# 4.8 New Approaches for Effective Mutation Induction in Gamma field

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## Abstract

The purpose of the report is to clarify the effects of chronic irradiation using in vitro culture on inducing the mutation of two model plants.
Culture technique combined with irradiation can overcome the problem of chimera formation and provided 10 times greater mutation efficiency than conventional method. Proper mutagenic treatment using cultured materials is indispensable to effective mutation induction.

The chronic culture method showed the widest color spectrum in chrysanthemum and extended toward not only the negative but positive direction. However, the acute culture methods indicated a relatively low mutation rate and a very limited flower color spectrum.

Flower color mutation of the regenerators could be induced more from petals and buds than from leaves. These facts is supposed that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture. It may be likely to control a direction of desired mutation.

One possible reason why the chronic culture methods showed higher frequencies is that most of the cells composing the tissue and organs continually irradiated into a cell division which was highly sensitive and more mutable to irradiation. Under these conditions, many mutated sectors may accumulate in the cells of the growing organs.

Regenerated mutant lines show remarkable decrease of chromosome numbers by irradiation. It is a proper indicator to monitor radiation damage.

In this study, the six flower color mutant varieties registered were derived from chronic irradiation. The combined method of chronic irradiation with floral organ cultures proved to be of particularly great practical use in mutation breeding for not only flower species but any other species.

## Introduction

Radiation breeding has yielded excellent results and is still developing as indispensable tool to improve varieties in many plants. So far, a number of radiations, beta ray, gamma rays, x-rays, neutron and so on have been investigated for mutation induction of plant species, ionized radiation, that is, gamma rays, x-rays has been utilized almost 80 % of the registered mutant varieties in the world.

There are two streams of gamma irradiation methods, acute and chronic irradiations. In the former case, acute irradiation facilities such as gamma room and gamma cell have been extensively operated treating with smaller materials like seeds, bulbs, tubers, scions, spores. In the latter, chronic irradiation facilities such as gamma field, gamma greenhouse and gamma phytotron have been utilized dealing with a large quantity of plant materials or big materials under low dose rates in the natural condition..

Since the Institute of Radiation Breeding (IRB) was established in 1960, it has open-door policy to not only public organizations but private sectors. Next year, a gamma field was installed in 1961 and operated for mutation induction for plant materials. Numbers of researches have been carried out to develop the effective methods of mutation induction and to breed useful mutants by using gamma field. During the quarter Century, much information on the following subjects had been accumulated in various plant species.

Radiosensitivity and optimal irradiation doses at chronic and acute irradiations.

Mutation induction methodology for major crops.

Alternation of mutation spectrum

On the other hand, in European Countries, Sweden researchers reported that the efficiency of the chronic facilities for mutation induction was less than that of seed irradiation in barley. The similar result was reported in durum wheat by Italian scientists. These negative results made the scientists and plant breeders disappointed and hindered them from further uses of gamma field.

More than 10 gamma field had been established in the world in 1960's, however, most of them were closed one after another owing to completion of the military mission or shortage of the expectation. Reflecting from disappointing atmosphere, radiation breeding works surrounding the gamma field had retarded for longer years. However, the gamma field in IRB has been continuously operated as an important tool and school for radiation biology and mutation breeding and as a symbol of peaceful use of atomic energy in Japan.

During the recent decade, however, the gamma field have successively produced numbers of useful mutant varieties of which Japanese pear varieties, "Gold Nijisseiki" and "Osa Nijisseiki" had been selected as resistant mutants against serious black spot disease and successfully released in the fruit growing districts in Japan.

It has already passed 30 years to recognize the hidden values of gamma field, since

biotechnology was introduced into the field of radiation breeding. The combined method of radiation with tissue and cell culture techniques was proved to be extremely efficient for mutation induction and confirmed to solve the problems in the mutation breeding. By using the combined method, useful mutant varieties have been selected at high frequency from the limited population regenerated from in vitro culture of chronic irradiated plants.

There still remain the problems to be solved on radiation breeding.

To increase the frequency of useful mutation.

To broaden the spectrum of mutation.

To control the direction of desired mutation.

The present report summarizes new approaches of the efficient use of a gamma field using new technology in some model plants. The primary purpose is to clarify the effects of chronic irradiation using in vitro culture on inducing the mutation of chrysanthemum and sugarcane, and to elucidate the effect of irradiation methods on mutation induction.

