REPORT NO. IAEA-R-1299-F

TITLE

Isolation and propagation of mutations by in vitro culture, (part of a coordinated programme on improvement of vegetatively propagated crops and tree crops through radiation induced mutations)

FINAL REPORT FOR THE PERIOD

1 December 1972 - 30 November 1974

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INTERNATIONAL ATOMIC ENERGY AGENCY

DATE December 1974

FINAL REPORT

Contract No. 1293 ~~1922/R1~~/RB

Isolation and Propagation of Mutation in Dahlia by in vitro Culture

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December 1, 1972 - November 30, 1974

Abstract

ISOLATION AND PROPAGATION OF MUTATIONS IN DAHLIA BY IN VITRO CULTURE

The present study was undertaken to search for a successful method for in vitro culture of mutated tissues in dahlia. Preceding the objective, the features of induced mutations and the effects of cutting propagation in dahlia were investigated, and the tissues easily regenerating plantlets in vitro were searched following the examination of effective condition of medium.

Induction of mutations : Tuberous roots of two cultivars, 'Kōsei' and 'Sunlight', were irradiated with 1,000 - 2,000 R of X-rays. Chlorophyll and flower-color mutations were successfully induced in both cultivars, but the frequency differed with genotypic constitution. The maximum frequency was observed at leaves and shoots on or from the fourth to fifth nodes from the base of plant. The use of M_1 tuberous roots seemed a way for isolating mutations though not so much efficient.

Tissue culture : In vitro cultured basal parts of ray florets, exactly the ovary, differentiated shoots. No shoot formation occurred in receptacle and leaf cultures, while roots were differentiated in leaf culture. Supplements of auxin and adenine to the medium besides cytokinin appeared to be necessary for inducing shoots. It is a serious problem in the tissue culture of dahlia that a large number of explants are endogenously comtaminated with bacteria.

Taking into consideration low rates of surviving and regenerating explants, it seems difficult at present for dahlia to conclude whether or not the tissue culture may become efficient in mutation breeding as compared with cutting propagation.

I. INTRODUCTION

It is important for improvement of vegetatively propagated plants to establish a procedure for raising new clones from desirable portions of the chimera induced through mutation. In vitro culture of mutant tissues for regenerating new plants seems to be a successful way for this purpose (1). On the course from induction of mutations to regeneration of new plants, however, there remain various unsolved problems, such as selection of tissue for culture, search for effecient condition of shoot-inducing medium and culturing technique for newly induced plantlets.

From these points of view, the present study was undertaken to establish an effective method for in vitro culture of the mutated tissues in leaves, receptacles and florets of garden dahlia. While a number of vegetatively propagated ornamental plants have been treated by radiations and used for mutation breeding (2, 3, 4, 5, 6, 7, 8, 9, 10, 11), garden dahlia (Dahlia variabilis Desf.) was considered as one of the most available for the study, because its high polyploidy and its great number of gene loci responsible for flower color must bear a high possibility for preparing very various sorts of mutations (12).

Preceding the objective, however, induction of mutations and detailed information on features of induced mutations in dahlia were needed. And besides, the effect of cutting propagation on isolating mutations was also considered worthy to be ascertained for evaluating the effect of in vitro culture on regenerating solid mutants, that is, wholly mutated plants. Furthermore, it was necessary to find out the tissues easily regenerating plantlets in vitro and to decide the kinds and concentrations of growth substances supplemented in the culturing medium. According to these

considerations, the present study is reported, being divided into two parts: the first part is concerned with the induction of mutations, and the second is as to the in vitro culture of tissues.

II. INDUCTION OF MUTATIONS

1. Materials and Methods

Two decorative dahlia cultivars, the medium inflorescenced, scarlet flowered 'Kōsei' and the large inflorescenced, yellow flowered 'Sunlight', were used for materials, which had been proved for somatic mutations to be rather easily induced by radiations in the preliminary studies.

In spring of 1972, twenty tuberous roots of each of these cultivars were irradiated with 1,500 and 2,000 R of X-rays and planted in field. The mutations induced on leaves and flowers were investigated with regard to the frequency, position within plant, biochemical property, and so on. As to the flower color mutations obtained in 'Kōsei', 14 leaf-bud cuttings were taken from the mutated shoots and propagated in order to separate and fix the mutation. Roots newly formed in the irradiated generation (M_1) were harvested in autumn and prepared for the experiment in next year.

In spring of 1973, the tuberous roots harvested from M_1 plants were planted as the vegetative second generation (VM_2) for detection of mutations. As to the plants developed through the cuttings of mutated M_1 shoots, leaf-bud cuttings were taken again and propagated in the same way as used in 1972, to examine whether the chimera could be completely dissolved or not.

