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DEPARTMENT: Pomology   Project No. 42

TITLE: The induction and testing of somatic mutations in apples, grapes and other economic plants.

DATE INITIATED: July 1, 1953.   DATE OF COMPLETION: The preliminary phases of the project, the induction and isolation of mutations, will require a minimum of five years.

LEADERS: John Einset and Karl Brase

ASSISTANTS: Charlotte S. Pratt and Mohammad Zahur

COOPERATORS: Members of the staff of the Brookhaven National Laboratory.

PROGRESS FOR THIS YEAR: Gamma irradiation: Apples: Acute gamma irradiation: In 1955 an experiment was set up to determine the effect of acute gamma irradiation on apple trees in late summer. Beginning on July 25, 101 two-year-old trees of Spy, Rome and Red Delicious growing in pails were moved into the gamma field at the Brookhaven National Laboratory for periods of 5, 10, 20 and 40 days during which they received a total dose of 5,000 r or 10,000 r. In November they were transported to Geneva and in May they were planted at G.F.B.F., Orchard 1, Row 10.

Buds were fixed prior to irradiation to determine their stage of development. Branches of Delicious/I exposed to the various dosages were forced in the spring following irradiation and buds were fixed in order to study the areas of radiation damage. These have not yet been examined.

During the summer of 1956 an attempt was made to measure differences in response to the three varieties that were exposed in 1955 to varying rates and duration of delivery of gamma rays. The number of growing shoots per unit of leader and per tree were counted. The growing shoots were scored as long shoots (those with internodal elongation and terminal buds) and as short shoots or spurs (those with little or no internodal elongation) with terminal buds or without them. The occurrence of flowering was noted.

There was no correlation of the number of shoots with dosage or duration of exposure. The lethal effect of radiation on buds might better have been measured as the ratio of the number of buds growing to the total number of buds.

An indication of the lethal effect of radiation on the growing point of the bud was given by the percentage of spurs which bore a few leaves but formed no terminal bud in the total number of shoots. In general, this percentage increased with increasing dosage. Budless spurs were lacking or rare in controls. In Spy this figure was about 20% in plants exposed to 5,000 r and 10,000 r for five days. It was 19-49% in plants of Spy receiving 5,000 r in 10-40 days and 80-90% in plants receiving 10,000 r in 10-40 days. Rome showed high percentages of budless shoots: 66-88% for

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plants receiving 5000 r and 80-97% for plants receiving 10,000 r, without a clear relationship with duration or amount of dose. Delicious had 90% or more budless spurs in all groups except that receiving 10,000 r in 5 days (73%).

The proportion of shoots developing into long shoots in all varieties decreased with increasing periods of exposure in each group receiving 5,000 r and 10,000 r, but had no consistent relationship to that of controls.

There was a conspicuous difference in the degree of "leafing out" observed in the groups of Delicious trees receiving 5,000 r and 10,000 r. This was recorded by photographs and was related to the greater size of the leaves in those plants receiving 5,000 r than in those receiving 10,000 r for the same periods of time. These differences were less conspicuous in Spy and Rome than in Delicious.

Bifurcated long shoots and double terminal buds on short shoots were occasionally found.

No Spy trees flowered. Flowers appeared on Rome trees which received no radiation and less frequently on trees receiving 5,000 r, but did not occur on trees receiving 10,000 r. Control Delicious trees flowered, but the treated ones did not.

This experiment suggests: 1) Potted apple trees survive higher acute doses of gamma rays delivered at the conclusion of the active growing season than those of X rays which dormant apple scions survive.

2) Degree of lethality of gamma radiation to buds varies with variety.

3) Both total dose and duration of exposure appear to affect the degree of lethality to buds, but the present data does not make the relationship clear.

Observations on apples in the gamma field: Miss Pratt worked at the Brookhaven National Laboratory May 14-18 and July 30-August 3 and made observations on the apples and grapes growing under chronic gamma irradiation. This group of 84 trees of six varieties were set in spring, 1954, in arcs 32-52 M from the cobalt 60 source. In 1956 they received 55-19 r per 20 hour day during the growing season. The observations of 1955 and 1956 on the apple trees planted in the gamma field in 1954 have been centered on the failure of spurs to form buds, the enlargement of long shoot tips and spurs, and on flowering and fruiting.

