GENETICAL, CYTOLOGICAL AND PHYSIOLOGICAL STUDIES ON THE INDUCED MUTANTS WITH SPECIAL REGARD TO EFFECTIVE METHODS FOR OBTAINING USEFUL MUTANTS IN PERENNIAL WOODY PLANTS*

H. Kukimura*, F. Ikeda*, H. Fujita*, T. Maeta*, K. Nakajima**, K. Katagiri**, K. Nakahira***, M. Somegou***

* Institute of Radiation Breeding, N.I.A.S., M.A.F., Japan.
** Sericultural Experiment Station, M.A.F., Japan
*** Governmental Forestry Experiment Station, M.A.F., Japan.

ABSTRACT

The aim of this research project is to elucidate the biological aspects of artificially induced mutations in perennial tree crops and to promote utilization of such mutations to practical breeding programmes. a number of mutants were obtained in various tree crops by use of gamma-rays and they have been examined for their usefullness in practical breeding. From the results obtained so far in *Cryptomeria* and mulberry (*Morus* spp.) mutants will be discussed.

Cryptomeria:

In Sugi, *C. japonica* D. Don.,morphological mutations such as dwarf, abnormal needle shape and waxless needles were induced by acute and chronic gamma-rays irradiation since 1962. Microscopic examination on both meiotic cell division and somatic cell division in 17 mutant clones was carried out. Aberrant division was detected at meiotic cell division in only 2 mutant clones. The aberrations were precedence of chromosome at the first meiotic division and multi-nuclear division also at first meiotic division. Somatic cell division of all the mutant clones was normal. In the other 4 mutant clones having very low pollen fertility, pollen-like grains which seemed to be PMC themselves were found simultaneously with normal pollen grains. In other mutant clones, the morphological changes are possibly due to gene mutations since no cytological anormality was observed.

Mulberry:

Rooting ability and shoot growth after transplanting of cuttings are very important traits in mulberry since approximately one-half of the saplings are multiplicated by cuttings in Japan. Six gamma-rays

* Research supported under IAEA Research Contract No. 1336.

induced mutant strains together with the original variety were checked on these points. No depressions on rooting ability of the mutant strains were observed and in some mutant strains more increased shoot growth than the original variety was observed. Especially, IRB 240-4 which obtained by re-irradiation treatment of a mutant showed remarkable increase of rooting ability and shoot growth. The fact that vital mutant was obtained through somatic mutation in mulberry is a matter of interest.

Entire leaf mutants were investigated for their breeding behavior. Tester plant of entire leaf shape with genotype *ll* was employed for the breeding test. The originals, being lobed leaf with heterozygous genotype *Ll*, were crossed with the tester and F_1 progenies segregated to lobed and entire leaf in 1:1 ratio. Also crossing of the entire leaf mutants with the tester resulted in the similar ratio. It is thought that the change to entire leaf from lobed leaf is not determined by only a single gene mutation.

In heavy snowfall region, mulberry is always attacked by die-back disease (*Diaporthe nomurai* HARA). It is feasible to induce the resistance by mutation since the susceptibility to the disease is likely to be determined by considerably small number of gene. Fifty mutant strains induced so far by gamma-rays from cultivars Ichinose, Kairyo-nezumigaeshi and Kokuso No. 21 were investigated for their resistance by applying an inoculation method with toxin of the fungus. But the test revealed none of the mutant strains any resistance. Also 165 grafts after gamma-rays irradiation 7.5-15kR were tested and two resistant strains were found, though their field resistance is not yet investigated.

Eight morphological mutant strains derived from two different cultivars by use of gamma-rays were examined for their peroxidase isozyme patterns by electrophoresis in order to know whether the mutations were due to genic origin. Mutant strains derived from Ichinose exibited a variation in number of isozyme bands from 7 to 12 while the original showing 9 bands. Also in the mutant strains from Kokuso No. 21 different isozyme patterns from the original were detected. Not only the number of isozyme band but different staining intensity was observed in all the mutant strains from the originals. The increase and decrease in the number of the band in the mutant strains may be resulted in gene mutations.

1. INTRODUCTION

The aim of our research project is to elucidate the biological aspects of artificially induced mutations in perennial woody plants and to promote utilization of such mutations to practical breeding programme.

A number of mutants have been obtained through gamma-rays irradiation at our laboratories in vegetatively propagated woody plants [1]. We are now engaged to analyse these mutants cytologically, histologically, physiologically and genetically. From the results obtained so far, Sugi and mulberry mutants will be discussed here.

2. SUGI

In Sugi, Japanese cedar (Cryptomeria japonica), a number of drastic and morphological changes such as dwarf, slender branch, pendulus branch, adnate needle, twining needle, waxless needle and so on were found in gamma-irradiated materials (Fig. 1.). These mutants were examined for their cytological abnormality if the changes were due to chromosomal aberrations.

