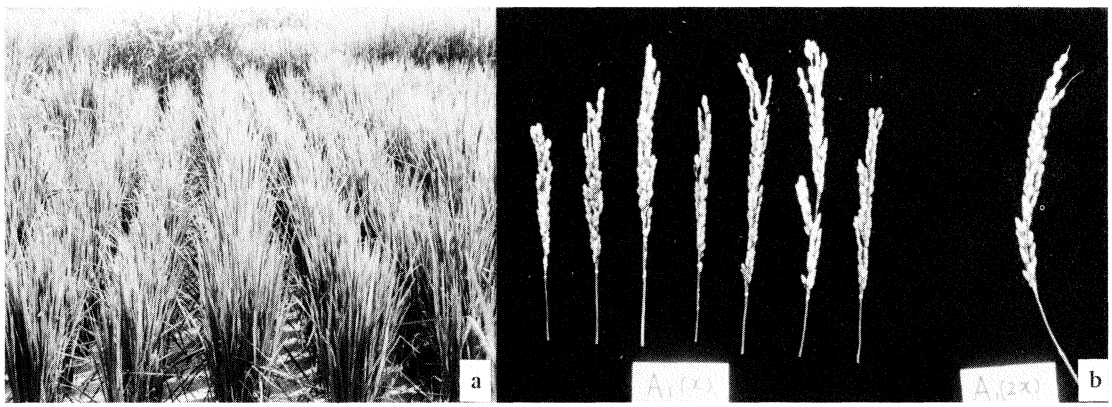

Fig. 8. Comparison of 15 vegetatively propagated haploid lines obtained from a pollen callus.
a : Difference in plant height in the field
b : Panicles of different haploid lines.

plants. Analysis of mutation was performed on ca. 800 D_1 and their progenies D_2 and D_3.

Fig. 4 shows the seed fertility of D_1 plants where 58.7% of D_1 plants had a decreased seed fertility of less than 80% (although, some of them were not genetical mutants). Table 4. shows the chlorophyll mutants in D_2. Mutation rate was 8.4%, and this rate was comparable to the rates following X-ray and γ-ray irradiation. Fig. 5–7. show several agronomic characters of D_2 generation. Heading date of D_2 varied from Aug. 19 to Oct. 7 (average date; Sep. 2.4 ± 2.9 days) with continuous distributions, compared with the control of Aug. 28 to Sep. 7 (average date; Sep. 1.7 ± 1.7 days). Both early and late maturing variations were induced in culture (Fig. 5). Analysis between the pedigrees of seed calluses showed that some pedigrees were significant in variance against control. Plant height varied significantly in D_2 (average height; 107.9 ± 11.2 cm) against control (116.9 ± 6.8 cm) (Fig. 6). Frequency of D_2 plants with less than 90 cm in height was 4.1%, and dwarf mutants (less than 50cm) were mostly sterile or displayed malformations. Seed fertility of D_2 in sampling inspection varied from 0 to 99% compared with 80 to 99% in the case of the control. Thirty percent of plants were mutated to less than 80% of seed fertility and 8.5% of the plants to less than 40% of seed fertility (Fig. 7).

Analysis of mutations for chlorophyll, flowering, plant height, fertility and morphology in D_1, D_2 and D_3 generations reveals that only 28.1% of regenerated plants were normal in all these characters. Mutation frequencies depended partially on medium conditions. Sectorial analysis of these mutated characters in plants originating from the same seed callus showed that mutations were induced repeatedly in the cells as in clonic irradiations.

Variations were also observed in the haploid plants regenerated by anther culture(Oono 1975). Fig. 8 shows the haploid lines from one pollen callus which showed variations in plant height and panicle length compared with vegetatively propagated lines. These variations could be caused by polygenic mutations. The frequencies of mutations in culture were higher than expected. The control of mutation frequencies through culture conditions is an interesting problem which needs further investigations.

Selection

Mutant cell lines were selected by culture in several crops (Table 5). However, only a few reports analysed the retained traits on regenerated plants. As described before, high frequency of gene mutations was induced repeatedly in rice tissue culture. It is therefore, expected that efficient selection will be possible by adequate selection pressure on cultured cells. For instance, many varieties were studied for the screening of saline resistant genes (IRRI 1975, El-Keredy *et al.* 1976).

However, if the tolerance to salinity were to increase by continuous mutations and the selections by tissue culture for millions at the cell level, the tolerant cells could be selected even if the mutation rates were as low as the rate of 10^{-6} in one gene like in spontaneous mutations. Actually, the rice calluses are growing on a medium with 1% NaCl (unpublished data), and further studies are needed to establish whether the saline resistance can be maintained in intact regenerated plants.

The cell mutant selection area in plant tissue culture has just begun and the mutants to be selected will afford many varied and important uses in the future.

Future prospects for test tube breeding

Investigations at the molecular, organelle and cell levels have experienced a rapid development in the recent years. As shown in Table 1, plant breeding methods will be efficiently improved with these developments.

Pure line induction by anther culture has reached the level of practical breeding in several crops, and the success of isolated pollen cultures (Nitsch 1976) will also increase the efficiency of breeding. Broadening the genetic variability by protoplast fusion will be successful in culture of fused cells. The induction of interspecific hybrid plants obtained by protoplast fusion was successfully achieved between *Nicotiana glauca* and *N. langsdorffii* (Carlson 1972). In heterokaryocytes of soybean-*Nicotiana*, a chromosomal reduction similar to that of fused animal cells was reported by Kao (1977), also combined manifestations of genetic characters after six month culture were reported by Wetter (1977), and the parasexual hybrid plants might even regenerate in the near future. Uptaking of heterogeneous organelles is also under investigation (Cocking 1975, Giles 1976). Modification of photosynthetic pathway of C_3 to C_4 in chloroplast is also interesting for improving the production of plants through organelle breeding.