Club-shaped shoot tips which set no buds at the end of the first growing season, and, although leafless, enlarge during subsequent seasons was a striking feature of Golden Delicious, and to a less degree, of Red Delicious, Spy, McIntosh and Cox, but not of Macoun. Club tips occurred most frequently on trees which are not flowering. They are most frequent and conspicuous on varieties which bear flowers terminally and which have prominent flowering shoots, such as Golden Delicious. Leafless one- or two-year-old spurs on trees with club tips also enlarged.

The proximity to the source at which flowering and formation of buds on spurs occurred varied in 1955 and 1956. Factors which may be related to this variability may be the youth of the trees, their recent establishment in the field, size of the fruit crop in previous years, and the fact that counts of all buds were not made. However, in respect to flowering Macoun, McIntosh and Spy appeared to be more resistant to radiation than Golden Delicious and Red Delicious; Cox Orange was intermediate. With reference to budded spurs, Macoun appeared to be most resistant and Golden Delicious least resistant. The wide differences in resistance of individual trees in the two years suggest that counts of total shoots and budless shoots might be made each year to determine if this variability continues as the trees become better established. Such counts were made on Golden Delicious in 1956.

Bifurcations occurred most frequently on vigorously growing shoots. Counts of bifurcations and of the long shoots were made both for the bifurcations of 1954-1955 and for 1956. No correlation between the ratio of bifurcations to long shoots and dosage was obtained. The ratios varied widely between trees of the same variety and in the same row.

A severe frost on May 24, 1956, killed most of the flowers. No fruit was harvested.

Propagations of apples from gamma field: Dr. Einset collected budwood on August 2 from 62 of the 84 identified trees planted in 1954 and this was budded into 96 two-year old trees in G.F.E.F., Orchard 6, Row 17.

Observations on grapes in the gamma field: In May, 110 Concord grapes were planted in the gamma field to replace plants which had died. Fifteen plants were planted in a control field at Brookhaven. Measurements of shoot growth, counts of nodes and classification of terminal buds were made on the new plants on July 31, 1956, in an attempt to study the effects of chronic gamma radiation on the first season's growth. The plants made uneven growth and no correlation between average length of shoots per plant or average length of internodes and daily dosage of gamma rays was observed. However, the percentage of budless shoots which was 71-78% at 32 - 40 M (55 - 34 r per day) showed a sharp decrease at 42 M. From 42 - 58 M (31 - 15 r per day) 23 - 56% of the shoots lacked terminal buds. No budless shoots were observed on the new vines set at 73 M (9 r per day). Among the plants in the control field, 0.34% of the shoots appeared budless and these may have been due to mechanical injury.

Three shoots on vine #20 in the 73 M row bore late-ripening clusters consisting of unusually large berries and many "shot" berries. The large berries did not have unusually high seed counts, for 16 such berries contained 1-3 seeds each, while 20 large and medium berries from normal clusters had 1-2 seeds. Seeds from the large berries will be grown for chromosome counts. It is planned to propagate the large-berried portions of this vine.

Seeds from grapes borne in the gamma field: Seeds from Concord grapes growing in the 40, 44, 46, 54 and 73 M rows in the gamma field were germinated and 86 seedlings planted in the nursery below Vineyard 21. The young seedlings were irregular in vigor, as may be expected in seedlings of Concord. Mottled or distorted cotyledons and bifurcated epicotyls occurred occasionally. These seedlings will be grown to fruiting to find out if new types will occur.

Seeds from vines in the 42, 50, 52, 53, 57, 58 and 73 M rows in the gamma field were harvested and will be grown.

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X ray and neutron irradiation. Apples: Irradiation of scions. McIntosh apple scions were treated with 200 r, 1000 r, 2000 r, and 4000 r of X rays for 30 min., 1 hour, 2 hours, 4 hours and 6 hours in the thermal column. Lower dosages were used to permit higher survival than had been obtained in previous years. The scions were grafted into 16-year old seedling trees, one-half of them being done on April 4-11, and the other half May 14-15. The percentage of survival of all the scions grafted in April was higher than that of scions done in May, particularly in the case of scions which received the higher dosages. For example, the percentage of survival of scions receiving 200 r was 77% of those grafted in April and 72% of those grafted in May; that of scions receiving 4000 r was 21% of the April lot and 11% of the May lot.