## Combined Effect of Irradiation Methods and Explant Sources on Mutation Induction in Chrysanthemum.

### Materials and Methods

In chronic irradiation, ten rooted cuttings of the chrysanthemum cultivar "Taihei" (pink flower) were transplanted at six points various distances from the $^{60}$Co source (88.8 TBq) in a gamma field. The growing plants were irradiated at dose rates ranging from 0.25 to 1.5 Gy/day for 20 hours every day. The total treatment doses of the plants were from 25 to 150 Gy for 100 days. The explant sources of leaf, bud and floral petal dissected from each irradiated plants were incubated on the callus induction medium after sterilization.

In acute irradiation, the explant sources of leaf, bud and floral petal dissected from unirradiated plants were incubated on the callus induction medium after sterilization. After three days of incubation, the explants were irradiated at a dose rate 10 Gy/hr, for total doses ranging from 20 to 100 Gy in a gamma room (44.4 TBq., $^{60}$Co source). Soon after irradiation, the segments were transferred to a new callus induction medium. The induced calluses were subcultured on the callus medium and then on the regeneration medium.

The regenerated plantlets were acclimatized in a greenhouse then transplanted to a field nursery to investigate mutation induction. The plants used as the control were established by cutting back twice from the lateral shoots of the chronically irradiated plants. In addition, regenerated plants derived from each explant source of unirradiated plants were treated as the comparison.

The mutation rate was calculated based on flower color mutants selected from the regenerated plants in each treatment. Moreover, the mutation efficiency at different

irradiation doses in each treatment was calculated using the following formula.

Mutation efficiency (%/Gy) = No. of mutated plant x 100 / Total no. of plants x Total irradiation dose (Gy)

Then after, to confirm chimera and secondary mutation induction on mutant lines, flower color mutation were investigated on regenerators from flower petals and buds taken from the selected mutant lines without any mutagenic treatment.

## Results

### Mutation Rate and Efficiency by All Combined Methods

All methods were evaluated to find which combination of irradiation and explant source yielded the best mutation rate and/or mutation spectrum. The method using a chronic petal culture gave the highest mutation rate, followed by chronic bud culture and acute petal. The methods using a leaf with either chronic or acute irradiation provided a lower mutation rate than those using petals and buds.

The mutation efficiency of all of the above methods using cultured materials was much higher than with the conventional method using chronic shoots. However, methods using any unirradiated materials, such as shoots, leaf, petal, and bud cultures, produced almost no mutants. The most variability of flower color on the regenerators was induced not by a culturing process but by gamma irradiation.

The acute petal culture method had the highest mutation rate, which increased 1.36% with a 1 Gy radiation dose. The other methods, using irradiation and explants, had somewhat similar mutation frequencies, ranging from 0.79 to 0.48%/Gy, whereas the one using chronic shoots was 0.076%/Gy. Thus, the culture methods using irradiation were about 10 times more effective than the conventional chronic cutting method.

### Flower Color Spectrum of the Mutants

The mutated flower colors (except the original pink) were categorized into seven types, e.g. white, light pink, dark pink, orange, yellow, bronze, and striped. Although a total of 550 mutants (14.91%) were obtained from 3689 plants, 79% of the mutants fell into light and dark pinks similar in color to the original variety. The mutation rate of the flower color of the regenerators, for all colors and ones excluded the light and dark pinks, indicate which methods extended the mutated color spectrum. The results reveal that the highest mutation rate was obtained by the chronic petal culture, and the chronic bud culture followed. The other methods chronic shoot, chronic leaf culture, and all acute irradiation methods proved to have lower mutation rates, with a narrow color spectrum.

## Color Analysis of Mutant Flower

The Hunter's color space analyzed by spectro photometer demonstrated that mutant flower colors were categorized into four groups, pink, white, yellow, and orange. Each group of pink/white or yellow was distributed over linear cluster indicating the light and dark directions. The orange color, however, was isolated from the above two groups. The mutant colors induced through chronic petals fell into three color groups, pink, white and yellow (except orange), those through chronic buds into pink and white, and those through chronic cutting into orange and pink. In particular, the orange was the specific color exhibited by the coexistence of pink and yellow layers on a periclinal chimeric mutant induced by chronic shoot. The above results suggested that the variety genetically possessed two colors. It was concluded that color analysis by the spectro photometer was effective for numerically expressing the relations among continuous colored mutants and indicating clearly unique clones.

## Discussion

All of the mutants induced by conventional chronic irradiated shoots had chimeric formation in the first vegetative stage; two of the three flower color mutants reverted to the original color and one stabilized as a color mutant. Contrarily, almost all of the flower color mutants derived from the in vitro cultured callus were apparently non-chimeric. Therefore, in vitro culture can overcome the problem of chimera formation.