In 1972, fifteen mutant clones, as can be seen in Table 1, which were induced by either chronic or acute gamma-irradiation were employed for microscopic examination. Firstly, mitotic cell division was checked in root tips of the mutant clones and their originals. In all of them, neither chromosome breakage nor deletion was observed (Fig. 2). Secondly, pollen mother cell division in eight mutant clones was examined. Of these mutant clones, six showed normal meiotic division with closely paired 11-bivalent formation, while other two showed abnormal division. In IRB 601-65, preceded orientation of two bivalents was observed at the first metaphase (Figs. 3, 4). Frequency of such cells was approximately 50% of all the pollen mother cells. Nevertheless, these two preceded chromosomes joined to the chromosome complements at the telophase. And no abnormality was observed at the second division.

In another mutant clone, IRB 601-22, lagging chromosomes were detected in some cells at the first metaphase and they did not join to daughter nuclei at the telophase. This resulted in the formation of micro nuclei.

In 1973, seven mutant clones were reiterated for the cytological observation of pollen mother cell division. Meiotic cell division was normal in all the mutant clones including IRB 601-22 which showed lagging chromosomes and micro nuclei in 1972's observation. In four mutant clones, IRB 601-3, IRB 601-6, IRB 601-14 and IRB 601-22, pollen mother cells which had pollen-like shape and completely matured pollen grains were found simultaneously in a given anther (Figs. 5,6). And these clones produced abnormal pollen grains with smaller size and thicker exine wall than those of normal ones and had low pollen fertility (Table 2). These irregularities are seen in Fig. 6.

Based on the cytological observation on the Sugi mutants, it was concluded that the morphological changes as well as low pollen fertility and abnormal development of pollen grains which were observed in IRB 601-1, IRB 601-3, IRB 601-6 and IRB 601-14 proved not to be caused by chromosomal aberrations. With these clones further studies are needed to make clear whether they are of genic origin.

3. MULBERRY

In mulberry (Morus spp.), experiments dealing with rooting ability of mutants, breeding test of leaf shape mutants, screening mutants resistant to die-back disease and variations of peroxidase isozyme patterns in morphological mutants were carried out.

3.1. Rooting ability of mutant strains

Rooting ability is very important in mulberry culture since approximately one-half of saplings are multiplied by cutting in Japan.

Six mutant strains derived from Ichinose which is the most prevailing cultivar in Japan were tested for the rooting ability. These

mutant clones were induced by either acute or chronic gamma-irradiation from the cultivar at the maximal exposure of 10kR with the exposure rates 8.8R/day to 5kR/h. To test for the rooting ability, one-year-old branches over 1cm diameter were gathered and stored at 2.5°C for one-month. The lower part of a branch was divided into three cuttings, each cutting bearing two buds. Thirty cuttings for each clone were used. Basal ends of the cuttings were immersed in 150ppm NAA solution for 24-hour. Then, all the cuttings were placed into moistened sand and kept at 30°C in a growth chamber for 20-day. During the latter ten days the cuttings were illuminated 13.30 day-length. The result is shown in Table 3. Percentages of rooted cutting were low in No. 158 and No. 3198. Ichinose, IRB 240-1, IRB 240-2 and No. 115 were similar in number and weight of root, while No. 158 and No. 3198 were much fewer and lighter. IRB 240-4 resulted in a large number and heavy weight, probably due to the fact that the cuttings of large size with an average diameter of 1.8cm were compelled to use in this experiment [2]. As can be seen in Table 3, two mutant clones, IRB 240-2 and IRB 240-4, both of which were obtained by re-irradiating IRB 240-1, did not exibit any decrease in the rooting ability. In addition, shoot growth in three mutant clones was tested (Table 4). Vigorous shoot growth nature of IRB 240-1 is maintained in IRB 240-2 in case of summer pruning, and also in IRB 240-4 in cases of both spring and summer pruning. Both the mutant clones showed increased leaf area compared with the original and IRB 240-1.

It is, therfore, probable to induce somatic mutations without any decrease in rooting ability and shoot growth by re-irradiation in mulberry although no information is available concerning the matter in other vegetatively propagated plants.

3.2. Breeding test of leaf shape mutants

Entire leaved mutant clones, artificially induced ones together with a spontaneous one, were investigated for their breeding behaviour.

IRB 240-1 is an entire leaved mutant clone derived from 5-lobed cultivar Ichinose by chronic gamma-irradiation. No. 3183 is an entire leaved mutant clone derived from 5-lobed cultivar Kairyo-nezumigaeshi by acute gamma irradiation. The entire leaved spontaneous mutant from Kairyo-nezumigaeshi was found in 1961 at the Tohoku Branch of Sericultural Experiment Station. These mutant materials were crossed with a tester plant cultivar Shiromekeiso which is true-breeding entire leaved. Originals were also crossed with the tester. Result is summed up in Table 5. The seedlings from these cross combinations segregated to lobed leaf and entire leaf. Segregation ratios were almost similar in all the crosses.