X-rays were newly given to the tuberous roots of 'Kōsei' in spring of 1973, to elucidate the effect of cutting back on preventing chimera formation. One hundred tuberous roots of 'Kōsei' were irradiated with 1,000 R of X-rays and planted in the boxes filled with sand and vermiculite. And about a month later, chlorophyll mutations revealed on leaves of main shoots were scored in every leaf position (Fig.1). Then each of unfolded leaves of all individuals was separately cut out with the respective axillary bud, and the axillary bud was grown to an intact plant. Since this plant corresponds to a lateral shoot of the maternal plant (Fig.1), it is hereinafter referred to as 'lateral shoot' for the convenience of description. Chlorophyll and flower color mutations manifested in these 'lateral shoots' were investigated

to examine the effect of cutting back on the release of mutants.

2. Results

i) Induced mutations

By X-irradiation of tuberous roots in 1972, chlorophyll mutations, such as virescent, were induced as mutated sectors on leaves of M_1 plants in both cultivars. The frequency of these mutations varied with the position of node to which the mutated leaves belonged. The maximum frequency was observed on the leaves of fourth to fifth nodes numbered from the base of plants, and was remarkably higher in 'Sunlight' than in 'Kōsei'. Flower color mutations were also observed in both cultivars, but wholly mutated shoots, namely, bud mutations were recognized only in 'Kōsei', which occurred on two plants of 1,500 R (10 %) and on three plants of 2,000 R (15 %), involving four sorts of changed color ; purple red, orange yellow, orange -yellow striped in scarlet, and yellow, against scarlet of the original. These results as well as those from the preliminary experiments may suggest that somatic mutations can be easily induced in dahlia, but the genotypic constitution of subjected materials fairly affects the occurrence of mutation.

The mutant flowers induced in 'Kōsei' were analysed on their pigments by paper chromatography (Fig. 2). The original cultivar comprised 13 major flavonoid pigments consisting of four pelargonins (spot nos. 3, 5, 6, 8), three cyanins (spot nos. 1, 2, 4), three buteins (spot nos. 7, 10, 12) and three flavons or flavonols (spot nos. 9, 11, 13). On the other hand, some of the pigments were proved to be missing in the mutant flowers, namely, buteins in purple red flower, most anthocyanins in orange-yellow flower, all anthocyanins in yellow flower, and all flavonoid pigments in white flower, which was obtained not as bud mutation but as probably spontaneous mutation of a mere capitulum. Thus, it was concluded that the changes of flower color were all due to some lack of the pigments contained in the original cultivar. The results obtained from cytological observations, where all the mutants possessed less number of chromosome (2n = $63 \sim 65+1f$) than the original (2n = 66), seemed to support this interpretation.

ii) Dissolution of chimera

Dissolution of chimera, which involves the prevention of chimera formation in broad sense, is of a fundamental necessity for isolating mutation, and its skill may decide the result of mutation breeding. From this viewpoint, a few examinations for dissolving chimera were tried for checking the practical meaning of in vitro culture of mutated tissue.

According to the observation of VM_2 generation (Table 1), the frequency of mutants, which included both real mutants and partially mutated plants, was generally low as compared with that in the M_1 generation, but the percentage of real mutants was considerably high. It was noticed that some of the mutaions which were manifested in the M_1 generation disappeared as observed in purple-red and orange-yellow flower mutations of 'Kōsei', and those not observed in M_1, e.g., white flower mutants of 'Sunlight' appeared newly. These results indicate that the use of M_1 tubers is a way for isolating mutations though it may not be so much efficient.

Among 14 plants grown after the first leaf-bud cutting, two plants reverted to the original type of flower color. However, all 47 plants raised through the second cutting exhibited respective changed colors (Table 2). The few-striped flower mutants found even after the second cutting are considered due to some unstable genetic factor different from other mutations. Thus, leaf-bud cutting seems to suggest an effective procedure for isolating mutation.

As the results on the effect of cutting-back, the frequencies of chlorophyll mutation in leaves of main shoots and of chlorophyll and flower color mutations in 'lateral shoots' are shown in Fig. 3 and Table 3, respectively. In leaves of main shoots, the highest frequency of mutated leaves was seen at the fourth to fifth leaf positions as well as observed in 1972. According to the previous observation, leaf of the fifth position was the youngest leaf primordium at the time of irradiation. In 'lateral shoots', chlorophyll and flower color mutations manifested themselves as spots or sectors on leaves of 'lateral shoots' or as wholly mutated 'lateral shoots'. The frequency of these mutated 'lateral shoots' was highest in the fourth and fifth nodes of the maternal plants for the both kinds of mutations. This trend quite agrees with the case of mutated leaves of main shoots. This means that 'lateral shoots' with the highest frequency

of mutations also correspond to the youngest leaf primordia at the time of irradiation. These findings seem to be firmly supported by LAPINS (13) and LAPINS et al. (14) : they stated that secondary buds (axillary buds in this paper) which were associated with the youngest leaf primordia at the time of irradiation gave rise to the highest frequency of mutations in secondary shoots (lateral shoots in this paper). From a practical stand point, these results suggest that it is recommended for effective isolation of mutations to cut back the main shoot slightly above the mutated leaf and to develop the axillary buds just under the cut site.

