INDUCTION AND ISOLATION OF SOMATIC MUTATIONS IN VEGETATIVELY PROPAGATED PLANTS*

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Introduction

Spontaneous somatic mutations have played a considerable role for the improvement of vegetatively propagated plants, but their rate is too low to be adequately used. Mutagenic treatments have been recognized as a valid tool to increase the frequency of somatic mutation by inducing chromosomal changes or by causing rearrangements of cell layers in the shoot apical meristem. Moreover, it is a peculiar characteristic either that the induced somatic changes will affect only few characters, without altering deply the genotype of an outstanding cultivar, or that the useful mutations can be directly propagated vegetatively.

It implies that the genetic variety assortment, induced by mutation, is much faster to reach than by cross breeding, due to the long pre-reproduction period of clonal plants, being also favoured by their heterozygosity which, on the contrary, makes unpredictable the results of recombination.

For these consideration, mutation breeding in fruit trees has been stimulated during the past 30 years and, in several countries, a wide number of experiments for the induction of useful mutations even though the results obtained have been not so numerous and moreover only five new varieties induced through mutations are reported in the list of F.A.O./I.A.E.A. of 1972. Therefore, the question raises that the success of any work with radiation-induced mutations requires a considerable effort and it is not simply a matter or irradiating few buds and hoping that few casual observations plus the magic of nuclear energy will do the rest.

Being conscious that positive results can be obtained through mutation breeding in vegetatively propagated plants, several authors have faced the problems of technique of mutagenic treatment as well as the technique of handling the material. The researches carried out in our Laboratory since 1963 follow these aspects and mainly cover the problems of methods of exposure, types of radiations, conditions during and after irradiation, mechanism of mutation induction and methodology of isolation of somatic mutations.

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Technique of mutagenic treatment

a.) Methods of exposure:

Both acute x-rays and gamma rays and chronic exposures of gamma rays in the gamma field (^{60}Co source) have been used to study fruit plant tolerance as well as mutation induction. Table 1 gives the list of treated material, the exposure rate and the total exposures used.

In order to estimate the radiosensitivity of acute irradiation, several exposures were applied on dormant scions, the basal part of which were shielded and the buds have been allowed to sprout by forcing the scions in water in the greenhouse. The data on the number of growing buds and the length of new shoots were recorded for a period from one week to two months and compared with the control.

In the gamma field, several species of fruit plants have been grown under chronic irradiation at different exposure rates, for a long time from one to four years and they showed different response from normality to lethality depending on the exposure rate applied.

It is relevant to note that both under acute and chronic exposures, the range of radiosensitivity of the investigated species is rather narrow. A total exposure between 2.5 and 5.5 kR is required in order to reduce sprouting of buds by 50% after acute treatment whereas exposure rate between 15 and 30 R/day affected the plant growth under chronic irradiation. From Table 2, it can be seen that trees of peaches, apricots, cherries and grapes are more radiosensitive than apples, pears and Corylus ones.

Until now we have not collected definitively data on the relative efficiency of acute and chronic exposure in inducing somatic mutations. However, for some materials, we have the evidence about the greater efficiency of acute treatment (i.e. in grapes, olives); whereas in one cultivar of peach we obtained two mutations (for earliness and nectarine types) under chronic irradiation, but after acute irradiation of thousand of buds, we never obtained any such mutations.

b.) Type of radiation:

It would be expected by using different kinds of radiation a different efficiency of the treatment both in terms of frequency and spectrum of mutations. We used for acute irradiation an x-ray machine operating at 250 kV, 15 mA, a gamma ray facility of ^{60}Co source (150 Ci) and thermal and fast neutrons of the TRIGA MARK II Reactor, while for chronic irradiation, we used a gamma ray facility of ^{60}Co source (250 Ci) operating in a gamma field.

The results obtained have shown a more uniform response and a higher biological efficiency at comparable total exposure, with densely ionizing radiations, as compared to sparsely ones. Because of the difficulty due to the size of the material and the limited space at disposal in the reactor only few material, plantlets of Fragraria, rooted cuttings of Antirrhinum, small scions of Vitis europea, have been irradiated with neutrons for mutagenesis purposes.

Interesting results of increasing the rates of mutations have been obtained in cultivars of Prunus avium, Vitis europea and Antirrhinum majus when given a second irradiation of the V_1 shoots derived from a treated bud.

c.) Material treated and stages:

The plants growing in the gamma field were chronically irradiated at the different developmental stages of dormant and actively growing buds, primordia differentation and floral buds. Some plants have been transplanted out of gamma field after receiving progressive total exposure. Sometimes dormant scions or summer buds, collected from the chronoically irradiated plants have been grafted on rootstocks.

The analyses of somatic mutations carried out on apple, pear, olive and peach plants, which were allowed to recover on the grafted buds taken from chronically irradiated plants, showed a very low frequency of mutations and usually narrow somatic sectors.