Data on the growth of the scions was recorded for those grafted early in the season (Table 1). It was found that the percentage of survival decreased with increasing X ray dosage, with a sharp downward trend at 4000 r. The percentage of survival for neutron-treated material was variable but showed marked lower survival at the 6 hour treatment.

Table 1. Survival of irradiated McIntosh scions grafted April 4-11, 1956, and average length of internode and percentage of budless shoots on the new growth.

Dosage	No. scions	% survival	Ave. cm./node	% budless shoots
X ray 200 r	43	77	1.6	3
1000 r	51	78	1.0	0
2000 r	47	72	1.2	5
4000 r	43	21	0.8	53
Nth ½ hr.	38	45	2.0	2
1 hr.	37	87	2.1	0
2 hrs.	45	42	1.8	0
4 hrs.	49	69	1.3	48
6 hrs.	51	24	0.7	96
Brookhaven control	44	64	2.2	3
Geneva control	40	71	2.2	0

The reduced growth which is characteristic of irradiated plants was studied in some detail. It was noticed that the length of the node, calculated from the total length of all shoots divided by the number of nodes, decreased with increasing dosage, with sharp downward trends at 4000 r and Nth 6 hours. This was largely related to few long shoots from scions treated with these dosages.

At the highest dosages used, 4000 r of X rays and 6 hours of neutrons, the percentage of survival and the length of the node sharply decreased and the percentage of budless shoots increased markedly. Dr. Zahur's anatomical studies showed the most severe damage to the apical meristem at these dosages also.

Among the scions irradiated in 1953, shoots from a Macoun scion which had been treated with neutrons for 6 hours, bore small, slightly irregular leaves throughout 3 year's growth. No fruit has yet been produced. It has been propagated, and buds will be checked for chromosome number. It is the first consistent, visible change which has been observed in shoots from irradiated scions.

The fruit borne by the scions irradiated in 1953 showed no conspicuous deviations from the normal.

Attempts to induce dwarfing in apples: At the suggestion of Dr. Arnold Sparrow of the Brookhaven National Laboratory, an attempt to induce dwarfing by irradiation of a band two inches wide around the trunk was initiated. Two-year-old trees of Milton were treated with 12,000 r, 18,000 r, 24,000 r, 30,000 r and 36,000 r of X rays. Ten of each lot and 13 controls were planted at C.F.B.F., Orchard 1, Row 10. From 5 to 11 trees in each group of treated plants and of controls planted at Geneva had green shoots at mid-summer, while others had dead or yellowing shoots. The survival of the trees was not correlated with dosage. The bark of the irradiated band was more or less brown, rather than the yellow-green or yellow-brown of the similar area of the controls. Sprouts appeared below the irradiated girdle more frequently than in the controls. It appears that the dosages used may have caused considerable damage to the trunk.

Trees of Monroe on Malling I, similarly treated, were grown at Brookhaven and were reported by Dr. Sparrow to have died above the area of irradiation and to bear sprouts below this area.

Grapes: Irradiation of cuttings. A total of 1134 dormant rooted cuttings were exposed to 200 r, 500 r, 1000 r, 2000 r, and 4000 r of X rays and to thermal neutrons in the larger instrument chamber in the reactor where they received doses equivalent to those received in 20 min., 1 hour, 2 hours and 4 hours in the chamber used in previous years. 252 cuttings were not treated, one-half being sent to Brookhaven and one-half being retained at Geneva. The cuttings were 16 inches long, instead of 7 inches as in former experiments. Lower dosages were used to allow greater survival of cuttings. Survival of all lots was better than 84%, except for those receiving 4000 r of X rays, of which 15% were living at mid-summer.

As in previous years, plants receiving 4000 r and the equivalent of 4 hours of thermal neutrons showed the most severe radiation damage - reduction in the number and length of internodes and high proportion of budless shoots. Plants of these lots grown 4 months in the greenhouse to study recovery showed little or no development of axillary buds on the new shoots or of dormant buds after the initial growth period. Plants receiving lower dosages showed symptoms of radiation damage which increased with increasing dosage. Those receiving 200 r of X rays and 20 minutes of neutrons did not differ markedly from the controls.