Especially, recent remarkable progress in molecular biology should enable to introduce bacterial nitrogen fixation gene (*Nif* gene) to plant cells using plasmid as a vector (Shanmugam and Velentine 1975). These studies offer great opportunities of broadening the genetic variabilities and of introducing in plant breeding useful genes from all over the biological world. We expect that this progress will greatly contribute to the efficiency of plant breeding.

Table 5. Selection and characteristics of mutants by tissue culture.

Species	Mutagen treatment	Selection	Characteristics	Reference
Acer pseudoplatanus	nitrosoguanidine	azaguanin	resistant cell lines reduction of hypoxanthine phosphoribosyl transferase	Bright & Northcote (1975)
Capsicum annuum (haploid & diploid)		NaCl	resistant cell lines	Dix & Street (1975)
Capsicum annuum (haploid & diploid)	ethyl methane sulfonate (EMS)	chilling	resistant cell lines	Dix & Street (1976)
Daucus carota	EMS ultraviolet light	DL-5-methyl-tryptophan	resistant cell lines resistant to other tryptophan analogues resistance trait was carried from culture to plant and back	Widholm (1972)
D. carota		p-fluorophenylalanine	resistant cell lines	Widholm (1974)
Glycine max	N-methyl-N'-nitro-N-nitrosoguanidine	5-bromodeoxyuridine (BUdR)	resistant cell lines	Ohyama (1974)
Nicotiana sylyestiris (haploid & diploid)		NaCl	resistant cell lines	Dix & Street (1975)
N. sylvestris	EMS	chilling	resistant cell lines	Dix & Street (1976)
Nicotiana tabacum (haploid)	EMS	BUdR	leaky auxotrophic line	Carlson (1970)
N. tabacum	EMS	methionine sulfoximine	regenerated plants were less susceptible to *Pseudomonas tabaci*	Carlson (1973)
N. tabacum (doploid)	N-methyl-N'-nitro N-nitrosoguanidine	theonine	resistant cell lines	Heimer & Filner (1970)
N. tabacum (diploid)		8-azaguanine	resistant cell lines	Lescure (1973)
N. tabacum (haploid)		streptomycine sulfate	resistant plants maternal inheritance	Maliga, Breznovits & Maiton (1973)
N. tabacum (haploid)	EMS	cycloheximide	non-genetic drug resistance	Maliga, Lazar, Svab & Nagy (1976)
N. tabacum		BUdR	resistant cell lines	Maliga, Marton & Breznovits (1973)
N. tabacum			BUdR resistance in regenerated plants is controlled by a simple Mendelian factor	Marton & Maliga (1975)
N. tabacum	EMS	NaCl	resistant cell lines	Nabors, Daniels, Nadolny & Brown (1975)
N. tabacum		carboxin	plants regenerated	Palacco & Palacco (1977)
N. tabacum	EMS, UV	DL-5 methyltryptophan	resistant cell lines	Widholm (1972)
N. tabacum		p-fluorophenylalanine	resistant cell lines	Widholm (1974)
Petunia hybrida (haploid)		streptomycine sulfate	resistant cell lines	Binding, Binding & Straub (1970)
P. hybrida (diploid)			not obtained	Binding (1972)
Zea mays		toxin of *Helminthosporium maydis*	resistant cell lines	Gengenbach & Green (1975)

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Discussion

T. Oinuma, Japan: I am most interested in the wide range of variation of chromosome number among anther derived rice plants. This phenomenon may be due to the formation of callus in the anther culture. Is it possible in rice to produce haploids without passing through the stage of callus formation?

Answer: Yes, it is possible. Embryoid formation in rice has already been reported by Dr. Guha-Mukherjee in 1973.

K. Sakai, Japan: If tissue culture methods are used for practical breeding, can we expect to obtain desirable genotypes as effectively as with traditional methods?

Answer: One of the limitations of anther culture is that the frequency of recombinants may be low. The additional use of irradiation might be effective to increase the recombinants.

Y. Hojo, Japan: I think that the variation in chromosome number induces irregular differentiation or morphogenesis in cells and tissues. Do you trace the process of differentiation or morphogenesis in the cell clusters?

Answer: No, I don't. Although the variation of chromosome number in callus tissues was wide, that of regenerated plants by rice anther culture led to haploid and diploid tissues with a small number of polyploid and aneuploid ones. Some selection mechanism to eliminate plants with abnormal chromosome number could be considered. Also, mutations in differentiated plants increased by subculture of callus.

J. T. Rao, India (comment): This new technique can be considered from two points of view. First, it should be possible to isolate disease-free tissue from a diseased somatic tissue.

For instance we have been able to isolate a mosaic free tissue and raise a plant from the diseased tissue. Such technique could also contribute to maintain a large collection of germ plasms in a small area.

Answer: Thank you for your comment.

G. S. Khush, The Philippines: Have you been able to obtain a single cell suspension culture of rice from the callus? Mutagenic treatment of single cell suspension culture should result in higher mutation frequency as compared with mutagenic treatment of the callus.

Answer: We haven't tried it yet. Dr. T. Kawai has tried to obtain single cell culture of rice by suspension culture but this experiment failed.