III. TISSUE CULTURE

1. Materials and Methods

i) Culture of leaves and receptacles

Preceding the culture of mutated sectors, explants from the leaf and receptacle of non-irradiated dahlia plants were cultured in vitro in order to search for effective concentrations of growth regulators in the medium.

Recently unfolded leaves of the two cultivars mentioned above were prepared for explants. Receptacles of capitula from the small inflorescenced, pink flowered, decorative cultivar 'Shin-kamome' were obtained when the outer ray florets of the capitula had just unfolded. These leaves and receptacles were sterilized by dipping in 70 % ethanol and immersing in a sodium hypochlorite solution containing 1 % active chlorine for 15 minutes. After two rinses with sterile water, leaf segments, about 5 mm square, and cubed receptacle segments of about 3 mm were excised aseptically. Each of these explants was then planted on a medium of 15 ml in a culture tube.

The basal medium comprised the major elements of MURASHIGE and SKOOG (15) and the following minor elements and organic substances (in mg/l) : $MnSO_4.4H_2O$ (25), H_3BO_3 (10), $ZnSO_4.7H_2O$ (10), $Na_2MoO_4.2H_2O$ (0.25), $CuSO_4.5H_2O$ (0.025), myo-inositol (100), nicotinic acid (5), L-glycine (2), pyridoxin-HCl (0.5), folic acid (0.5), biotin (0.05), sucrose (20,000), and 5 ml per liter of a solution containing (per liter) 4.47 g of Na_2EDTA and 5.57 g of $FeSO_4.7H_2O$. This basal medium was supplemented with one of 16 combinations of 2,4-D (0, 0.05, 0.5 and 5 ppm) and N^6-benzyladenin (BA)

(0, 0.05, 0.5 and 1 ppm).

The pH of the media prepared was adjusted to 5.5 before adding 0.8 % agar and autoclaving. The cultures were placed under continuous lighting at 25°C. Lighting was provided by fluorescent lamps giving an intensity of about 300 ft-c at the outside of the culture tube. Culturing was continued for eight weeks, consisting of two four-week subcultures, which were carried out on the medium with the same constituents.

After culturing for eight-weeks, segments of the callus produced were succesively transplanted to two types of media aiming at inducing shoots. The basal medium was supplemented with the combinations of 2,4-D (0 and 0.05 ppm) and BA (0.5, 1 and 5 ppm) for the first subculture, and with those of IAA (10 ppm), kinetin (1 and 10 ppm) and adenine-sulphate (0 and 10 ppm) for the second subculture. Each of these cultures consisted of two four-week subcultures, which were carried out on the medium with the same constituents.

ii) Culture of virescent leaf-sectors

Segments of virescent leaf-sectors induced by mutation were taken from fully developed variegated leaves of 'Sunlight', which had been irradiated with 1,000 R of X-rays, and were then subjected to culture. For this culture, 0.05 and 0.5 ppm 2,4-D were added to the basal medium together with 0 and 0.05 ppm BA.

iii) Culture of basal parts of florets

Basal parts of outer ray florets were taken from the presently unfolding capitula of non-irradiated 'Shin-kamome' and 'Kōsei'. Separated ray florets were washed with a non-ionizing detergent and sterilized by immersing in a sodium hypochlorite solution containing 0.5 % active chlorine for 15 minutes. Upper parts of these florets were then cut off and only the basal parts including ovary with a small portion of the petal were planted on the medium.

In the case of 'Shin-kamome', the explants were cultured on the medium containing IAA (10 ppm), kinetin (0.1, 1 or 10 ppm) and adenine-sulphate (1 or 10 ppm) for six weeks, and then surviving explants in the initial culture were transplanted randomly to the medium added with NAA (0, 0.05 or 0.5 ppm), BA (0.05, 0.5 or 1 ppm) and adenine-sulphate (0 or 20 ppm). For 'Kōsei', they were cultured on the medium supplemented with NAA (0, 0.05 or 0.5 ppm), BA (0.5, 1 or 5 ppm) and adenine-sulphate (0 or 20 ppm) for six

weeks, and then transplanted to new media with the same supplements.

2. Results

i) Culture of leaves and receptacles

Growth of cultured tissues: In the initial culture of leaf and receptacle explants, a wide range of concentrations of auxin and cytokinin was incorporated to the medium to investigate the growth response of the explants.