In mulberry, the lobed leaf is dominant over the entire leaf and it is proposed that the lobed is determined by a single gene action [3]. Kairyo-nezumigaeshi, if inbred, will not breed true and its selfed seedlings segregate in 3 lobed : 1 entire ratio, accordingly its genotype may be represented as Ll, while that of the tester Shiromekeiso as ll. Progenies from the cross Kairyo-nezumigaeshi with Shiromekeiso segregated lobed and entire to fit 1:1 ratio. There were no differences between the mutant and the original in segregation ratio. Similar result was also obtained in the case of the crosses Ichinose and its mutants with the tester, although the chi-square values were considerably large. If it is presumed that IRB 240-1, No. 3183 and the entire leaved spontaneous mutant were induced through a single gene mutation, that is, from Ll to ll, all the seedlings between the mutants and the tester must be entire leaved plants. Contrary to expectations, in every cross, segregation of lobed and entire fitted 1:1 ratio statistically at 0.05 probability level. From the result above mentioned, it is concluded that the L2 layer in these mutant clones did not changed through recessive mutation of a single gene. It is likely to explain that L1 and/or L3 layers which may have effect on leaf shape might have changed by gamma-irradiation.

3.3. Screening mutants resistant to die-back disease

In heavy snowfall regions in Japan, mulberry is always attacked by die-back disease, Diaporthe nomurai. Breeding resistant varieties to the disease is very important and urgent matter. Although there are some resistant varieties bred by conventional cross breeding, their low foliage yield and poor feeding value acount for less reliability. Inheritance of the susceptibility is rather simple and occurs in major genic ways. It is, therfore, expected that mutants resistant to the disease might be induced through either gene mutation or small deletion of chromosome which carries the susceptible gene(s) [4].

The fungus, D. nomurai, was cultured on sterilized mulberry shoot at 23°C in an incubator. Five races of the fungus isolated from infected mulberry were used. Three pieces of spore horns which covered over the surface of infected shoot were removed and dissolved in 2ml of distilled water for conidia suspension. Four or five mulberry shoots of 20cm long were prepared from each material to be tested. Conidia suspension from the five races was seperately inoculated on to the same shoot by a vaccinator. Inoculated shoots were kept in moistened sawdust at 20°C. Ten days after, investigation on development of necrosis was carried out on the inoculated shoots. In case of necrosis developed, it was grouped as susceptible and when no necrosis or scarce necrosis were observed, it was grouped as resistant [5].

Twenty-five mutant clones from cultivar Ichinose, 18 mutant clones from cultivar Kokuso No. 21 and 7 mutant clones from cultivar Kairyo-nezumi-gaeshi were tested. But, the inoculation test revealed none of the 50 mutant clones any resistance to the fungus.

In addition, the same test on gamma-irradiated shoots originated from buds of two clones, Tani No. 4693-2 and Tani No. 4711-3 were tested. The irradiation was given on scions in spring of 1972 with 7.5, 10 and 15kR exposures at two different exposure rates of 250 and 500R/h. The irradiated scions were grafted in April and grown in a field in 1972 and

the materials for the inoculation test were taken. Result of the test on the shoots of the grafts is shown in Table 6. The more exposure and exposure rate were given, the lower percentage of successful grafting was produced. From the inoculation test, two shoot were screened as resistant from among 684 shoots. These screened two were derived from Tani No. 4711-3 grafts which were irradiated by 7.5kR at 250R/h. In order to investigate the field resistance of these two shoots, multiplication is now being carried out. As for screening the resistant mutant shoot, the method employed here is considered useful and reliable.

3.4. Variation of peroxidase isozyme patterns in morphological mutants

Isozyme technique was applied to know whether mulberry mutants which were obtained by gamma-irradiation were due to gene mutation. In this experiment, variations of peroxidase isozyme patterns were investigated in induced mutants with morphological changes.

Six mutant clones induced from 5-lobed cultivar Ichinose and 2 mutant clones from entire leaved cultivar Kokuso No. 21 were used along with originals. All the mutants were induced by gamma-irradiation to buds of dormant shoots. Description of the mutant clones are seen in Table 7.

Cutting was made with moistened sand at 30°C under 12 hours day-length. After 30 days, when about seven leaves expanded, leaf blades locating at between the third and sixth leaf were used as a sample for gel electrofocusing devised by Hirano and Sekiyama [6]. For peroxidase staining, Endo's method was employed [7]. All the samples were run with three replications. For the pH determination of the gel, an identical gel was run in parallel. After the electrophoreisis, the gel was sectioned into 15 pieces. Each piece was soaked in 2ml of deionized water for two hours and pH of the solution was measured. And then pH of corresponding location in the other gels were determined.