Both summer buds at resting stage and dormant buds of several fruit trees have been irradiated; the results have clearly shown in cherry and apple that at the same total exposures, both the primary effects in V_1 and the somatic mutations observed in V_2 and V_3, were lower after summer buds irradiation.

It must be underlined that the summer buds which were irradiated and then grafted are going to dormancy til the next season. The buds of dormant scions irradiated are ready for sprouting immediately in the spring.

The irradiations which have been performed in cultivars of grape, apple, olive, Corylus and cherry by treating dormant scions or one year old grafted trees or rooted scions, have shown a better tolerance of the rooted material. It implies that we could easily increase the exposure from 500 R to 1000 R over the dose used for dormant bud which had to be grafted, by treating grafted material with shielded root apparatus and thus to enhance the rate of mutations.

In order to increase the size of mutated sector, irradiation was carried out in grapes by treating the dormant buds and removing at sprouting the main shoot so that the V_1 shoots will originate from a xilary buds which were already present in the treated bud but with a comparatively reduced cell population.

To avoid chimeras produced by treatment of a multi-cellular structure are a shoot apex, some preliminary experiments have been carried out by treating the root cuttings of Cydonia vulgaris, Malus communis, Prunus avium, Corylus avellana and Phlox, which have the potentiality to form buds from roots.

Starting from few cells with the same purpose, the apomictic plants, such as species of apples and cultivar of orange, have been irradiated at single cell stage, the initial of nucellar embryo formation. We have explored the suitable conditions which, during irradiation, might reduce the physiological effects and thus enhance

the genetical effects of the treatment. The effect of O_2 and N_2 conditions have been studied on dormant scions of Malus communis, Prunus avium, Ribes nigrum treated with gamma rays. A greater tolerance of buds, estimated in terms of growth in N_2 conditions relative to O_2 and air, have been ascertained especially by increasing the total exposure (Figures 1 and 2).

After the treatment, to give better chance to the mutated cells to grow and to be competitive with the unmutated ones, the rooted scions of cultivars of Vitis europea and Olea europea were grown in V_1, both under normal and controlled environmental conditions.

Mechanism of mutation induction

Somatic mutations might be raised by true one-cell mutational event which occur in the shoot apex layer, or by cell layers rearrangement of a genetical preexisting chimeric structure.

We have investigated such possibilities by using a heterozygous clone for flower colours of Antirrhinum majus, peach cytochimeras 2n-4n-4n and 4n-2n-2n and a genetic marker chimeras of peach and of Corylus avellana atropurpurea.

Treatments were given in Antirrhinum by applying the chronic exposure of 200 R/d in the gamma field on growing plants and the acute exposure ranging from 2 to 4 kR on rooted cuttings.

Periclinal mutated sectors have been induced and through clonal propagation and growth, we were able to isolate several mutants with a periclinal mutated layer. Further investigations were aimed to ascertain the number of cell layers involved in the induced periclinal mutants and in the forthcoming ones. Hence, we are carrying out the analyses of the segregation of the self-pollinated mutants and controls. The comparative segregation behaviour would indicate, if the somatic mutation induced resides or not in the tissue, where the sex cells are formed. Besides, irradiations of some induced mutants have been performed and they have shown a high frequency of somatic sector of the original colour, which clearly reveals the chimeric structure existing in the shoot apex. By using in vitro culture technique, we are trying also to regenerate plants coming from the inner layers in the induced somatic mutants of Antirrhinum and to verify the genetic constitution of such layer. Dormant scions of the two types of peach cytochimeras have been irradiated at the total exposures of 300, 600, 900, 1200, 1500 R and histological analyses have been carried out on buds fixed at successive developmental stages.

These analyses indicate that the radiation affects the stability of these cytochimeras, 4n-2n-2n and 2n-4n-4n and even at lowest exposure of 400 R the 50 and 80 percent of the observed apices respectively had changed from the initial structure. The results obtained by the irradiation of such cytochimeras and of

the genetic chimeras of Corylus and Prunus persica allow to state that the cells might shift from one to another layer and there is chance of cells shifting from outer to the inner layers and vice versa. The irradiation has a different influence in the stability of the two types of cytochimeras studied.

The shifting of cells from one layer to another has been explained either as a result of change of cell division plane (f.i. from anticlinal to periclinal) or radiation damage of cells and their substitution by the adjacent ones. The cell layers rearrangement produced by radiation treatment of shoot apex leads to new types of chimeras and reveals the chimeric structure of spontaneous or induced mutation.

Methodology of somatic mutation isolation

The successful use of mutation breeding in vegetatively propagated plants mainly resides in the adapted technique of handling the material after the treatment. Whereas the detection and isolation of the induced mutations may not present difficulties when the treated tissue consists of only a single cell, difficulties arise, on the contrary, by irradiation of an organized and multicellular structure such as a shoot apex, which leads to sectorial or mericlinal chimeras and during growth the mutated tissue might form a constant periclinal layer. The chance of a genic or chromosomal mutation induced in one or few cells to give rise to a somatic sector will depend on the result of competition of mutated cells with the unmutated neighboring ones. Even if proliferating mutated cells form somatic sectors, they may be again lost because they appear in part of the plant that normally do not develop into further growth. In our material, the guideline criteria adopted for somatic mutation isolation are based on the histological analyses of treated shoot spices which gave the indication of primordia number already present in such buds and on the plant growth behaviour (apical or basal).