Studies of "recovery" of vines from primary radiation affects suggest that this occurs principally by the development of an axillary bud into a shoot, usually with normal leaves and a terminal bud. This occurs frequently in mid-summer on budless shoots at doses less than 4000 r of X rays and 4 hours of thermal neutrons.

Anatomical study of effect of radiation on shoot apices of Concord grape. Budless shoots from plants receiving 2000 r and 4000 r of X rays and 2 and 4 hours of thermal neutrons in 1955 were sectioned and compared with the normal apical meristem of control plants. The normal shoot tip consisted of a dome-shaped apical meristem surrounded by a graduated series of primordia. When a budless stem tip was sectioned, it showed differentiated leaves around a flattened area. The cells in this area showed various degrees of damage, which may be divided into three types; 1) The surface cells were more or less collapsed, but the nuclei were still discernible. 2) The surface cells were completely collapsed and subsurface cells were enlarged. Divisions sometimes took place within such a large cell. 3) In two meristems which showed inactive but living cells in the whole shoot apex or in part of it, certain cells in the upper four layers had spirally thickened walls.

This preliminary study suggests that the meristematic cells of the young leaf primordia and of the upper layers of the apical meristem are more sensitive to radiation than the more differentiated cells. The field observations support this. The budless shoot usually has 1-6 leaves, which may be assumed to be differentiated in the dormant bud at the time of irradiation. These expand but are variously distorted, suggesting the death or retardation of groups of cells, such as those in the marginal meristem. No new leaves or stem are produced.

Field data also showed that the percentage of budless shoots produced following irradiation of dormant buds was a fairly consistent indication of radiation damage. Dosages which produced a high number of budless shoots, especially when axillary meristems were also damaged, were useless as means to induce somatic mutations.

Seeds of apples and grapes: Seeds of Golden Delicious, Rome and Spy apples and Steuben grapes, all open-pollinated, were irradiated with 20,000 r, 40,000 r and 60,000 r of X rays and with thermal neutrons for 10, 20 and 40 hours in attempts to determine a suitable dosage for the induction of somatic mutations. At each dose 300-500 seeds were irradiated soon after harvesting (25-33% water content in seeds) and after after-ripening 10 weeks in damp sphagnum in a polyethylene bag (12-34% water content in seeds).

These dosages were all more than a 50% lethal dose. The frequency of germination decreased with increasing dosage. Grape seeds were more sensitive than apple seeds. No germination occurred except at doses of 10 and 20 hours of neutrons. Although the separation of the cotyledons occurred, shoot and root meristems mostly failed to function. Only 107 apple seedlings from seed exposed to 20,000 r of X rays and for 10 hours in the thermal column grew enough to be potted.

The germination of seeds irradiated after after-ripening was frequently lower than that of seeds irradiated before after-ripening, but this difference was not consistent.

Split cotyledons, irregular leaves and bifurcated shoots were occasionally observed in the seedlings.

Seeds will be irradiated at lower dosages and the resulting seedlings grown in order to look for mutations.

Anatomical studies of radiation damage in the apical meristem of apple (M. Zahur): Although it is generally recognized that the shoot apex is susceptible to radiation damage, there is little information on the area of greatest damage. There are conflicting views on whether dividing cells are more sensitive than resting cells. The anatomical study of the shoot apex of some apple varieties exposed to both acute and chronic irradiation was undertaken with the hope of throwing some light on these problems.

Effect of acute irradiation: Dormant scions of McIntosh and Red Delicious were subjected to a wide range of acute doses of X rays and thermal neutrons at Brookhaven National Laboratory, as shown below:

Variety	Year	Thermal neutrons	X rays
McIntosh	1955	2 hours	4000 r
		4 hours	5000 r
		6 hours	
McIntosh	1956	½ hour	500 r
		1 hour	1000 r
		2 hours	2000 r
		4 hours	4000 r
		6 hours	
Red Delicious	1955	2 hours	4000 r
		4 hours	5000 r
		6 hours	

After treatment the scions were forced in water in the greenhouse at Geneva. The buds were collected as they started to grow (1955) or at weekly intervals for three weeks (1956). They were fixed in F.A.A. or in CRAF, dehydrated in t-butyl alcohol, embedded in Tissuemat, sectioned transversely and longitudinally at 8-10 u, and stained in safranin and fast green, Foster's iron chloride and tannic acid, or Heidenhain's hematoxylin.

