TOBACCO BREEDING BY ANTHER CULTURE

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Introduction

Because of its great usefulness in genetic studies and practical breeding, experimental haploidy has attracted much attention on the part of geneticists and plant breeders. Guha and Maheshwari (1964) first reported the *in vitro* production of embryos from anthers of *Datura*. Since then, a number of studies on anther culture in several crops have been published.

In tobacco (*Nicotiana tabacum*), the technique of anther culture has made such great progress in the past ten years that we can now produce a large number of haploid plants without any difficulty. Other important problems such as the chromosome doubling method, characteristics of haploids and doubled haploids, and agronomic adaptability of doubled haploid lines were studied to establish the haploid method of tobacco breeding. These experimental results are systematized and now contribute to the development of new tobacco varieties in Japan.

Tobacco breeding by anther culture was formerly reviewed by Nakamura *et al.* (1974). In this paper, recent studies made at our Institute are reviewed, and the haploid method of tobacco breeding will be outlined.

Anther culture technique

Nakamura and Itagaki (1973) examined some anther culture media and found that the medium shown in Table 1 gave the best result. We plate the anthers of pollen at the uni-nucleate stage aseptically on the medium and keep them at a constant temperature of 25° C and under continuous fluorescent lighting. One week after plating, the anthers turn brown, and four to five weeks after plating, haploid plantlets develop from the anthers.

Table 1. Composition of the anther culture medium (mg/litre of water)

KNO_3	250.0	IAA	2.0
$Ca(NO_3)_2\ 4H_2O$	300.0	Myo-inositol	100.0
KH_2PO_4	100.0	Thiamine hydrochloride	0.4
$MgSO_4\ 7H_2O$	100.0	Adenine sulphate	10.0
$MnSO_4\ 4H_2O$	10.0	Active carbon	3g
$FeSO_4\ 7H_2O$	27.85	Sucrose	30g
Na_2EDTA	37.35	Agar	6g

The pH of the medium is adjusted to 6.7 with HCl or NaOH before adding agar.

Chromosome doubling techniques

Three methods of chromosome doubling have been developed. The first one is the tissue culture method developed by Kadotani (1969). By culturing pith or root of haploids, we can double the number of chromosomes at a high rate. However, this method is difficult. The

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second is the colchicine treatment of inflorescence of haploids. When haploids are grown up to the flowering stage, the inflorescence is soaked in a 0.2% colchicine solution for 48 hours. The soaked inflorescence dies after treatment, but the chromosome-doubled shoots develop to give fertile flowers. By this method, we can expect that the rate of chromosome doubling will be 30% on the average. The third is the colchicine treatment of haploid plantlets. Burk *et al.* (1972) reported that the treatment of haploid plantlets with a 0.4% colchicine solution for three to four hours was effective for chromosome doubling. We tried their method several times, but failed to obtain satisfactory results. Then we examined the various combinations of concentrations of colchicine solution and hours of treatment for enhancing the chromosome doubling rate.

Table 2 shows results of the experiment. In this experiment, haploid plantlets obtained from the anthers 45 to 50 days after plating were treated with the nine combinations of colchicine concentration and treatment hours at 25° C. Some of the combinations gave a doubling rate as high as 50%, and there was a tendency for the doubling rate to increase with the increase of colchicine concentration and treatment hours. There seemed to be the same tendency for the induction of tetraploids. From these results, the combination of a 0.2% colchicine solution and 48-72 treatment hours is recommended. We used this method in recent breeding programs.