The further consideration is that a mutational event will occur by chance and with the same probability in the cells of the different meristematic territories. As a consequence, the number of mutated cells will be higher in the larger primordia; but, on the contrary, the size of the mutated sector will be larger in the primordia with lower number of cells.

For these reasons, the V_1 shoots have been grown and cut back at the end of the season leaving two to three basal buds; the other V_1 buds have been propagated taking into account their position in the shoot. This technique used in plants with apical dominance will give chance to all the V_1 buds to develop and to enlarge the mutated sector. The total number of V_1 buds which

has been forced to growth is based on the number of primordia already present in the irradiated bud (V_0). However, such primordia number might be more or less numerous depending on the developmental stage of the bud, position (main or auxillary) and the variety or species considered.

Following these criteria, a suitable methodology was used for isolation of somatic mutations in grapes, olives, peaches and cherries (Figures 3 and 5). After the irradiation of dormant buds of several sweet cherry varieties, the extensive analyses carried out on the V_2 shoots, which have been grown by recording their position in the V_1 indicated that the higher frequency of mutations ("spur types") are coming from the bud primordia originating in the area immediately below the apical meristem of the treated bud.

Similar results were obtained in two cultivars of olives. A further indication came out from the irradiation of dormant plants of Prunus avium rootstock $F_{12/1}$ at the total exposure of 3500 R and 4500 R.

The highest somatic mutation frequency was found in the V_2 plants coming from 4^{th} to 12^{th} bud and 1^{st} to 3^{rd} bud of the V_1 shoots respectively after lower and higher total exposure. It would indicate that either the primordia number of the treated bud or the total exposure applied had to be considered in the propagation of the V_1 buds for a more efficient isolation of somatic mutations.

Practical objectives of mutation breeding in fruit plants

The researches carried out in our Laboratory deal mainly in the exploration of better techniques of mutagenic treatment and the more efficient methodology of somatic mutant isolation.

Moreover, in cooperation with the Institutes of Horticulture of Ministry of Agricolture and Forestry, of National Research Council and of University, experiments are in progress for the induction of useful mutations in several fruit species (Table 3). The main practical objectives are:

a.) Induction of "spur types" in cultivars of cherries, rootstock of cherries, olives and Italian apple cultivars (Figure 6 and 7).
b.) Induction of mutations for disease resistance in grapes, peaches and apples.
c.) Induction of mutations for fruit qualitative characteristics in peaces, grapes, apples and Eriobotrya japonica.
d.) Induction of self-fertility in olive and cherry cultivars.

Table 1: List of irradiated plant species and treatment data.

SPECIES AND CULTIVARS	PLANT OR ORGAN TREATED	TYPE OF RADIATIONS	EXPOSURE RATES	TOTAL EXPOSURES	TECHNICAL REMARKS	PURPOSE
Pirus communis 6 cultivars	young trees	γ ^{60}Co	16 - 31 R/d.	16 - 32KR	Chronically exposed for 50 months	Radiosensitivity
	dormant scions	γ ^{60}Co	500-600 R/hr.	300 R-6 KR	Plants allowed to recover	
	floral buds	x-Rays	185 R/min.	2 - 6 KR	Summer buds grafted	Mutation breeding
		thermal neutrons	0,1 Rad/sec.	0.3-0,5 KR	Scions grafted	
					Pollen used for pollination	
					Scions forced in greenhouse	
Malus communis 5 cultivars	young trees	γ ^{60}Co	16 - 31 R/d.	14 - 30KR	Chronically exposed for 44 months	Radiosensitivity
	dormant scions		284-1047R/hr.	2 - 7 KR	Summer buds grafted	
					Plants allowed to recover	Mutation breeding
					Scions grafted	
					Scions forced in greenhouse	
Vitis europea 14 cultivars	young trees	γ ^{60}Co	11 - 23R/d.	10 -20 KR	Chronically exposed for 34 months	Radiosensitivity
	dormant scions		235-550R/hr.	1 - 4 KR	Plants allowed to recover	
	seeds	x-Rays	185 1008R/min.	2 -20 KR	Scions grafted on root stock	Mutation breeding
		thermal neutrons	2×10^9 n/cm^2 sec.	0,3-0,5 KR	Scions forced in greenhouse	
					Seeds germination	
Olea europea 5 cultivars	young trees	γ ^{60}Co	11 - 23R/d.	10 - 20 KR	Chronically exposed for 34 months	Radiosensitivity
	rooted scions	x-Rays	187-281 R/hr.	3 - 4 KR	Plants allowed to recover	
	dormant scions		104 185R/min.	1 6 KR	Scions forced in greenhouse	Mutation breeding
					Plants transplanted in field	
Corylus avellana 3 cultivars	dormant scions young trees	γ ^{60}Co	620R/hr.	2 - 7 KR	Scions forced in greenhouse	Radiosensitivity
					Plants transplanted in field	Mutation breeding
Rubus idoeus	dormant scions	γ ^{60}Co	620R/hr.	2 - 6 KR	Scions forced in greenhouse	Radiosensitivity
Ribes nigrum	dormant scions	γ ^{60}Co	620R/hr.	2 - 6 KR	Scions forced in greenhouse	Radiosensitivity