The normal shoot apex: It was thought worthwhile to study the morphology and accidental damage, if any, in about 200 shoot apices in dormant buds of Golden Delicious and green tip buds of McIntosh and Red Delicious. No differences between varieties or stages of development were observed. The normal vegetative shoot apex was found to be a flat or slightly elevated dome. The height of the dome depended upon the stage of development of the shoot apex. It was narrowest and almost flat in the minimal phase of the plastochron (the phase at which the elevation of the youngest leaf primordium is detectable) and attained the greatest width and height in the maximal phase (the phase before the emergence of the youngest leaf primordium). The number of mantle or tunica (outer) layers, to be indicated as T_1, T_2, T_3, T_4, also varied with the developmental phase. The maximum stratification (four layers) was identifiable either in the maximal phase of the plastochron or just after that phase. The minimum stratification (two layers) could be seen in the period following

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the minimal phase to the mid-plastochron phase. The distinction between tunica and corpus based upon cell size did not hold in apples. The cells of T_1 and T_2, the usual tunica layers, showed the same size variation as those in T_3 and T_4 and deeper lying layers.

The term corpus or central mother cell zone, as used in the present study, was applied to only those cells which lay between the mantle layers and the pith rib meristem, whose upper limit was usually 8-10 cells below the surface of the apex. The central mother cells divided in all planes and at any time the cells could be traced to a single cell lying in the middle.

Accidental damage to the cells of the shoot apex, as determined by counting collapsed cells in the median or near median sections, was found to be negligible. In a very few cases a cell here and there showed some damage. In only two cases were all the cells in the shoot apex collapsed. The damaged shoot apex in both these cases showed signs of uncontrolled growth resulting from the activity of pith rib meristem.

Radiation damage: The microscopic examination of 200 shoot apices subjected to a wide range of doses of ionising radiations showed three stages of radiation damage: 1) The cytoplasm of the damaged cell stained very lightly, but the nucleus retained its normal appearance. 2) The cytoplasm was lightly stained, and the nucleus had lost its normal structure, i.e., the nuclear membrane was indistinct and the chromatin was clumped. 3) The cell walls collapsed. This was related to pressure exerted by the growth of adjacent tissues.

The degree of radiation damage varied widely among buds exposed to the same dosage. This may be related to physiological variation in buds or to variations in dosage received due to distance from the source of radiation, shielding by the woody stem, etc. Despite this variation in degree of damage, a trend toward increasing frequency of damaged buds with increasing dosage was observed.

The radiation damage in the apex became more evident in buds of later collections from forced scions. In material exposed to mild and severe doses of ionising radiation the degree of damage in buds collected three days after forcing was similar, whereas buds from the same scions collected 10-16 days after forcing showed increasing damage with increasing dose. With the growth of the bud the radiation damage became more apparent.

The immediate shoot apex was the site of radiation damage. However, the various layers of the shoot apex designated as T_1, T_2, T_3, T_4, the corpus and the pith rib meristem showed varying degrees of response.

a. T_1 (the outermost layer of the shoot apex) which usually gives rise to the epidermis of leaves and stems, was found to be the most resistant to ionising radiation. The degree of resistance differed in different varieties. In McIntosh eight shoot apices out of 167 showed damage to T_1, whereas 24 out of 33 shoot apices of Red Delicious exposed to comparable doses showed radiation damage in T_1.

b. Resistance markedly decreased in the deeper lying layers. Layers designated as T_2, T_3 and T_4 showed approximately the same amount of radiation damage.

c. Corpus cells or the cells of the central mother cell zone were much more sensitive to radiation damage than any of the four superficial layers.

d. The pith rib meristem showed marked resistance to radiation damage. In McIntosh 21 out of 167 shoot apices showed entirely or partially damaged pith rib meristem. In all the rest of the cases the cells of this region were either normal or composed of slightly enlarged cells. In a few cases more than one growth center could be distinguished below the damaged corpus. It is postulated that these growth centers may be the origin of the bifurcated stems which occur commonly following irradiation. An interesting feature of the damaged or partially damaged pith rib meristem was the presence of oversized cells which occurred singly or in groups.

e. Procambium was affected only at heavier doses. Following 4000 r of X rays and 6 hours' exposure to thermal neutrons the entire procambium was destroyed. At 5000 r of X rays even the differentiated vascular elements showed radiation damage. The protoxylem elements became clogged with a highly refractive material, whereas phloem elements were completely obliterated.