Table 2. Effect of the colchicine concentration and treatment hours on the chromosome doubling rate of haploid plantlets

Treat. hour	Colchicine %	No. of plants			% of 2n
		treated	2n	4n	
24	0.05	62	6	1	9.7
24	0.10	55	13	2	23.6
24	0.20	40	16	3	40.0
48	0.05	57	11	0	19.3
48	0.10	35	11	1	31.4
48	0.20	33	15	3	45.5
72	0.05	8	1	0	12.5
72	0.10	18	6	1	33.3
72	0.20	25	14	5	56.0

Selection of doubled haploids

It has been required to distinguish diploidized plants from haploid plants in the early stage after colchicine treatment. We examined the possibility to distinguish them by counting the number of chloroplasts in the guard cells of stomata. The epidermis of haploid and diploid plants was stained with the iodine-potassium-iodine solution and the number of chloroplasts in the guard cells was counted microscopically.

Fig. 1 shows the results of the experiment. There was a clearcut difference between haploids and diploids as far as the number of chloroplasts contained in a pair of guard cells was concerned. Haploid guard cells had 12 chloroplasts and diploid guard cells had 20 chloroplasts as a mode.

This method was then applied to the population of anther-derived plants treated with the colchicine solution. The fifth leaf from the top of the plant was subjected to the chloroplast counting before budding. After flowering, 11 out of 26 plants proved to be diploid, and the others were haploid. Only two plants were misclassified. The plantlet colchicine soaking method

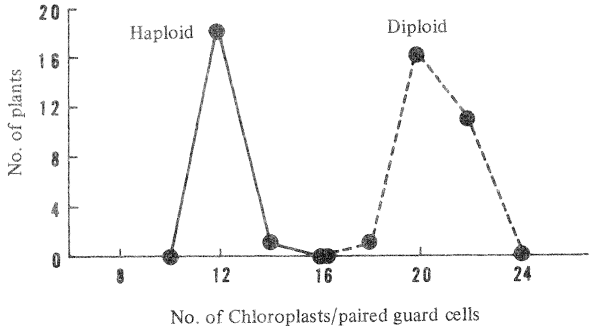

Fig. 1. Frequency distribution of chloroplast numbers in a pair of guard cells of stoma

mentioned in the previous section and the selection of doubled haploids by counting the number of chloroplasts will contribute to save the greenhouse space required for breeding.

Performance of doubled haploid lines

Since the doubled haploid lines are considered to be completely homozygous, the question is to know whether the doubled haploid lines show the same growth habits as those of ordinary cultivars which have some genetic variations.

Reduction in vigor. Doubled haploid lines were compared with the ordinary diploid lines in the field experiments conducted by several investigators (Legg and Collins, 1968; Kadotani and Kubo, 1969; Burk *et al.*, 1972; Oinuma and Yoshida, 1974; Nakamura *et al.*, 1974). It was evident from these reports that some of the doubled haploid lines showed some reduction in vigor. However, the amount of reduction in vigor varied, depending on the investigator. Nakamura *et al.* (1974) found that only one of 14 doubled haploid lines was inferior to the parental variety in yield. On the other hand, one third to half of the doubled haploid lines showed a reduced vigor in other experiments (Burk *et al.*, 1972; Oinuma and Yoshida, 1974). To obtain more detailed information on this problem, we made an experiment in 1976.

A plant of flue-cured variety Bright Yellow 103 (BY 103) was chosen as the parent, and 30 doubled haploid lines were derived from it. At the same time, 30 selfed lines were obtained from the same BY 103 plant. These 60 lines were tested in a randomized block design with three replications. Fig. 2 shows the frequency distribution of average plant yield of the tested lines.

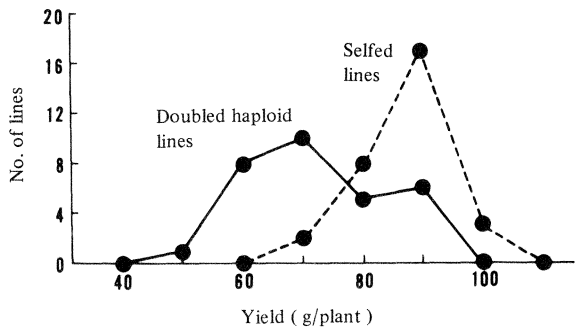

Fig. 2. **Frequency distribution of yields in doubled haploid and selfed lines of fluecured cultivar BY103** (Kumashiro *et al.*, 1977)