In Ichinose and its 6 mutant clones, a total of 12 bands was detected as can be seen in Figs. 7 and 8. Bands located at pH 4.9, 5.0, 5.1, 5.8,

7. 8.5 and 9.8 were common to the original and all the mutants, although there were slight differences of staining intensity. Mutant clones, Ic. No. 1, Ic. No. 3 and Ic. No. 4 had a band which was not present in the original. Mutant clone Ic. No. 2 showed three more additional bands than original, that is, the bands at pH 5.5, 5.6 and 6.8. Mutant clone Ic. No. 5 was characterized by the loss of two bands of pH 5.7 and 6.0 which existed in the original and the other 5 mutant clones. This seemed to have any connexion with a change in leaf shape from 5-lobed to entire. Further investigation in this point must be done using several entire leaved Ichinose mutants. In Kokuso No. 21 and its 2 mutant clones, a total of 11 bands was identified. Bands located at pH 4.9, 5.0, 5.1, 5.8, 7.7, 8.5, 9.1, 9.5 and 9.8 were common to the original and the 2 mutants with little differences in staining intensity. Mutant clone Ko. No. 1 showed the band at pH 5.6 which was not observed in the original. Mutant clone Ko. No. 2 did not show the band at pH 6.0 which was present in the original.

Isozymes are prevailingly considered to be the products of structural genes. Therefore, the increase and decrease in number of peroxidase isozyme bands appeared in the mutant clones may be resulted in mutation of gene(s) responsible for the peroxidase isozyme production.

REFERENCES

[1] NISHIDA, T., NAKAJIMA, K., OHBA, K., TAKATO,S., Radio-sensitivity and induction of somatic mutations in woody perennials under chronic gamma ray irradiation, Gamma Field Sympo. 6(1967) 19

[2] HONDA, T., Studies on the propagation of mulberry trees by cutting, Bull. Sericultural Expt. Sta. 24(1970) 133

[3] AIDA, F., NAKAJIMA,K., MATSUSHIMA, M., A cross experiment regarding the breeding behavior of leaf shape in mulberry, J. Sericult. Sci. Japan 31(1962) 182

[4] NYBOM, N., KOCH, A., "Induced mutations and breeding method in vegetatively propagated plants" The Use of Induced Mutation in Plant Breeding (Report of the FAO/IAEA Technical Meeting, Rome, 1964), Pergamon Press, Oxford 661

[5] MIYAYAMA, K., OKABE, T., Studies on the test for the resistance of die-back disease by inoculation of conidia, J. Sericult. Sci. Japan 41(1971) 230

[6] HIRANO, H., SEKIYAMA, F., Detection of peroxidase isozyme in mulberry, Report, 24th Conv. Kanto Br. Sericult. Soc. Japan, 8(1973) (In Japanese)

[7] ENDO, T., Application of the Nadi Reaction to Rice-Peroxidase Isozyme Stain, Bot. Mag. Tokyo, 85(1972) 147

Table 1. Origin and characteristic of Sugi mutants

Mutant	Original	Gamma-rays irradiation			Detected in	Character	Remark*
		Method	Exposure	Exposed in			
			R				
IRB 601- 1	Kumasugi	Acute	1,440	1962	1963	Slender branch, adnate needle	1972,1973
IRB 601- 2	"	"	"	"	"	Slender branch, slightly twining needle	"
IRB 601- 3	"	"	"	"	"	Needle twines at sprouting	"
IRB 601- 4	"	"	600	1963	1964	Pendulus branch	"
IRB 601- 6	"	"	"	"	1965	Dwarf	"
IRB 601-11	"	"	580	1965	1966	Short, twining needle	"
IRB 601-14	"	"	600		1964	Dwarf; short and light green needle	1973
IRB 601-15	"	"	700	1964	1965	Straight, fine, adnate needle	1972,1973
IRB 601-17	"	"	580	1965	1966	Waxless needle	"
IRB 601-17'	"	"	"	"	"	Argute needle	"
IRB 601-21	"	Chronic	1,456	1962	1963	Waxless needle	"
IRB 601-22	"	"	"	"	"	Dwarf; waxless needle	"
IRB 601-23	"	Acute	2,280	"	1964	Waxless needle	"
IRB 601-26	"	"	1,200	1964	1965	"	"
IRB 601-32	"	"	580	1965	1965	"	1973
IRB 601-65	Bokasugi	Chronic	4,120	1962	1967	Dwarf; short, fine needle	1972,1973
IRB 601-70	Tateyamasugi	"	1,659	"	1963	Dwarf; waxless needle	"