The highest concentration of 2,4-D (5 ppm) quickly gave rise to discoloration and browning of explants. Calli were induced at the concentrations of 0.5 and 0.05 ppm of 2,4-D, and the former (0.5 ppm) initiated callus formation earlier than the latter (0.05 ppm). But the final quantity of callus was larger in the latter, regardless of BA concentrations incorporated. Without 2,4-D incorporation, any growth of the explants did not occurred. Finally, the best growth of callus was observed at 0.05 ppm 2,4-D with 0.05 ppm BA both in the leaf culture (Fig. 4) and in the receptacle culture (Fig. 5). In these cultures, it was noticed that compact and green callus was produced from receptacles, while fragile and pale callus from leaves. Incorporation of BA accentuated the firmness of the callus with elevation of the concentration.

In some of leaf explants cultured on the medium added with 0.05 ppm 2,4-D alone, root formation was observed firstly at three weeks after the beginning of culture, and the rooting rate of the explants reached 70 % by the termination of eight-week culture (Fig. 6). On the contrary, no shoot formation was observed in any medium both for leaf and receptacle cultures throughout the eight-week culture.

Subculture of callus: Following the initial eight-week culture, segments of the calli produced on the best medium for their growth were transplanted to the media which were available for inducing the shoot. In this first subculture, only the calli, transplanted to the medium with 0.05 ppm 2,4-D and 1 or 5 ppm BA, proliferated both in leaf and receptacle cultures. And the difference of callus appearance due to its tissue source got diminished, because the initial light-brown color of the calli from leaves turned to green and their structures became more compact. However, no shoot formation was observed in this first subculture.

In the second subculture, where the other constitution of growth

regulators was applied for the supplement of the medium, the best growth of green callus was observed on the medium added with 10 ppm IAA and 10 ppm kinetin, regardless of the incorporation of adenine (Fig. 7). No shoot formation could be recognized also throughout this second subculture.

ii) Culture of virescent leaf-sectors

The segments of mutated virescent leaf-sectors did not grow even on the medium that was most suitable for the growth of explants from normal leaves in the preceding experiment.

It could not be ascertained whether or not this is due to lower viability of the mutated tissue, because the explants used had been infected endogenously with bacteria and died off shortly after planting.

iii) Culture of basal parts of florets

In the initial culture, the petal portion of the explant discolored within two or three weeks after planting and became necrotic by the termination of this culture. The ovary, however, swelled without callus formation and kept its color green (Fig. 8). This swelling was better with the increase of auxin concentration and the decrease of cytokinin concentration. The addition of adenine promoted the swelling.

In the subculture of the ovary, differentiation of shoot primordia was recognized in both the cultivars within three weeks after transplanting. In the case of 'Shin-kamome', the shoot formation occurred on the media supplemented with 0.5 ppm BA and 20 ppm adenine-sulphate, 0.5 ppm BA alone, and 0.05 ppm BA and 20 ppm adenine-sulphate. When NAA was incorporated to the medium, however, no shoot primordium was formed at any concentration of BA. As for 'Kōsei', on the other hand, shoot primordia differentiated on two kinds of the media added with 5 ppm BA, 20 ppm adenine-sulphate and 0.05 ppm NAA, and 0.5 ppm BA, 20 ppm adenine-sulphate and 0.5 ppm NAA. Three weeks after having differentiated the shoot primordium, the explants were transplanted on the basal medium supplemented only with 1 ppm gibberelline (GA_3), aiming at further development of the shoot (Fig. 9).

The regenerated shoot primordia of 'Shin-kamome' were transplanted on the basal medium without any growth regulators intending to raise new intact plants, but they died shortly after the transplanting.

As far as the present results are concerned, supplement of both auxin and adenine to the medium besides cytokinin seems to be indispensable for

the differentiation of shoot primordium. Though, as for 'Shin-kamome', the shoot primordium was differentiated even on the medium supplemented with cytokinin alone, it should be noticed in this case that both auxin and adenine had been supplemented into all of the media used for the initial culture. It cannot be yet concluded, however, what constitution of the growth regulators is most suitable for shoot formation in the ovary culture of dahlia, because the rate of the explants that differentiated shoot primordia was very low, namely, only one of five explants on the same shoot-inducing medium was differentiated in each case through the two different series of culture.

IV. DISCUSSION

Experimental results indicated that inducing somatic mutations by radiation was rather easy in dahlia, though the genotypic constitution of subjected material fairly affected the occurrence of mutation. This trend may be dominant in the vegetatively propagated plants that carry a number of heterozygous gene loci responsible for relevant characters like dahlia. In such plants, therefore, one of the most important problems connected with mutation breeding is considered the isolation of induced somatic mutations, in other words, the dissolution of chimera as pointed before.