The average yield of 30 doubled haploid lines which was 71.7 g/plant was significantly lower than the average 86.5 g/plant observed in selfed lines. The variation among doubled haploid lines was greater than that among selfed lines. Seven doubled haploid lines were lower in yield than the average of selfed lines by three times the standard deviation. They were considered to be the lines with the reduced vigor. This result is in agreement with the recent report by Burk and Matzinger (1976). The cause of the reduced vigor is not known. However, we can assume that it was due to the occurrence of genetic changes during the anther culture and/or chromosome doubling.

Uniformity of growth. Table 3 shows the mean values and variances of some traits calculated from single plant measurements for cultivars and doubled haploid lines of flue-cured tobacco.

Table 3. Mean values ($\bar{X}$) and variances (V) of cultivars and doubled haploid lines

		Days to flower		Plant height		No. of leaves	
	D.F.	$\bar{X}$	V	$\bar{X}$	V	$\bar{X}$	V
Cultivars							
BY 4	73	69.2	4.36	147.6	120.47	21.7	2.23
BY	59	72.5	4.10	176.1	76.83	23.9	3.17
Doubled haploid lines							
KO 32	37	75.2	2.91	179.0	64.05	27.5	1.77
KO 71	37	75.8	4.68	155.1	76.82	28.3	2.15
KO 72	34	78.1	4.25	179.3	56.50	30.7	2.33
KO 73	33	74.6	2,85	176.3	53.54	26.3	1.78
KO 75	30	76.1	6.75	168.8	70.71	30.2	5.97
KO 76	38	74.0	3.39	164.6	72.07	26.5	2.05
KO 77	38	73.8	5.09	171.3	118.64	28.0	6.42
KO 79	35	75.7	2.83	169.6	63.34	27.7	2.05
KO 33	33	73.7	4.87	170.3	82.29	24.0	1.88
Homogeneity of variance (χ^2)			12.9		16.5		42.8 **

** Significant at the 1% level.

Bartlett's test for homogeneity of variances proved that the variances were homogeneous for days to flower and plant height, but not homogeneous for number of leaves per plant. However, there was no evidence that the doubled haploid lines had smaller or greater variances than those of the cultivars. We can conclude that the doubled haploid lines showed, as a whole, a uniform growth comparable to that of the cultivars.

Agronomic adaptability. It is required that a cultivar should have an agronomic adaptability to a wide range of environments. Since the genetically heterogeneous populations are expected to be more stable in performance than the homogeneous populations, it is necessary to evaluate the agronomic adaptability of the doubled haploid lines which are completely pure.

In this experiment, seven cultivars and ten doubled haploid lines of flue-cured tobacco were tested under four different environments (Oka *et al.*, 1976). The four environments covered one year at two locations and two years at another location. The doubled haploid lines tested were the promising selections bred from BY 4 X BY 103. For the evaluation of

adaptability, plant height and yield were measured because they represent the vigor of growth. Some methods have been proposed to evaluate the stability and adaptability to the different environments. Oka *et al.* (1976) used the method of "ecovalence" proposed by Wricke (1965) and partitioned the sum of squares for the genotype-environment interaction into the parts due to the respective entries. The lower value of contribution to the total genotype-environment interaction means a higher adaptability.