The mutation rates of all mutated colors indicated that the chronic petal and chronic bud culture methods yielded a much higher mutation rate than the chronic shoot method. In addition, the acute culture methods using petals, buds, and leaves held their positions between the chronic culture and the chronic cutting methods. Most of the cultured methods using irradiation provided 10 times greater mutation efficiency than the chronic shoot method. The above results demonstrate that the application of in vitro culture on radiation breeding markedly improves the mutation frequency.

A somaclonal variation often occurred on regenerators from callus and was used for mutation breeding without mutagenic treatment in some crop species (Larkin and Scowcraft, 1981). In this study, however, mutagenic treatment, such as irradiation, was essential for effective mutation induction since mutation induction through tissue culture was rare from any sources of explant in the variety. This result agrees well with the observation that changes in flower color are largely restricted to treatments involving irradiation (De Jong and Custers,1986).

In contrast, the mutation rates (excluding the original color) clearly indicated that the chronic petal culture method yielded the widest color spectrum, and induced seven mutant colors (except orange). The chronic bud culture method was second, followed by the chronic

leaf culture method.

It is likely that flower color mutation of the regenerators could be induced more from petals and buds than from leaves. In this respect, it is supposed that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture, which permits a high mutation frequency of flower characteristics in their regenerators.

Bush et al. (1976) also investigated plants regenerated from petal culture which exhibited many more mutant types than those from shoot tip cultures, and proposed that the mutable loci were most mutable in somatic tissue of inflorescence. These findings suggest that the mutation induction of the regenerator is dependent on explant sources.

It is worth investigating whether desired mutation could be obtained frequently on regenerators from explant tissue of the desired genes expressed. If it is true, a direction of desired mutation could be controlled by induction technique.

The acute culture methods indicated a relatively low mutation rate and a very limited flower color spectrum. In addition, the regenerators derived from acute irradiated plants definitely decrease in stalk elongation and number of petal per flower as the irradiation dose rose.

One possible reason that the chronic culture methods showed higher frequencies than the acute culture methods is that most of the cells composing the tissue and organs continually irradiated into a cell division which was highly sensitive and more mutable to irradiation. Under these conditions, many small mutated sectors may accumulate in the cells of the growing organs.

In contrast, when the explants composed of various cell stages were irradiated at high doses and dose rates, the dormant cells, less sensitive to radiation, could survive, but the more sensitive dividing cells were inactivated. Acute irradiation applied to cultured materials at much higher doses led to physiological damage and the ultimate death of cells according to their stage of the cell cycle.

When regenerated plants can be obtained through flower species culturing, the selection of a mutated colored petal under chronic irradiation should promote the induction of a wider color spectrum on the regenerators. Naturally, a high mutation frequency of flower color mutants can also be obtained from the petals with unchanged color from chronic irradiated plants. The appearance of mutated colors on chronic irradiated plants is a good prior indication of the color spectrum of the mutants, which can be extended to the regenerators through tissue and cell culture techniques.

In this study, the six flower color mutant varieties registered were derived from chronic irradiation. The combined method of chronic irradiation with floral organ cultures proved to be of particularly great practical use in mutation breeding of flower species. The method is also valid to obtain non chimeric mutants, to enhance mutation frequency, and to

enlarge the mutation spectrum not only in flower species but also in any crop species.

## Comparison of Combined Methods of Callus Culture with Chronic and Acute Irradiation in Sugarcane

### Materials and Methods

Sugarcane cultivar, Ni 1 were used for experiments of chronic or acute irradiation and tissue culture. In chronic irradiation, two plantlets developed from one-eye cuttings were planted on 40 cm diameter pots, and five pots were prepared each for 6 irradiation treatments in a gamma field, including non-irradiated plants (control) grown outside an irradiation facility.

The potted plantlets were irradiated chronically at 50, 100, 200, 300 and 500 Gy in terms of total dose for 90 days. When the irradiation scheme had terminated, four top portions of 100 and 300 Gy plots and control were served as explant sources for tissue culture.

In acute irradiation, explants excised four top portions of intact plants, were irradiated at 50, 100, 200, 300 and 500 Gy in terms of total dose for 20 hours in a gamma room.

Rolled youg leaves was dissected for callus induction on a modified Murashige and Skoog (MS) medium as reported in Heinz. Then, regenerated plantlets from callus were transferred to experiment field. Preliminary data were collected on agronomic characters such as stalk length, stalk diameter, number of stalks, leaf length, leaf width, weight per stool and some qualitative characters. Estimation of chromosome number of the mutant lines was investigated basing on nuclear DNA content by flowcytometry.