For obtaining wholly mutated shoots or plants without chimeral structure, there may be expected a few methods, such as cutting back the shoot growing from an irradiated bud, 'internal disbudding' of apical meristem by irradiation and natural use of latent buds in shoots and tubers irradiated. In the present study also, for instance, it was suggested that cutting back produced a significant number of apparently wholly-mutated 'lateral shoots'. And most of them have been proved to be real, solid mutants through observing the plants raised from tubers formed on those 'lateral shoots'. In spite of the effectiveness of these procedures, however, in vitro culture of mutated tissues to regenerate new plants appears to hold an intrinsically strong point for isolating mutation, because it can make use of a mutated part, even if small, quickly and directly without any operation for dissolving the chimeral structure. A remaining question seems to be how far the in vitro method can be achieved practically.

The tissue culture experiments carried out in the present study are based on this point of view. In these experiments, an accent was kept on detecting the tissues that could regenerate new plants with ease. SHIGEMATSU et al. (16) manifested that a new mutant clone of white flowered chrysanthemum could be raised through in vitro culture of receptacle segments of a white flowered capitulum induced from a pink flowered chrysanthemum by γ-irradiation. PIERIK et al. (17) and ROEST et al. (18) also indicated that shoots were induced from receptacle explants in gerbera and in pyrethrum, respectively. Besides, SHIGEMATSU (19) succeeded in regenerating new plants from in vitro cultured mutant petals of chrysanthemum and cornflower. In composite plants, thus it has been suggested that floral parts are successful for isolating mutations induced in flower chracters. In other ornamental plants, at least so far as dahlia is concerned, however, there are scarcely any information with this respect.

As to the improvement of flower characters as neccessary in dahlia breeding, on the other hand, it is a matter of course that the use of floral parts is most reasonable in case of tissue culture. It is considered, however, that culturing the tissues of some vegetative organs also may be helpful for the improvement of flower characters, because the tissues of mutated leaves, for instance, possess a high possibility of carrying mutated genes responsible for flower traits as suggested before, and those tissues can be easily distinguished and utilized at an early developmental stage of plant.

According to these considerations, florets, receptacles and leaves of dahlia plant were chosen as explant sources for the tissue culture experiments in this study.

Through these experiments, shoot primordia could be formed only on the basal part of floret, exactly on the ovary. It appears that the shoot primordia originated directly from the ovary wall, though this is not yet ascertained by a histological observation. In propagation by means of tissue culture, it is desired that a new plant is directly regenerated from the explant to escape the genetical variation which frequently occurs in the plant originating from callus. In this respect, the ovary culture seems to be available for the in vitro propagation of dahlia, though a culturing system to raise whole plants is not yet established. Ovary culture may be

suited also for speedy isolation of mutation, beacause mutant florets can be separated immediately after a mutational event appeared in the capitulum.

Shoot primordium was formed neither on leaf and receptacle tissues nor on the callus from these tissues, whereas roots were formed. It is said that auxins have an inhibitory effect on shoot formation while inducing callus, and the intensity and persistency of this inhibition vary according to their chemical structure. Viewed from this point, it may be a reason why no shoot was differentiated on the leaf and receptacle explants that the auxin used in the initial culture was 2,4-D. Hence, other auxins should have been tested for the induction of shoots.

In this series of tissue culture of dahlia, it was quite difficult to keep explants aseptic and healthy. Notwithstanding healthy appearance of the stock plant, a large number of explants had been endogenously infected with bacteria, and died shortly after planting in spite of careful aseptic procedures. So it seems necessary for tissue culture of dahlia to select uninfected tissues and stock plants. In this respect, it is important that the percentage of the infected explants varied with different organs, being higher in leaves and especially in receptacles and lower in florets. Thus in this meaning also, the ovary is a suitable material for the in vitro propagation of mutants in dahlia.

Taking into consideration the results that, even in the ovary culture, about a half of the explants died off during the initial six-week culture due to bacterial comtamination, and only 20 % of the surviving explants could develop shoots, it seems difficult at present for dahlia to conclude whether or not the tissue culture may become efficient in mutation breeding as compared with field techniques, e.g., cutting back.

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TABLE 1. MUTATIONS OBSERVED IN THE VEGETATIVE SECOND GENERATION (VM_2)

VARIETY	EXPOSURE (R)	No. OF PLANTS OBSERVED	No. OF CHLOROPHYLL MUTANTS 1)						No. OF MUTANTS IN FLOWER	
			4/4	3/4	2/4	1/4	S	SPOTTED	COLOR	SIZE OR SHAPE
KŌSEI	0	33	0	0	0	0	0	0	0	0
	1,500	33	0	0	0	0	1	0	1	0
	2,000	30	1	0	0	0	4	2	2	4
SUNLIGHT	0	34	0	0	0	0	0	0	0	0
	1,500	100	4	1	1	0	4	0	2	2
	2,000	84	5	0	0	1	2	1	2	6

1) Classified by the ratio of mutated part to whole plant: 4/4, real mutant: S, small than 1/4.