Table 4 shows the plant height and yield averaged over the four environments as well as the percentage of contribution of each genotype to the total genotype-environment interaction. With regard to the interaction for yield, the average of the percentage of contribution of cultivars was 4.9% and the range was 2.3% to 8.8%, whereas the average of doubled haploid

Table 4. Mean values of plant height and yield averaged over the four different environments and contribution of each genotype to the total genotype-environment interaction (Oka *et al.*, 1976)

Cultivar or line	Mean value				Contribution to interaction			
	Plant height		Yield		Plant height		Yield	
	cm		kg/10a		%		%	
Coker 319	132.7		229.7		2.6		8.8	
SC 72	131.0		222.2		6.9		6.6	
Sp. G 140	133.7		276.5		11.0		5.4	
Va. 770	132.0		242.7		9.4	5.7	2.7	4.9
McNair 944	127.2		22.60		4.2		5.8	
BY 4	140.2	152.2	192.5	226.5	0.4		2.3	
BY 103	164.2		260.5		5.6		3.0	
Line 1	154.2		212.5		15.0		20.6	
2	155.0		227.5		7.9		4.3	
3	142.2		189.0		9.8		2.0	
4	158.7		248.2		1.5		13.4	
5	156.0	151.2	200.7	221.1	6.4	5.9	8.1	6.5
6	142.2		186.7		4.8		1.8	
7	136.7		214.0		2.7		0.8	
8	151.5		268.7		3.7		7.5	
9	156.2		246.5		5.9		4.7	
10	157.1		246.5		1.9		2.2	

Line 1 ~ 10 are the selected doubled haploid lines from BY 4 X BY 103.

lines was 6.5% and the range was 0.8% to 20.6%. The doubled haploid lines had slightly higher values and a wider range than those of the cultivars. However, this result was not surprising, because these doubled haploid lines had not been selected for the adaptability. On the other hand, the cultivars and the doubled haploid lines tested were nearly the same in the average as well as in the range of the percentage of contribution for plant height.

From these results, we can conclude that the doubled haploid lines are not inferior to the cultivars as far as adaptability is concerned, though doubled haploid lines are completely pure in the genetical sense. In the unselected population of doubled haploid lines, the adaptability may differ from line to line, and we may be able to select the lines with high adaptability.

Application of anther culture to genetic analysis

Compared to an F_2 population, a haploid population derived from an F_1 hybrid will show a very simple segregation. When two pairs of genes located on the different chromosomes are involved, there will be a segregation for nine genotypes in the F_2 population; this segregation makes a contrast to the segregation for four genotypes in the haploid population. Using this property of haploid populations, we made the genetic analyses on alkaloid content and resistance to diseases such as black root rot and bacterial wilt.

The result of genetic analysis on bacterial wilt resistance is presented as an example. Bacterial wilt caused by *Pseudomonas solanacearum* is one of the major diseases in tobacco. The resistance to bacterial wilt is tested by artificial inoculation in the greenhouse or by natural infection in the infected field. However, the expression of the resistance is dependent on the environmental conditions, so that the evaluation of the resistance sometimes fluctuates. This fluctuation makes the genetic analysis difficult on a single plant basis. When the lines of F_3 or later generations are used in the studies, the genetic segregation in each line itself makes the analysis difficult. On the other hand, doubled haploid lines are expected to be completely homozygous, so that they will be more useful than the unfixed progenies in genetical analyses.

In this experiment, the resistant variety Hatano White Line was crossed to the susceptible variety LA Burley 21. Eighty-two doubled haploid lines bred from the anthers of the F_1 hybrid were tested for the resistance by the artificial inoculation method. Five plants were used for each of the lines and the tests were repeated twice. Tested plants were rated 0 (healthy) to 5 (heavily infected) by their symptoms.

Fig. 3 shows the frequency distribution of the average susceptibility indexes of the doubled haploid lines. There appeared three peaks. The left peak corresponds to that of the resistant parent Hatano White Line, and right peak corresponds to the susceptible parent LA Burley 21. The intermediate peak suggests that the resistance is controlled by more than one pair of genes. The 82 lines may be classified into three groups, each containing 38, 26, and 18 lines. We assumed that the resistance was controlled by two pairs of genes in this cross. When the resistance is controlled by two pairs of genes (A-a, B-b) and A gene has a greater effect than that of B, the segregation will be (AABB + AAbb) : aaBB : aabb = 2 : 1 : 1. The experimental result fits in with this hypothesis (P = 0.5 – 0.3). Doubled haploid lines will be useful for genetic analysis especially when the expression of genes is affected by the environmental conditions.