* Year of microscopic observation

Table 2. Pollen fertility of mutant clones of Sugi

Mutant	Number of pollen observed	Number of fertile pollen	Number of sterile pollen	Number of pollen-shaped PMC
		(%)	(%)	(%)
Control	305	300 (99.3)	5 (0.15)	0
601-1	206	122 (59.2)	79 (38.4)	0
601-3	205	38 (18.5)	119 (58.0)	45 (22.0)
601-6	206	22 (10.7)	139 (67.5)	45 (21.8)
601-14	150	28 (17.7)	116 (77.3)	6 (4.0)
601-22	340	124 (36.5)	197 (57.9)	19 (5.6)
601-23	172	158 (91.9)	14 (8.1)	0
601-32	169	159 (94.1)	10 (5.9)	0

Table 3. Percentage of rooted cuttings, number of root and weight of root in the cuttings of mulberry cultivar Ichinose and its gamma-rays induced mutants

Mutant	Exposure	Character	Percentage of rooted cutting	Number of root per cutting	Fresh weight of root per cutting
			%		g
Ichinose (Original)		5-lobed leaf	97	42.5	1.891
IRB 240-1	8.8R/day, 3 years	Entire leaf	97	34.3	1.440
IRB 240-2	10kR, 0.5kR/h to IRB 240-1	5-lobed leaf	100	32.4	1.342
IRB 240-4	"	"	100	63.2	2.627
No. 115	10kR, 0.5kR/h	Small leaf, short internode, short branch	100	36.6	1.902
No. 158	10kR, 5kR/h	"	67	13.4	0.336
No. 3198	"	Entire leaf, thick, deep green and coarse leaf, branch many and short	70	17.2	0.552

Table 4. Leaf area and shoot length of mulberry cultivar Ichinose and its gamma-rays induced mutants

Mutant	Average length of new shoot in mid-June		Average area per leaf
	Spring pruning	Summer pruning	
	cm	cm	cm^2
Ichinose (Original)	62.4	32.4	155.8
IRB 240-1	65.3	36.5	135.3
IRB 240-2	60.5	41.6	157.1
IRB 240-4	75.9	36.7	179.0

Table 5. Inheritance of leaf shape in crosses of mutants and originals with tester

Cross combination	Total number of seedling	Number of seedling with		
		Entire leaf	Lobed leaf	Both entire and lobed leaf
IRB 240-1 x Shiromekeiso	68	42	26	0
Ichinose x Shiromekeiso	68	41	27	0
No. 3183 x Shiromekeiso	66	34	31	1
Entire-leaved spont. mutant of Kairyo-nezumigaeshi x Shiromekeiso	21	7	13	1
Kairyo-nezumigaeshi x Shiromekeiso	39	22	17	0

Table 6. Result of inoculation test of dye-back disease on grafted scion

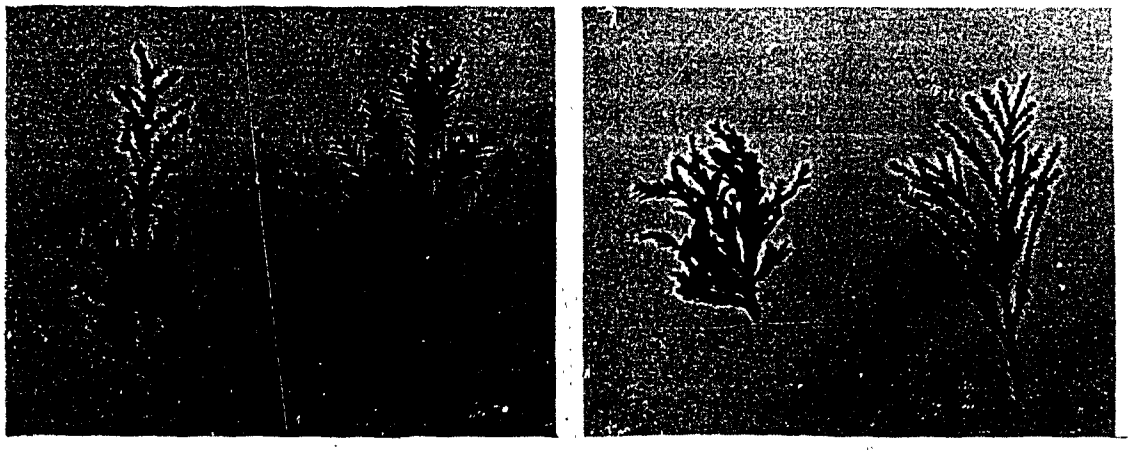
Exposure	Exposure rate	Number of investigated plant		Susceptible	Resistant
		Tani 4693-2	Tani 4711-3		
kR	R/h				
15	500	0	0	0	0
15	250	13	9	22	0
10	500	0	3	3	0
10	250	9	11	20	0
7.5	500	0	5	5	0
7.5	250	0	45	43	2*
0	0	57	13	70	0

* These are not ascertained whether they are truly resistant in a field.