SPECIES AND CULTIVARS	PLANT OR ORGAN TREATED	TYPE OF RADIATIONS	EXPOSURE RATES	TOTAL EXPOSURES	TECHNICAL REMARKS	PURPOSE
Prunus persica 11 cultivar	young trees dormant scions summer buds	$\gamma^{60}Co$ x-Rays	11 - 23 R/d. 612-2000 R/hr. 185 - R/min.	10 -20 KR 1 - 5 KR 400-1500 R	Chronically exposed for 34 months Plants allowed to recover Scions forced in greenhouse Buds grafted Plants transplanted in field	Radiosensitivity Mutation breeding
Prunus avium 18 cultivar	young trees dormant scions summer buds floral buds	$\gamma^{60}Co$ x-Rays	441-1000 R/hr 185 R/min.	0,4-4,5 KR 2- 6 KR	Plants transplanted in field Buds grafted Scions grafted Pollen used for pollination	Radiosensitivity Mutation breeding
Prunus armeniaca 1 cultivar	dormant scions	x-Rays	185 R/min.	1 - 5 KR	Scions forced in greenhouse	Radiosensitivity
Prunus domestica 1 cultivar	dormant scions	$\gamma^{60}Co$	612 R/hr	1 - 5 KR	Scions forced in greenhouse	Radiosensitivity
Ficus carica sativa	dormant scions	$\gamma^{60}Co$	620 R/hr	2 - 6 KR	Scions forced in greenhouse	Radiosensitivity
Juglans regia	dormant scions	$\gamma^{60}Co$	620 R/hr.	2 - 7 KR	Scions forced in greenhouse	Radiosensitivity
Eriobotrya japonica	young trees	$\gamma^{60}Co$	1000 R/hr	3 - 7 KR	Plants transplanted in pots	Radiosensitivity
Citrus nobilis	summer buds	$\gamma^{60}Co$	1000 R/hr	2 -10 KR	Buds grafted	Radiosensitivity
Citrus aurantium	seeds	$\gamma^{60}Co$	1000 R/hr.	1 -16 KR	Seeds germination	Radiosensitivity

Table 2: Radiosensitivity of fruit plants

Species	Chronic exposure	Acute exposure
	Severe effect on growth (over one year) R/day	Dormant buds LD/50 (40 days) KR
Ficus carica sativa	-----	2,5
Vitis vinifera	15	3,0
Olea europea	15	3,5
Prunus persica	15	3,5
Prunus avium	-----	4,0
Prunus domestica	-----	4,0
Armeniaca vulgaris	-----	4,0
Juglans regia	-----	4,2
Ribes nigrum	-----	4,5
Rubus idoeus	-----	5,0
Malus communis	25	5,0
Citrus nobilis	-----	5,5
Pirus communis	30	5,5
Corylus avellana	-----	7,0

Table 3: Mutations in fruit trees induced by irradiation

(Joint projects between the Agricultural Laboratory of C.N.E.N. and the Horticulture Institutes of M.A.F. and C.N.R.)

PLANTS	Mutations recorded	Character
PEACHES		
cv. Fertilia	3	Nectarine
cv. Fertilia	1	Early ripening fruits
cv. Favorita III	1	Narrow leaf and semiglabrous fruit
CHERRIES		
cv. Bigarreau Moreau	3	Spur types
cv. " Napoleon	3	" "
cv. " Burlat	1	" "
cv. Durona di Vignola I	4	" "
cv. " " " II	17	" "
cv. Mora di Vignola	5	" "
cv. Mora di Cassano	6	" "
cv. F 12/1	4	" "
GRAPES		
cv. Bonarda	1	Short internodes and thinning grapes
cv. Bonarda	1	Early ripening fruits
cv. Dolcetto	1	Leaf morphology
cv. Regina dei Vigneti	1	Small grapes and seedless
OLIVES		
cv. Ascolana	several mutations	Short internodes, leaf morphology
cv. Moraiolo	" "	" " " "
cv. Leccino	" "	" " " "