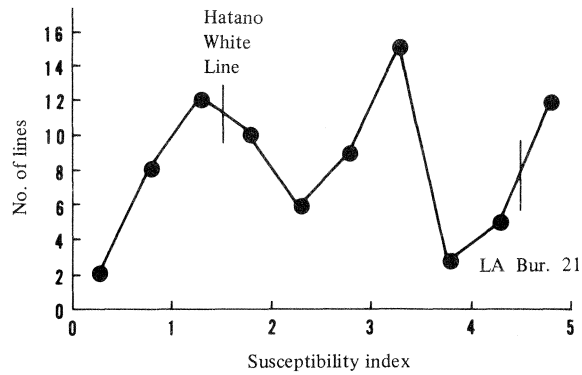

Fig. 3 **Frequency distribution of susceptibility indexes to bacterial wilt in the doubled haploid lines derived from Hatano White Line X LA Burley 21** (Itagaki *et al.*, 1977)

Development of new varieties

We released MC 101, the first variety in the world developed by the haploid method, in 1976 (Nakamura *et al.*, 1975). A number of tobacco breeding programs by the haploid method have been undertaken in Japan, and some breeding lines have been subjected to the variety test. Development of fluecured lines, KO 32 and KO 53, resistant to powdery mildew is presented here.

Powdery mildew caused by *Erysiphe cichoracearum* reduces both yield and quality of leaf tobacco. However, no commercial variety resistant to powdery mildew has been released in Japan. We made a breeding program to develop a flue-cured variety which had both high smoking quality and the resistance to powdery mildew, black root rot, and bacterial wilt. To fulfill these objectives we used four varieties as parents, whose properties are listed in Table 5.

Table 5. Properties of the parental varieties for breeding a new high quality variety resistant to powdery mildew and other diseases

	BY 4	Kutsaga E1	BY 103	Coker 254
Powdery mildew		R		
Black root rot		R	R	
Bacterial wilt			R	R
Yield			High	High
Quality	High			

R : Resistant

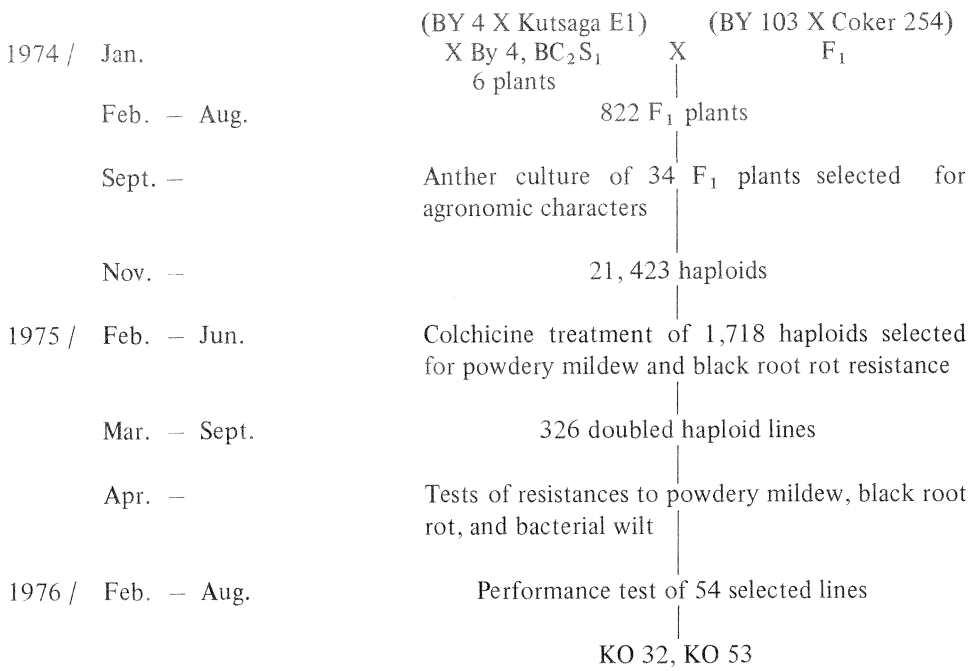

Fig. 4. Process of the development of flue-cured tobacco lines KO 32 and KO 53 by the haploid method (Okamura and Murai, 1977)