Table 7. Description of mutants used in peroxidase experiment

Mutant	Exposure	Character	Wide/length ratio of leaf blade
Ichinose (Original)		5-lobed leaf	0.78
Ic. No. 1	7.5kR, 5kR/h	Elongated leaf	0.62
Ic. No. 2	"	"	0.66
Ic. No. 3	"	"	0.68
Ic. No. 4	"	"	0.73
Ic. No. 5	10kR, 5kR/h	Elongated and entire leaf	0.70
Ic. No. 6	7.5kR, 5kR/h	Marginally curled leaf	0.72
Kokuso No. 21 (Original)		Entire leaf	0.75
Ko. No. 1	10kR, 5kR/h	Marginally curled leaf	0.60
Ko. No. 2	"	Shoot length is c. 1/2 of the original, dwarf growth	0.75

A B



C D

Fig. 1. Morphological mutants induced by gamma irradiation

A. Right, control (Kuma-Sugi).
Left, IRB 601-3, mutant with twining needle

B. Right, control (Iwao-Sugi).
Left, IRB 601-42, mutant with waxless, thin and densely attached needle

C. Right, control (Boka-Sugi).
Left, IRB 601-65, dwarf mutant with thin and short needle

D. Right, control (Tateyama-Sugi).
Left, IRB 601-70, waxless dwarf mutant with short and thick needle

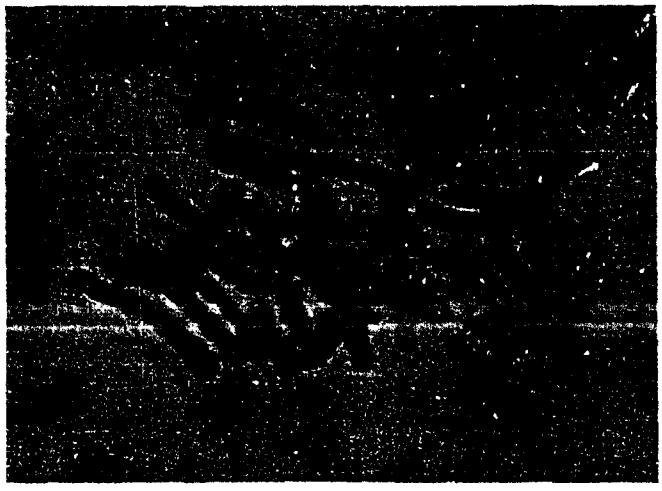


Fig. 2. Chromosomes in somatic cell at root tip of IRB 601-1, 2n=22

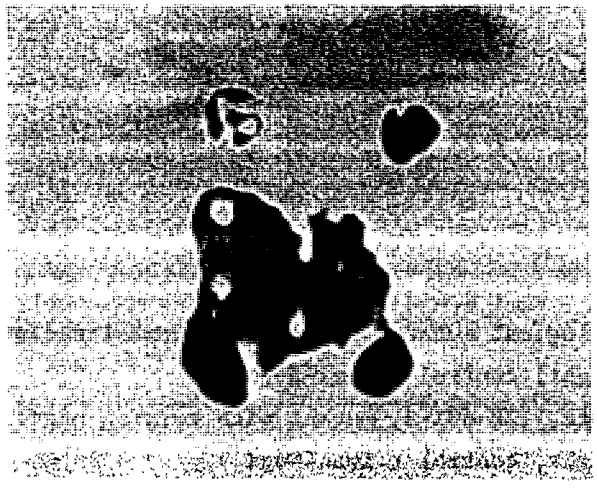


Fig. 3. Abnormal division at the first metaphase in pollen mother cell of IRB 601-65. Two chromosomes move towards each pole precededly to chromosomes orientation.

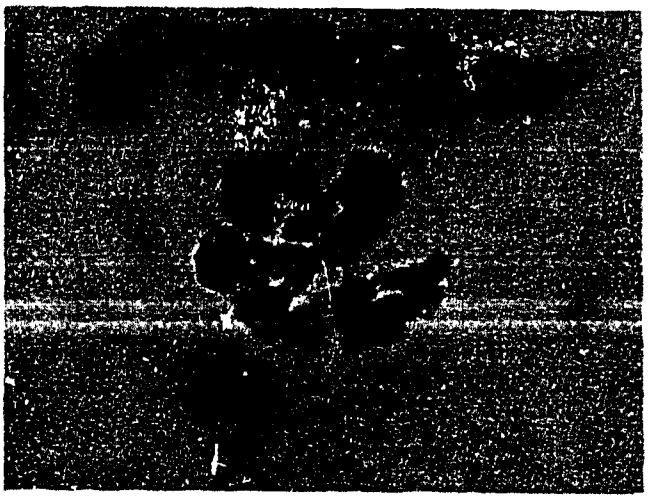


Fig. 4. First metaphase in pollen mother cell of IRB 601-1, 11 paired chromosomes are observed.