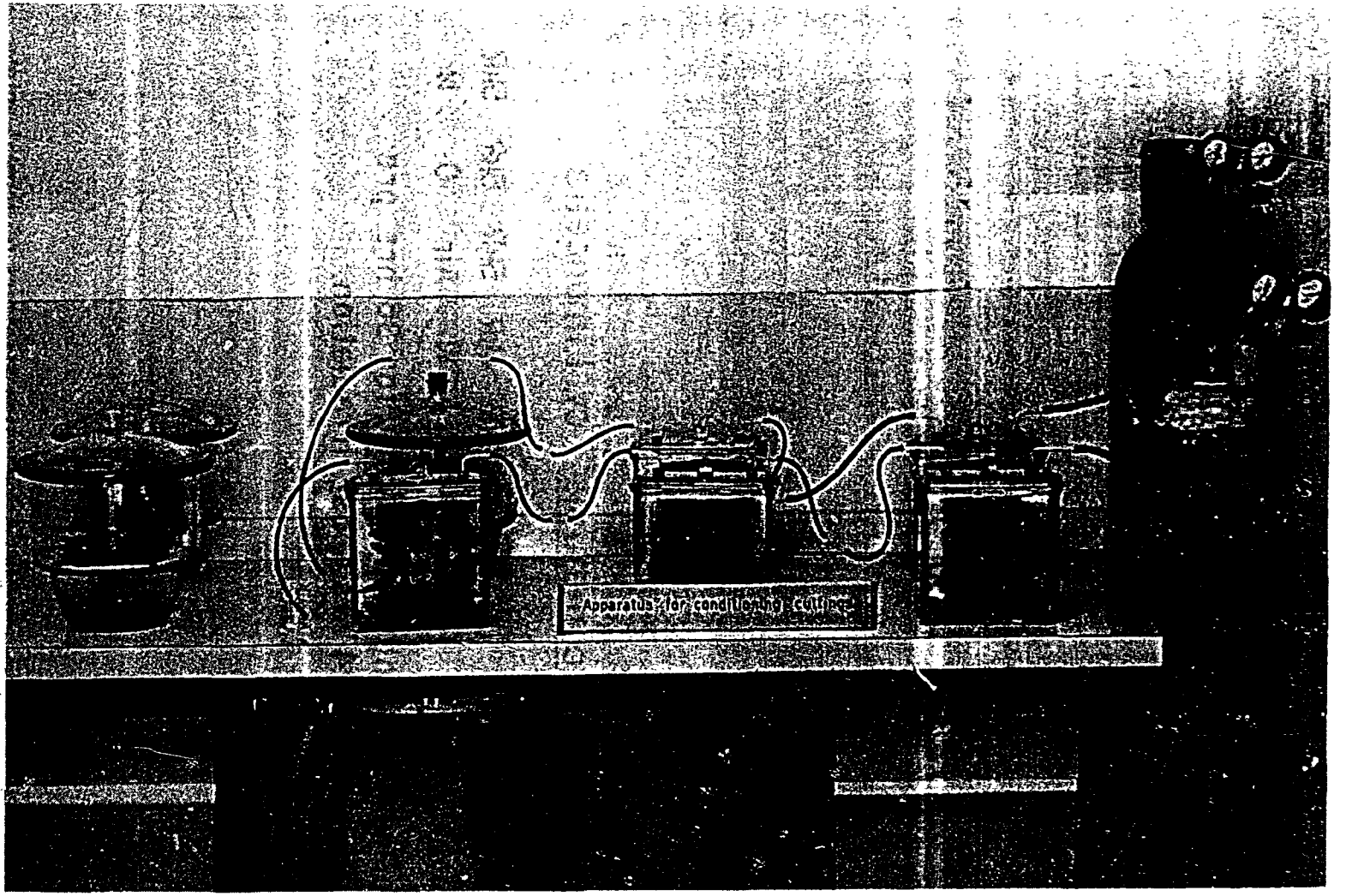

Figure 1: Apparatus for pre- and post-irradiation treatment of dormant scions with O_2 or N_2.

Malus communis cv. Limoncella

Gamma rays dose	0kR	0kR	0kR	4kR	4kR	5kR	5kR	5kR
Conditions	Air	O_2	N	O_2	N	Air	O_2	N

Dormant cuttings conditions for 36 hrs pre during and 24 hrs post irradiation.

Figure 2: Effect of pre- and post-irradiation treatment of dormant scions with O_2 or N_2.