The outermost layer of the leaf primordia behaved in the same way as T_1 in the shoot apex. It was covered with a thick layer of cuticle and was capable of rapid division. This layer was most resistant to the effect of ionizing radiation.

Effect of chronic irradiation: Budless spurs and club tips of Golden Delicious grown two years in the gamma field at Brookhaven National Laboratory at 32 M and 36 M from the cobalt source were fixed in F.A.A. and sectioned according to the method previously described.

One-year old spurs collected on August 1, 1956, produced leaves but no terminal bud. When sectioned, T_1 was found to be resistant, but T_2, T_3, and T_4 and the corpus were killed. The cells of T_1 proliferated and the surface was thrown into folds. No regularly organized leaf primordia were present. Divisions in different places in the pith rib meristem occurred and contributed to the enlargement of the spur.

Club tips one year old showed the characteristics of the spurs but had developed further. Cracks occurred in the folded surface layer because of the increase in bulk caused by the activity of the pith rib meristem. Periderm developed from cortical cells and eventually covered the surface of the tip.

A larger club tip presumed to be two years old showed persistent periderm. The bulk of the tip was composed of parenchyma with scattered short tracheids.

Conclusions: It appears from these preliminary observations that the relative sensitivity of the cells in the apple shoot apex is not determined solely by their comparative ability to divide. The four tunica layers are composed of cells having the capacity to divide actively, but T_1 is more resistant than the other layers. Periderm consists of dividing cells but it continues its activity under doses of chronic gamma irradiation which destroy the inner tunica layers. The cuticular coating of the cell wall, known to be present on cells of T_1, periderm and on pollens, which are notably insensitive to radiation, may be an important factor in radiation resistance.

PRESENT STATUS: The collection of irradiated apple and grape material, which must be grown to fruiting before evaluation, has been augmented in 1956. Studies on the lethal effects of ionizing radiations were continued in order to determine suitable dosages. Field observations and anatomical studies indicated that the site of greatest damage was the apical meristem. Extensive damage to the meristem stopped further growth.

The severity of primary radiation effects (alterations which occur after radiation treatment but which are not perpetuated in subsequent growth) is a guide to the tolerance of the plant to radiation, and therefore it is used as a basis for the selection of suitable dosages. However, the relationship between primary radiation effects and inheritable mutations has not been elucidated. The variety and the nature of the material and its treatment subsequent to radiation are important factors in radiation sensitivity. From the experience of past seasons, certain dosages which cause fair survival and limited growth disturbance appear to be practicable treatments for building up a population in which to look for mutations. Suggested dosages for apple scions are 1000 r - 3000 r of X rays and 3 hours of thermal neutrons. Better survival is obtained if grafting is done as early as possible. Whole trees seem to be more resistant to irradiation than scions, but are more difficult to handle. Concord grapes appear to be more sensitive than the apple varieties tried. 1500 r - 3000 r of X rays and 2-3 hours of thermal neutrons may be suitable dosages.

PUBLICATIONS: Einset, J., and C. Pratt. Problems and methods of handling irradiated, woody, vegetatively propagated plants. Talk presented at the Work Conference on Radiation-Induced Mutations, Brookhaven National Laboratory, May 24, 1956, to be mimeographed for distribution to the members of the conference.

Pratt, C. Can atomic energy improve fruits? Farm Research 22: 3:3. July, 1956.

Pratt, C. Effects of ionizing radiations on stem apices of Concord grape. Presented before the General Section of the Botanical Society of America, A.I.B.S. meetings, University of Connecticut, Storrs, Conn., August 28, 1956.

PLANS FOR THE COMING SEASON: The accumulation and testing of irradiated apple and grape material will continue as well as basic studies on the effects of radiation on the apical meristem. Seeds of apple and grape will be irradiated at lower dosages to permit better survival. Irradiation of apple scions at suitable dosages will be performed. Propagation of apples in the gamma field will be continued.

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