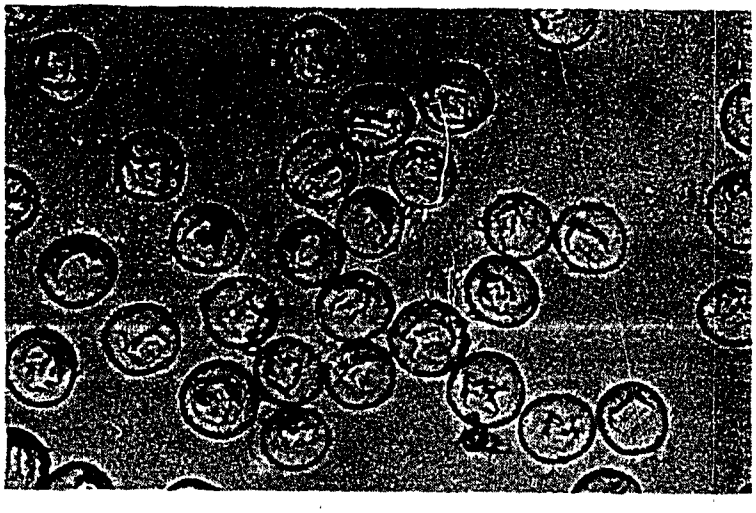


Fig. 5. Matured pollen of control plant in Sugi(Cryptomeria japonica).

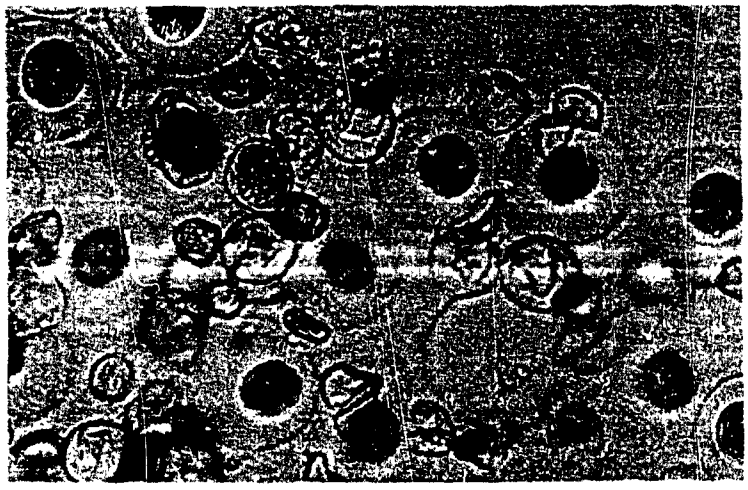


Fig. 6. Abnormal pollen of the mutant IRB 601-3, characterized by pollen shaped PMC which are stained deeply in the figure.