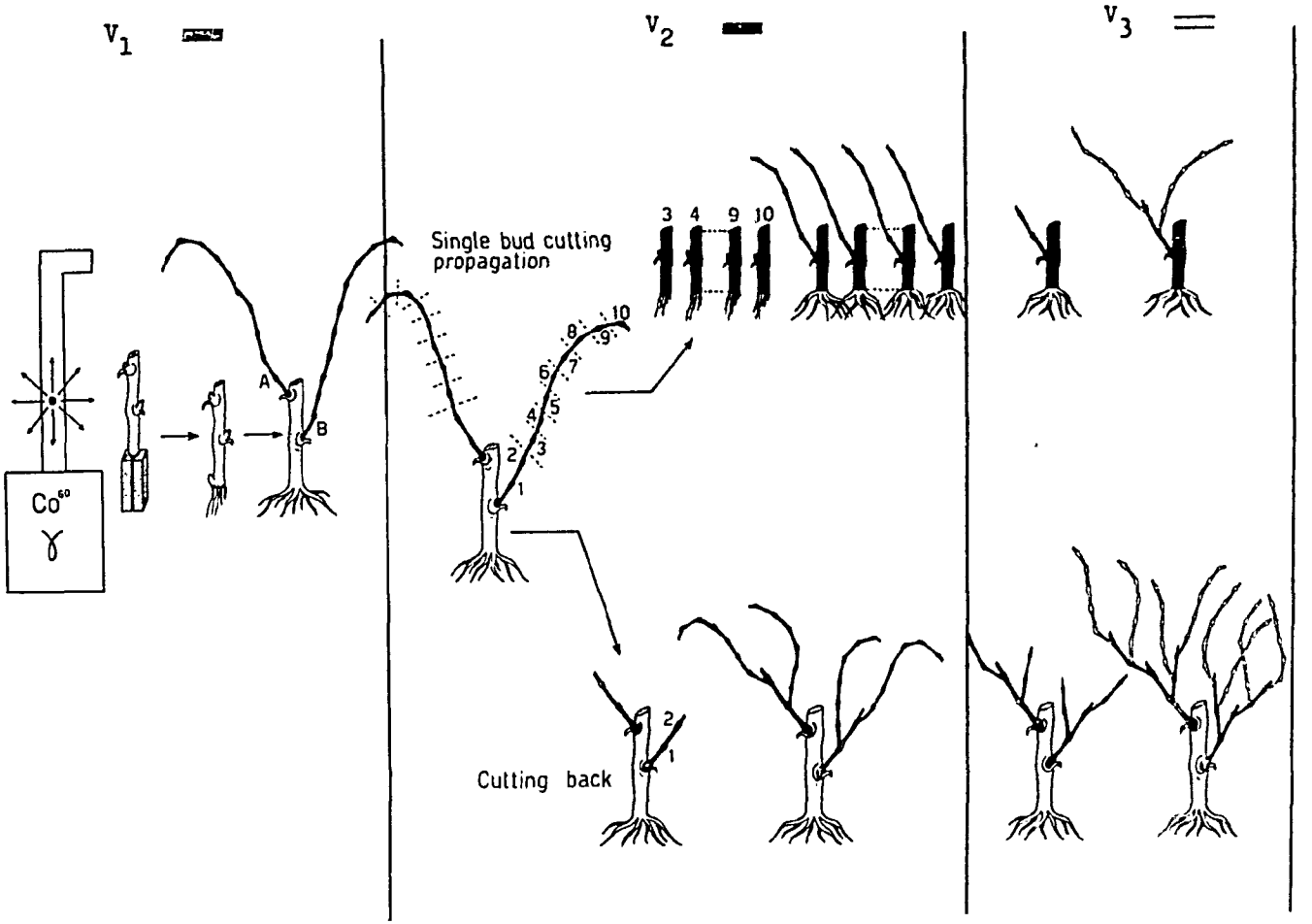

Figure 3: Methodology scheme used for isolation of somatic mutations in grapes

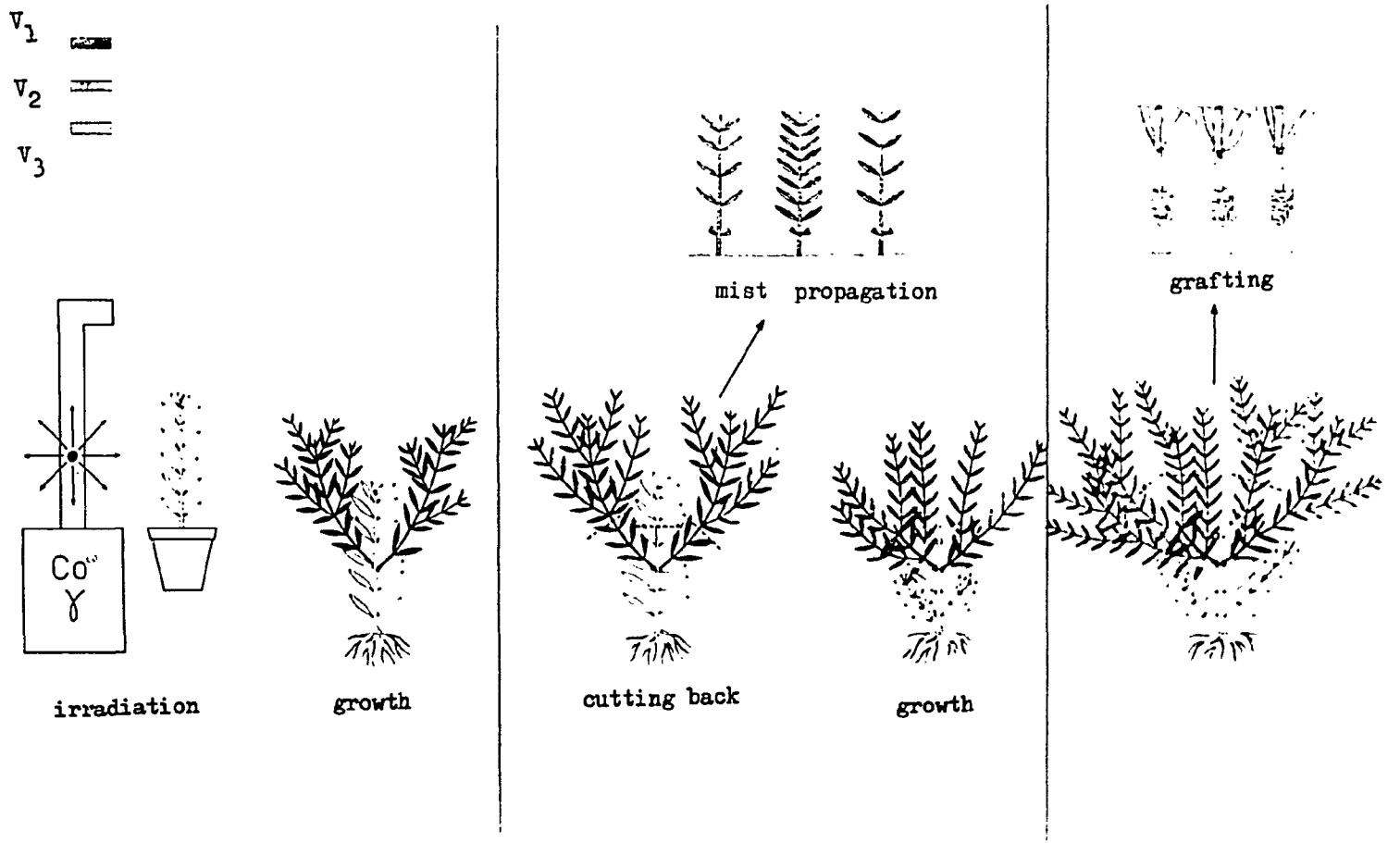

Figure 4: Methodology scheme used for isolation of somatic mutations in olives.