The breeding process is shown in Fig. 4. Kutsaga E1 is a donor parent of the powdery mildew resistance. The powdery mildew resistance is controlled by double recessive genes. If four varieties are crossed in an ordinary double cross fashion, the frequency of powdery mildew resistant genotype will be very low in their progeny. For this reason, the powdery mildew resistance was first introduced to the high quality parent BY 4 by two back-crosses. Then the powdery mildew resistant plants were crossed to the F_1 of BY 103 X Coker 254. Selections were made in the F_1 of this three-way cross for agronomic characters, and 34 out of 822 plants were selected as the anther source.

Anther culture of these 34 plants gave 21,423 haploids in total. These haploids were subjected to the selection for powdery mildew and black root rot resistance, and 1,718 haploids were selected. They were treated with colchicine by the inflorescence soaking method which gave 326 doubled haploid lines. These 326 lines were tested for the resistance to powdery mildew, black root rot, and bacterial wilt. From the tests of resistance, 54 lines were selected and their performances were tested in the field.

Finally two promising lines KO 32 and KO 53 were obtained. The characteristics of KO 32 and KO 53 are shown in Table 6. They are now under the variety test in the farmers fields.

Table 6. Characteristics of selected doubled haploid lines KO 32 and KO 53 (Okamura and Murai, 1977)

	KO 32	KO 53	BY 4 (Cont.)
Days to flower	76	73	71
Plant height (cm)	173	179	176
Number of leaves	21	20	19
Leaf length (cm)	59	56	56
Leaf width (cm)	22	22	21
Yield (kg/10 a)	239	223	199
Total alkaloids (%)	4.4	3.3	5.3
Powdery mildew	R	R	S
Black root rot	R	R	S
Bacterial wilt	R	R	S
Quality	High	High	High

R : Resistant, S : Susceptible

In the previous breeding programs by the haploid method, anthers of the F_1 hybrid of a single cross were cultured. When several breeding objectives are involved, a single cross may not cover all of the requirements, so that a three-way cross, a double cross or more complicated crosses will be needed. In the application of haploid method to such a complicated breeding program, it is recommended to select anther source parents in the segregating population. It should be emphasized that the genetical information on the cross combination is essential.

Discussion

The haploid method of breeding requires an efficient technique for the induction of haploids and for its diploidization. Following the establishment of the anther culture technique, we tried to develop an efficient technique for chromosome doubling. Colchicine treatment of haploid plantlets gave an acceptable rate of chromosome doubling as presented in Table 2. By this method, we were able to treat a large number of haploid plantlets at a time. In addition to this plantlet colchicine soaking method, the development of selected doubled haploids by chloroplast counting makes it easy to obtain doubled haploid lines. In the earlier breeding

programs, the selection was made first for the disease resistances in the haploid stage, and then for agronomic characters in the doubled haploid stage by a field test. The selection will be hereafter made mainly on the doubled haploid lines.

The characteristics of doubled haploid lines were studied from the view points of uniformity of growth, agronomic adaptability, and vigor. In the ordinary diploid method of breeding, selections are made in successive generations year after year, so that the selected lines may be expected to have adaptability to the climate conditions. In contrast to this, since doubled haploid lines are completely fixed and their selections are made usually only in one year, the question is whether the doubled haploid lines selected under only one environment really show the adaptability to the wide range of environmental changes. The results presented in Table 4 answer this question. We can expect that the doubled haploid lines show, as a whole, good agronomic adaptability comparable to that of the cultivars. Although a reduced vigor was observed in some of the doubled haploid lines, it was possible to select the promising lines with good performances (Tab. 4, 6).