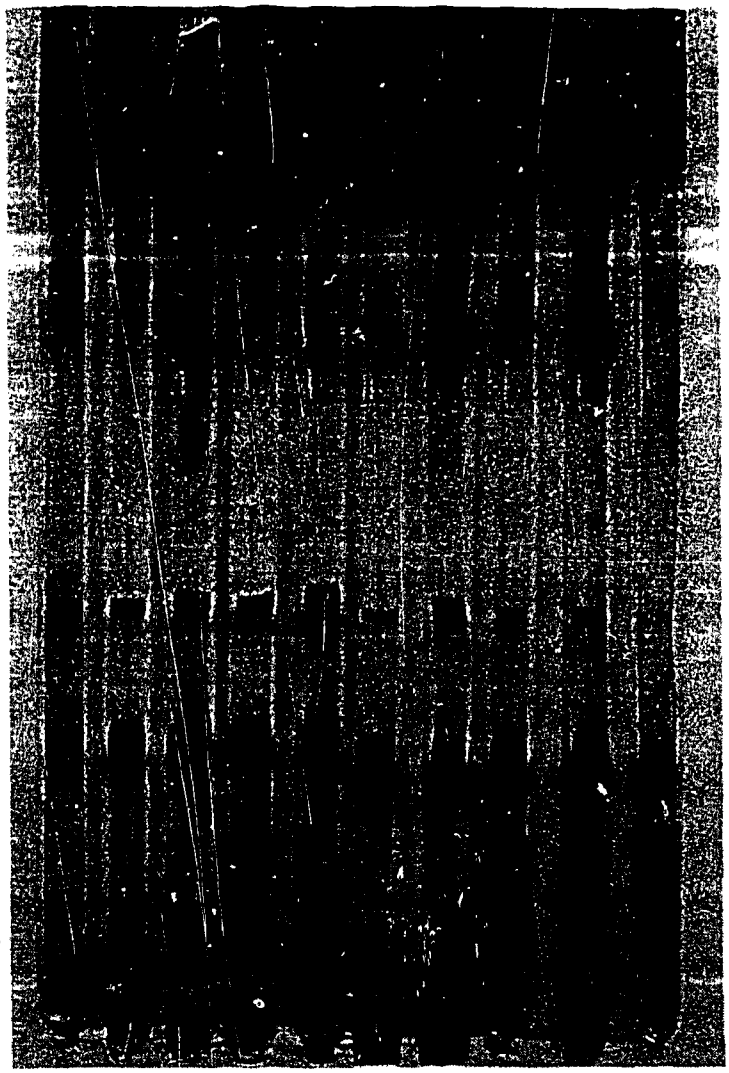


Fig. 7. Photograph of peroxidase zymograms in Ichinose, Kokuso No. 21 and their mutant clones.

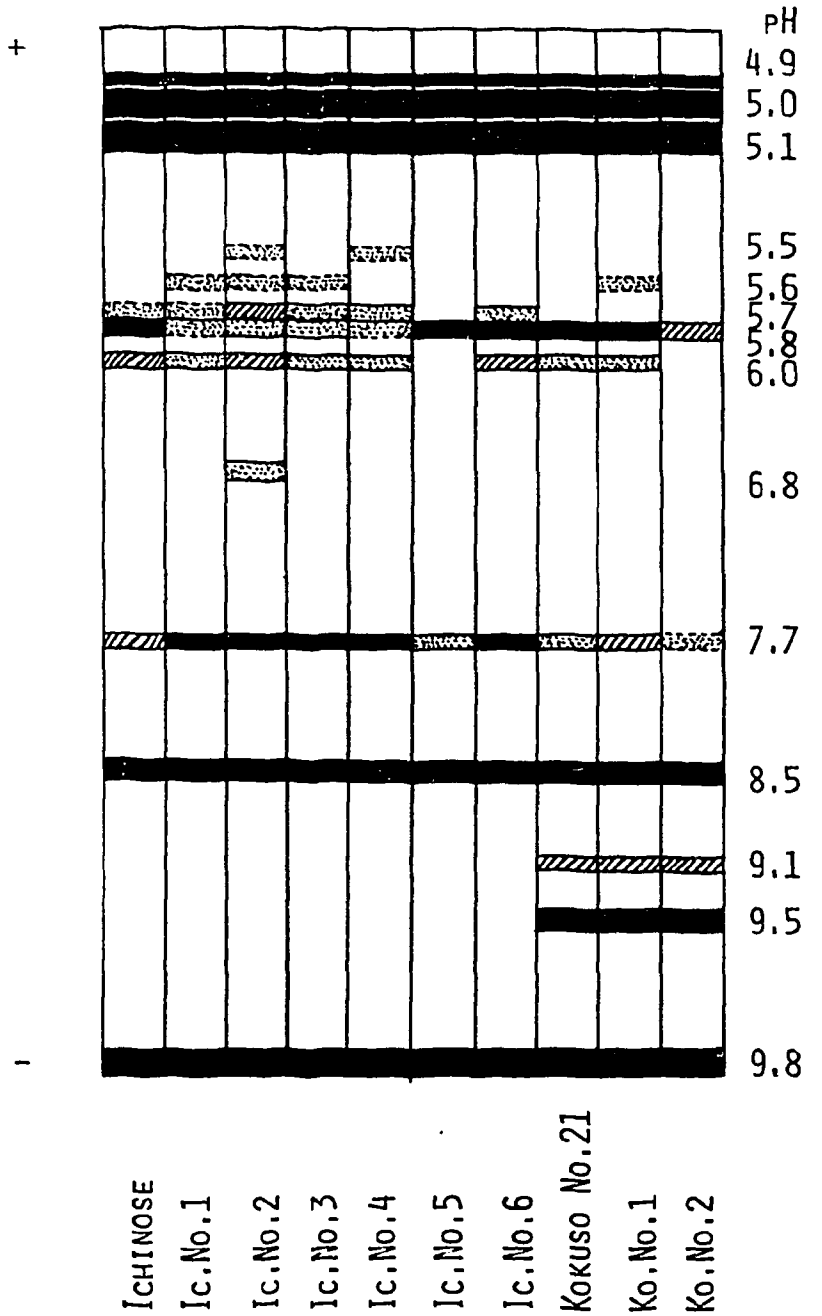


Fig. 8. Diagram of peroxidase zymograms in Ichinose, Kokuso No. 21 and their mutant clones.