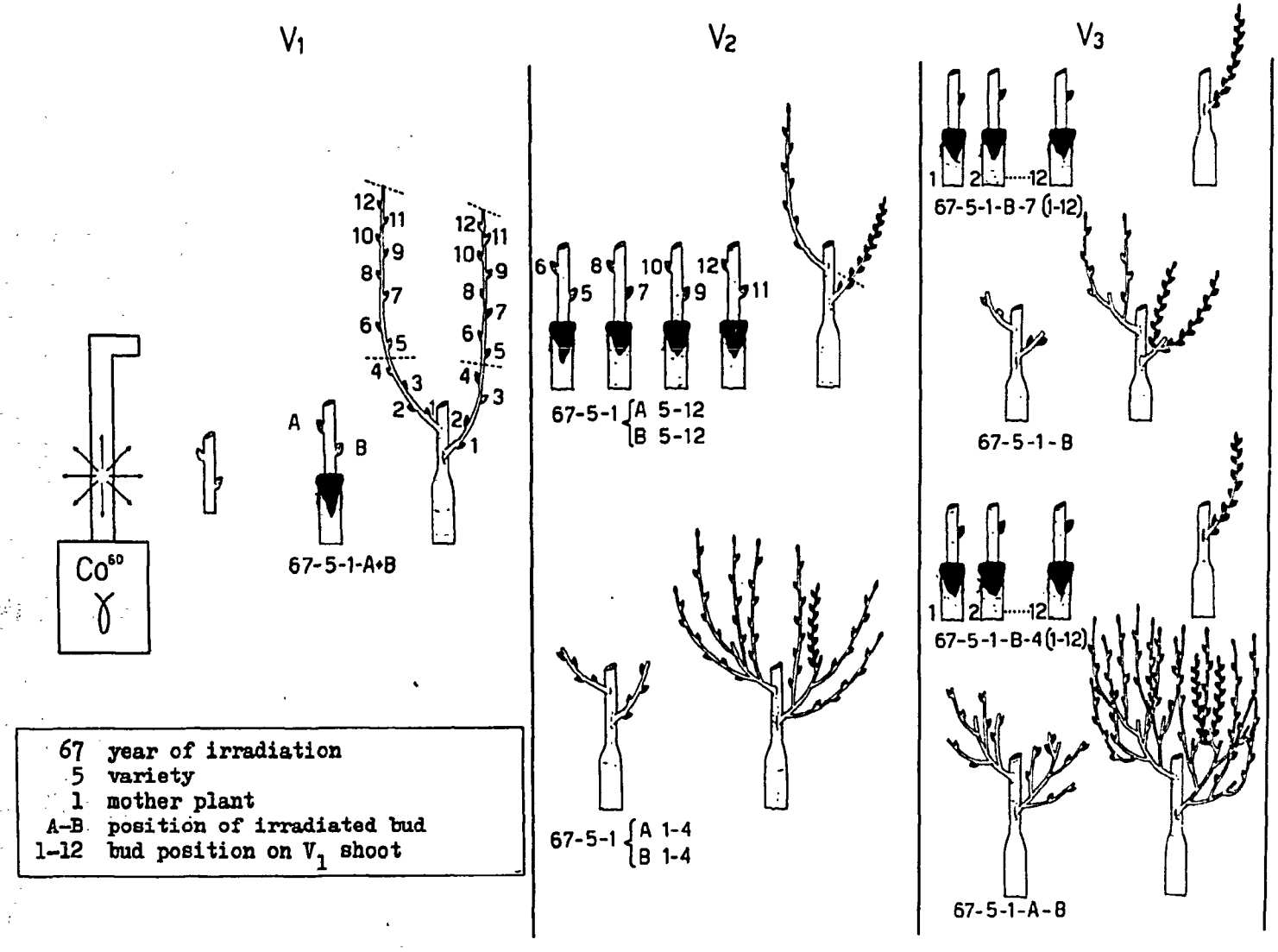

Figure 5: Methodology scheme used for isolation of mutations in cherries.

Figure 6: Four-year old cherry tree cv. Durona