TABLE 2. ISOLATION OF MUTATION BY CUTTING IN 'KŌSEI'

BUD MUTATION OBSERVED (SEPT. '72)	FIRST CUTTING (OCT. '72)		SECOND CUTTING (MAR. '73)	
	No. OF PLANTS GROWN	FLOWER COLOR	No. OF PLANTS GROWN	FLOWER COLOR
PURPLE RED	3	ALL WERE PURPLE RED.	10	ALL WERE PURPLE RED.
ORANGE YELLOW	5	ONE WAS SCARLET, THE OTHERS WERE ORANGE YELLOW.	10	ALL WERE ORANGE YELLOW.
ORANGE-YELLOW STRIPED	5	ONE WAS SCARLET, THE OTHERS WERE ORANGE-YELLOW STRIPED.	10	ALL WERE ORANGE-YELLOW STRIPED. 1)
YELLOW	1	YELLOW.	17	ALL WERE YELLOW.
CONTROL (SCARLET)	10	ALL WERE SCARLET.	17	ALL WERE SCARLET.

1) Three plants showed few stripes.

TABLE 3. FREQUENCIES OF CHLOROPHYLL AND FLOWER COLOUR MUTATIONS INDUCED IN 'LATERAL SHOOTS' (1,000 R of X-tays, 'Kōsei').

POSITION OF 'LATERAL SHOOT' IN MATERNAL PLANT	No. OF 'LATERAL SHOOTS' OBSERVED	No. OF 'LATERAL SHOOTS' MUTATED IN	
		CHLOROPHYLL (%)	FLOWER COLOR (%)
1 - 2	20	0 (0)	0 (0)
3	51	10 (19,6)	1 (2,0)
4	79	21 (26,9)	5 (6,3)
5	103	31 (30,0)	8 (7,8)
6	109	10 (9,2)	0 (0)
7	98	8 (8,2)	1 (1,0)
8	62	3 (4,9)	0 (0)
9	19	0 (0)	0 (0)
TOTAL	541	83 (15,3)	15 (2,8)

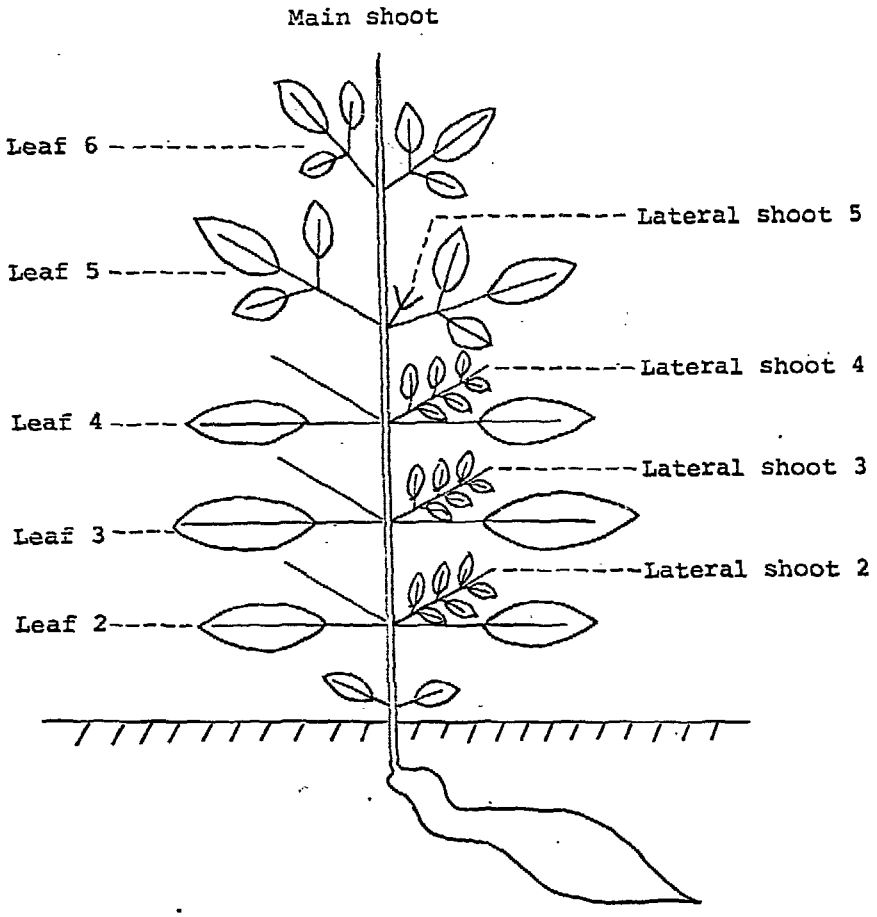

FIG. 1. Schematic presentation of leaves and lateral shoots in dahlia (2,3,4,5,--: positions of leaves or lateral shoots).