### Results

#### Variation of characters

The means of major characters in the chronic irradiation were similar between dosages in both generations. However, the coefficients of variation were greater in both generations as the irradiation dose increased. These results suggest that the regenerated populations from chronically irradiated plants showed rather wider variation than ones from non-irradiated. The ranges of variation extended not only in a negative but also in a positive direction.

On the other hand, the means of major characters were decreased remarkably as the irradiation dose rose. The coefficients of variation were relatively larger at lower dose but smaller at higher doses. From these results, regenerated mutant lines were adversely affected by high dose-rate radiation.

No significant correlation was found in 0 Gy and 100 Gy populations, while highly positive correlations for stalk diameter (+0.866***) and stalk length (+0.585***) were

revealed in 300 Gy. In addition, a significant positive correlation between two generations was found for stalk number per stool and cane weight per stool in any irradiations. Consequently, the results suggest that significant positive correlation could have arisen from the wider genetic variation among the subclones induced by gamma ray irradiation, and that such variation could be transmitted by clonal propagation. It was noted that the increased variances with higher heritability can be transmitted by clonal propagation and hence utilized as genetic sources in mutation breeding.

### Variability on synthesized characteristics

In order to elucidate variability of overall characteristics of the subclones, principal component analysis was computed based on a correlation matrix for 10 characters of 136 subclones. Contribution for the first, second and third components was calculated as 31.4, 22.8 and 10.6% of the total variation, respectively. The biological meaning of the first component represents low yield directing towards the positive side, and high yield towards the negative. The second component indicated whether the subclone was 'stalk weight type' or 'stalk number type'

A scatter diagram between the first (Axis $Z_1$) and the second component (Axis $Z_2$ ) indicates that the subclones from the control(0 Gy) distributed in relatively limited region around the origin. The subclones from the 100Gy and 300Gy are scattered over a broad region. It is suggested that chronic irradiation, combined with tissue culture, broaden the variation of general characteristics on the subclone populations. From the viewpoint of practical breeding, those subclones from 300Gy that lie in the $-Z_1$, $Z_2$ quadrant are of special interest, showing high yield with long, thick, and heavy stalks.

From the standpoint of actual mutation breeding, two subclones, were remarkably higher in cane weight over the donor. In addition, 5 clones from 300 Gy, 3 ones from 100 Gy, and one from 0 Gy had some potential to be improved cane yield under the commercial cane belts. It is much interest from viewpoint of increasing sugar content that 4 subclones from 300 Gy, 3 ones from 100 Gy and 3 ones from 0 Gy were markedly higher than the donor.

### Change of chromosome number in mutant lines

Average chromosome numbers estimated by DNA content show remarkable trend by irradiation methods. Regenerated lines from unirradiated plants show the same chromosome number as the original variety estimated for $2n = 115$. In acute irradiation, regenerated mutant lines show remarkable decline of chromosome numbers as the irradiation dose roses. There is close negative correlation between irradiation dose and chromosome number of each mutant lines.

On the contrary, in chronic irradiation, regenerated mutant lines indicate generally little decrease in chromosome number even by successive chronic irradiation for three years.

## Discussion

The subject to be discussed is variability of callus derived subclones induced by the process of tissue culture and by mutagenic treatment. In the present study, some extent of somaclonal variation was observed on the subclones by tissue culture without using irradiation. None of the quantitative characters such as stalk size and weight of cane showed significant variability. Also, no correlation was observed for stalk size and yield components between the first and second generation. It is concluded that only minor variations among tissue culture subclones had occurred.

The chronic irradiation on growing plants induced major variations on quantitative character such as stalk size and yield of cane in their regenerated subclones. Such somaclonal variations in the second generation extend toward not only the negative but positive extreme as the irradiation doses increased. These results suggest that chronic irradiation in the gamma field combined with tissue culture possessed great potential to induce useful genetic variation in mutation breeding.

On the other hand, the acute irradiation on cultured materials induced damaged and less vigor mutants on radiation dose exceeded. From the result, acute irradiation using in vitro materials seems to be risky, therefore, proper irradiation method and optimum dose should be considered.

Since mutagenic treatments of tissue and cell cultures had been attempted by Heinz et al., little other work has been reported. From the present study, it is evident that gamma irradiation plus tissue culture is a method of extending variation and increasing mutation frequency. Such technique may be profitable for sugarcane to enlarge somaclonal variations. In the present report, 300Gy was the highest chronic irradiation dose for survival of a viable meristem from which in vitro explants were taken and, later, seed prieces. The chronic irradiation method adopted in the study accumulated a relatively high dosage but with reduced radiation damage prejudicial to proliferation and differentiation of the callus.