II di Vignola (Control).

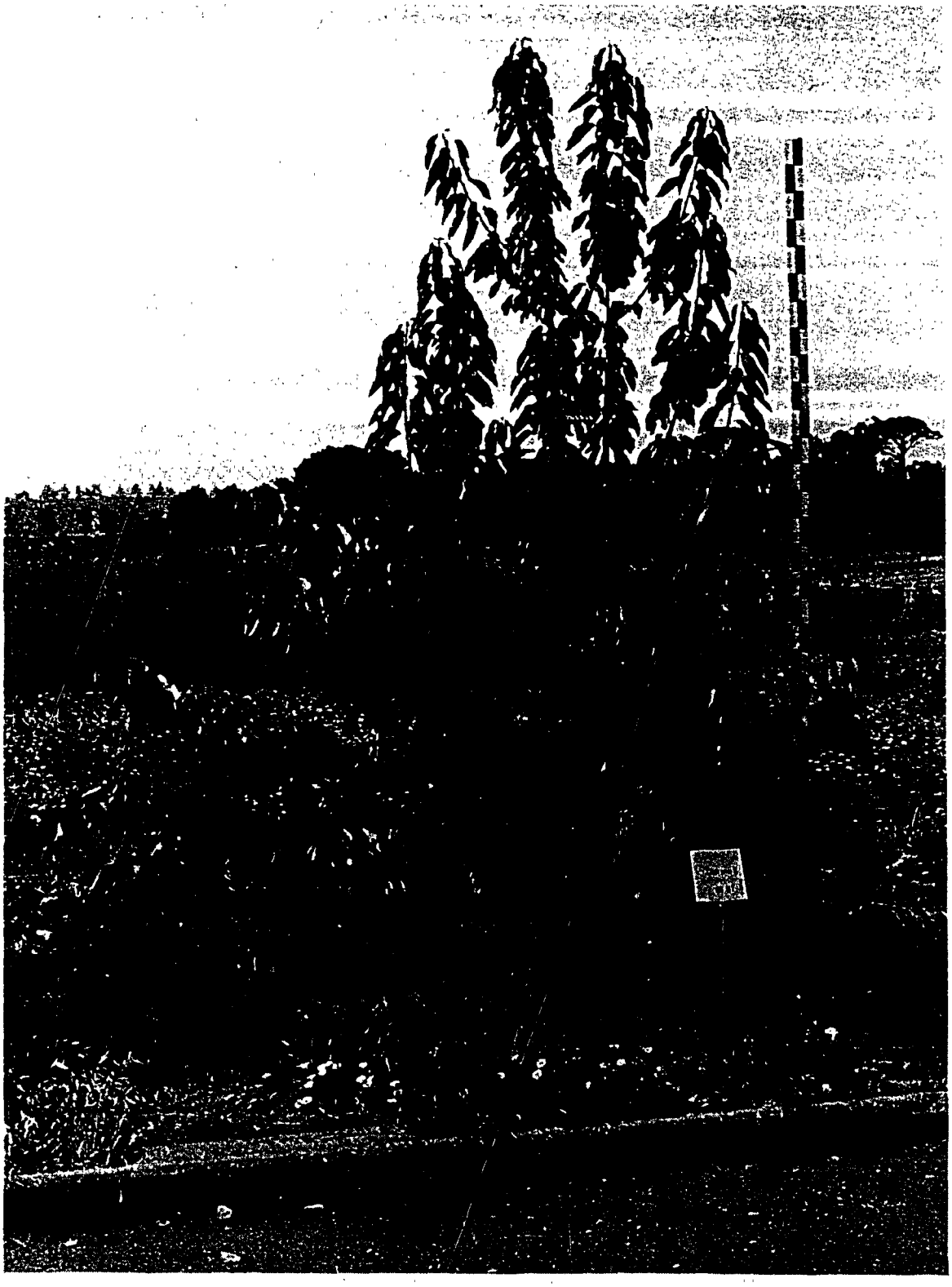

Figure 7: Four-year old compact radiation induced mutant cherry tree of cv. Durona II di Vignola.