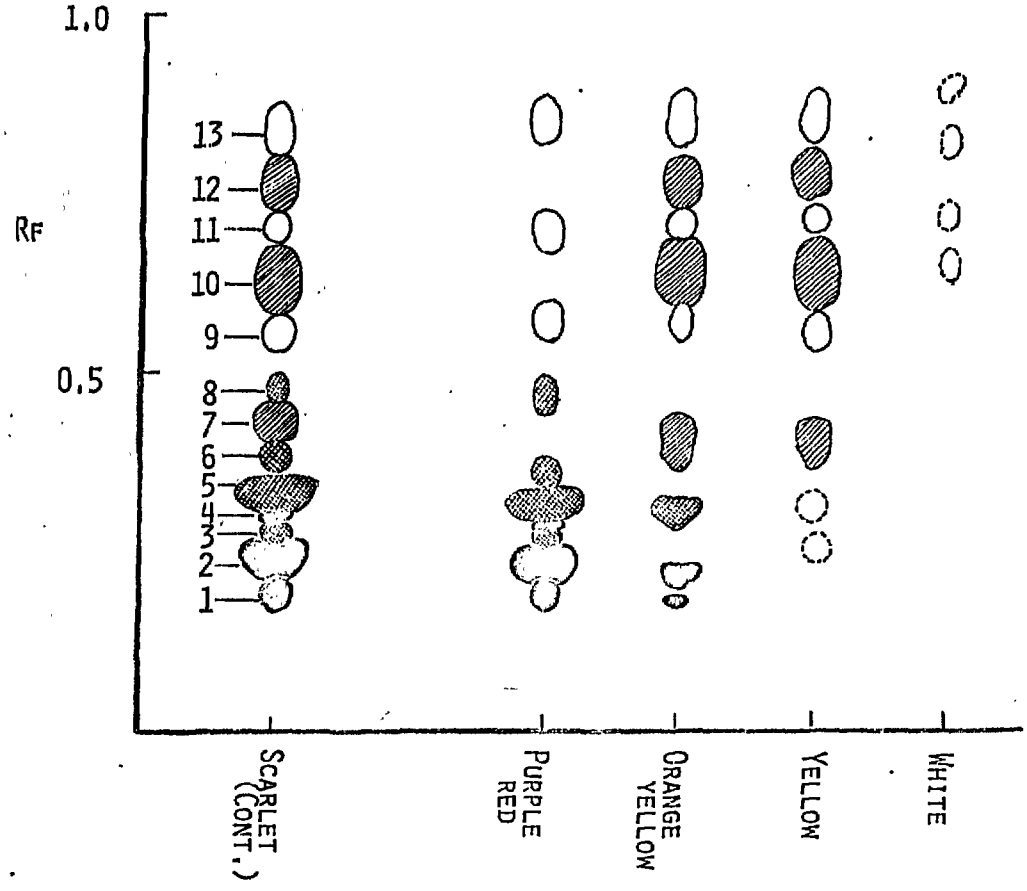

FIG. 2. Paper chromatograph on flavonoid pigments of the mutant flowers.