In other words, every cell in the growing plants under chronic irradiation is irradiated during the cell division process which is generally the most sensitive and critical phase for mutagenesis. Higher mutation frequencies among the subclones may be assumed to originate from one or few cells in which mutation have occurred during the cell division due to long chronic irradiation. Therefore, the tissue culture technique combined with chronic irradiation is an effective means to multiply mutated cells to regenerated mutants.

## Conclusion

From the studies on mutation breeding in two model plants, the followed conclusion on the effects of chronic irradiation has been obtained using in vitro culture and various gamma irradiations.

1. Culture technique combined with irradiation can overcome the problem of chimera formation. Most of the cultured methods using irradiation provided 10 times greater mutation efficiency than the chronic shoot method.
2. A somaclonal variation often occurred on regenerators from callus, however, none of significant variation was appeared from callus regenerators in both crops. Therefore, proper mutagenic treatment on cultured materials is indispensable to effective mutation induction.
3. It is clearly indicated that the chronic culture method yielded the widest color spectrum in chrysanthemum and extend toward not only the negative but positive direction. The acute culture methods indicated a relatively low mutation rate and a very limited flower color spectrum.
4. Flower color mutation of the regenerators could be induced more from petals and buds than from leaves. In this respect, it is supposed that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture. It may be likely to control a direction of desired mutation.
5. One possible reason why the chronic culture methods showed higher frequencies than the acute is that most of the cells composing the tissue and organs continually irradiated into a cell division which was highly sensitive and more mutable to irradiation. Under these conditions, many small mutated sectors may accumulate in the cells of the growing organs. In contrast, when the explants composed of various cell stages were irradiated at high doses and dose rates, the dormant cells, less sensitive to radiation, could survive, but the more sensitive dividing cells were inactivated.
6. In acute irradiation, regenerated mutant lines show remarkable decrease of chromosome numbers as the irradiation dose roses. There is close positive correlation between chromosome number and biomass of each mutant line. In chronic irradiation, regenerated mutant lines indicate generally little decrease in chromosome number. It is a proper indicator to monitor radiation damage.
7. In this study, the six flower color mutant varieties registered were derived from chronic irradiation. The combined method of chronic irradiation with floral organ cultures proved to be of particularly great practical use in mutation breeding for not only flower species but any other species.

## Reference


1. Broertjes, C. and A.M. Van Harten (1978) Application of Mutation Breeding Methods in the Vegetatively Propagated Crops. Elsevier, Amsterdam, pp 270-276.
2. Broertjes, C., S. Roest and G.S. Bokelmann (1976) Mutation breeding of *Chrysanthemum morifolium* Ram. using in vivo and in vitro adventitious bud techniques. Euphytica, 25:11-19.
3. Bush, S.R., E.D. Earles and R.W. Langhans (1976) Plantlets from segments, petal epidermis and short tips of the periclinal chimera *Chrysanthemum morifolium* 'Indianapolis'. Am. J. Bot., 63:729-737.
4. De Jong, J. and J.B.M. Custers (1986) Induced changes in growth and flowering of chrysanthemum after irradiation and in vitro culture of pedicels and petal epidermis. Euphytica, 35:137-148.
5. Heinz, D.H. (1973) Sugarcane improvement through induced mutations using vegetative propagules and cell culture techniques. In: Induced Mutations in Vegetatively Propagated Plants. IAEA, Vienna. pp53-59.
6. Heinz, D.H., M. Kirshnamurthy, L.G. Nickell and A. Maretzki (1977) Cell, tissue and organ culture in sugarcane improvement. In: Rennert & Bajaj (eds.) Aspects of Plant Cell, Tissue and Organ Culture, pp 3-17.
7. Larkin, P.J. and Scowcroft, W.R. (1981) Somaclonal variation, a novel source of variability from cell culture for plant improvement. Theor. Appl. Genet., 60:197-214.
8. Liu, M.C. and W.H. Chan (1987) Tissue and cell culture as aids to sugarcane breeding. II. Performance and yield potential of callus derived lines. Euphytica, 27: 273-282.
9. Micke, A., B. Donini and M. Muluzynski (1990) Induced mutations for crop improvement. Mutation Breeding Review, 7:1-41.
10. Nagatomi, S.(1991) Enlargement of induced variations by combined method of chronic irradiations with callus culture in sugarcane. Gamma Field Symp., 30:87-110.
11. Nakajima, K. (1977) Studies on the effective methods for induction of mutations of vegetatively propagated plants by the use of the gamma field. Bull. Inst. Rad. Breed., 4:1-105.
12. Yamashita, A.(1981) Twenty years research of chronic gamma-ray irradiation on seed crops. Gamma Field Symposia, 20:87-102.