The efficiency of the haploid method of breeding will also be discussed. The probability of obtaining a desired homozygous recombinant of independent genes is higher in the haploid method than in the ordinary diploid method. We will compare the frequency of desired homozygous recombinants in the F_2 population derived from the cross of two pure lines with that in the haploid population derived from the same hybrid. When n pairs of independent genes are segregating, the frequency of desired homozygous recombinants is $(1/2)^n$ by the haploid method and $(1/4)^n$ by the diploid method. The haploid method is 2^n times more efficient than the diploid method in obtaining desired homozygous recombinants.

In a practical breeding program using the diploid method, selections are usually made in the successive generations and the desired genes are fixed in the course of selections. Selections within a doubled haploid line are totally ineffective. Therefore the haploid method usually requires an initial population greater than that by the diploid method.

It may probably be said that the haploid method is suitable for breeding programs which involve a small number of segregating genes. When a number of genes are involved, it is practically impossible to select desired recombinants at once. In such a case, the selection of anther source plants may be effective when the overall efficiency of breeding system is considered.

Haploid method of tobacco breeding has the limitations characteristic to the method itself, and involves some problems which remain unsolved. However, this method is advantageous for the development of new varieties in a few years. It has also contributed to establish successfully MC 101 and KO lines. Haploid method will be used as a powerful tool in breeding and genetical analyses.

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Discussion

G. S. Khush, The Philippines: To generate greater variability you might like to obtain cell suspension from the anther calluses, treat the callus with some mutagenic agent and thus increase the variability. Have you tried this technique?

Answer: We have not tried that method. As I mentioned, genetic changes occur during anther culture as well as chromosome doubling. As the genetic changes are usually undesirable, we do not plan to use mutations in anther culture at the present time.

N. Murata, Japan: As regards the chromosome aberration problem raised by Dr. Khush, I should like to ask you whether the lack of chromosome aberration in tobacco is attributable to the technique whereby you induce plantlets directly from anther or is due to the intrinsic chromosomal stability of the tobacco plant?

Answer: I am not able to answer this question. However, as no calluses are formed in our anther culture medium, the haploids are considered to develop directly from pollen. This may be the reason why chromosomal aberrations are not frequently encountered in the anther derived plants.

G. S. Khush, The Philippines: Do you get any chromosomal instability in the doubled haploids?

Answer: We sometimes observed chromosomal aberrations in the anther derived plants and in the offsprings of colchicine-treated haploids. However, these aberrations are not frequent and once a doubled-haploid is produced, the chromosomes are completely stable in its progeny.

H. Fujimaki, Japan: How many generations do you save by adopting the anther culture technique, as compared with traditional breeding systems.

Answer: By the conventional diploid method, at least three to four years are required to fix the hybrid lines even when the accelerating generation advancement method is being used. On the other hand, we can obtain fixed lines only within one year by the haploid method, hence saving two to three years.

F. Kikuchi, Japan:

1. You mentioned that the haploid method was suitable for a breeding program which involves a small number of segregating genes but that when a larger number of genes was

involved conventional breeding methods were preferable. What is your criterion of selection of methods?

2. Judging from Table 4, it seems that selected doubled haploid lines such as line 10 are high-yielding and adaptable. Therefore, I think that even in anther culture breeding, adapted varieties can be selected. What is your opinion on this point?

Answer:

1. We have no special criterion. It depends on the situation. Generally speaking, we use the haploid method when the development of new varieties is requested to meet urgent demands in the tobacco production.

2. You are quite right. Adaptability and high-yielding ability differ from line to line and it is possible to select a promising line which has both a high-yielding ability and a high adaptability.