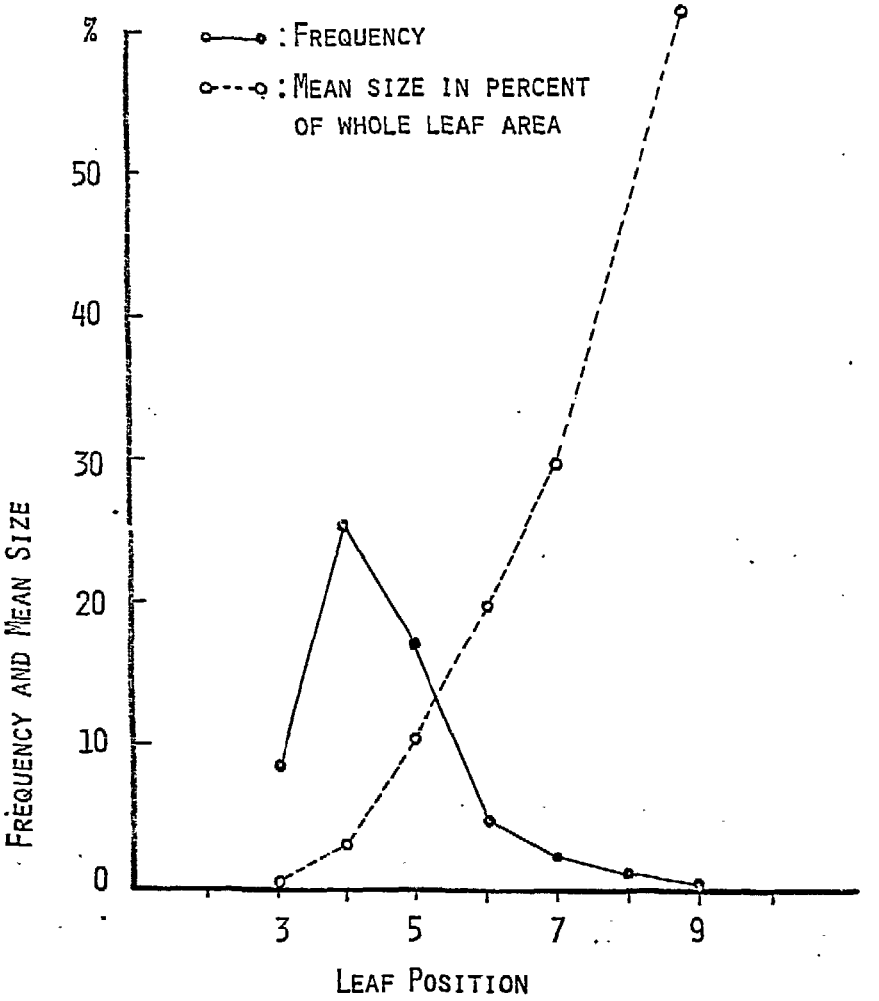

FIG. 3. Relationships of frequency of mutated leaves and mean size of mutated sectors to leaf position in plant.

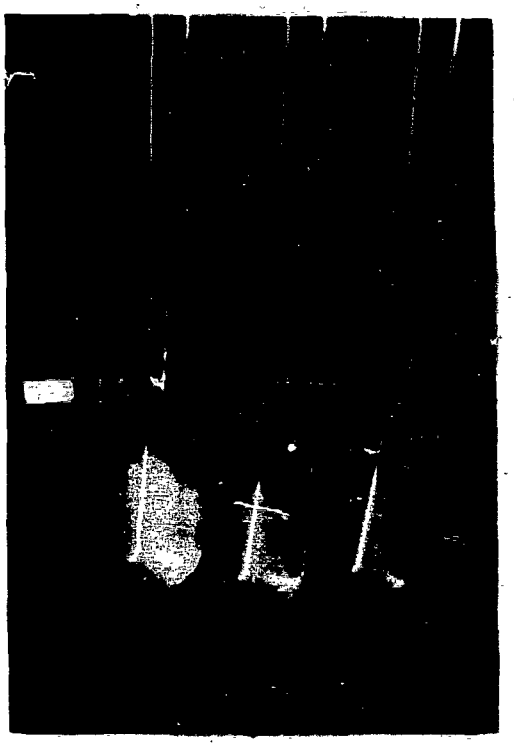

FIG. 4. Callus originated from leaf segments of 'Kosei' after 8-week culture on media containing 0.05 ppm 2,4-D and 0.05 ppm BA.

FIG. 5. Callus originated from receptacle segments of 'Shin-kamome' after 8-week culture on media containing auxin and cytokinin (left; 0.05 ppm 2,4-D and 0.05 ppm BA: right; 0.5ppm 2,4-D and 0.05 ppm BA).

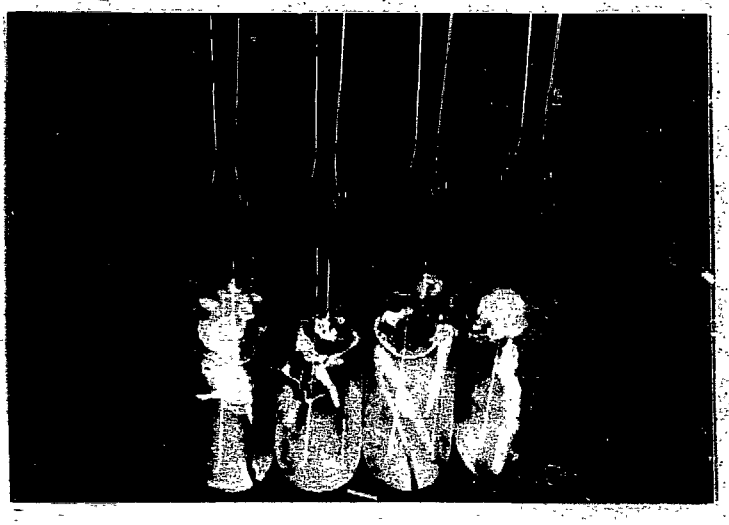

FIG. 6. Root formation from leaf segments of 'Kōsei' after 8-week culture on media containing 0.05 ppm 2,4-D.

FIG. 7. Callus growth subcultured for 4 weeks on media containing 10 ppm IAA and 10 ppm kinetin (left), and 10 ppm IAA, 10 ppm kinetin and 10 ppm adenine-sulphate (right).

FIG. 8. Swelled ovary of ray-floret explant of 'Kōsei' cultured for 6 weeks on medium containing 0.5 ppm NAA, 0.05 ppm BA and 20 ppm adenine-sulphate.

FIG. 9. Developing shoot on subculturing medium added with 1 ppm GA_3, which was defferentiated from ovary of 'Kōsei' cultured on medium containing 5 ppm BA, 20 ppm adenine-sulphate and 0.05ppm NAA.