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Somatic Crossing over in *Glycine max* L. (Merrill): Sensitivity to and Saturation of the System at Low Levels of Tritium Emitted Beta-Radiation*

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Summary. Seeds of varieties L65-1237 and T219 of soybean were soaked in 20-50 ml of tritiated water (HTO) containing between 0.01 and 50 μCi/ml of tritium (T) for various periods from 24 to 142 hr. Genetic damage was determined by counting the number of $Y_{11}Y_{11}$ - $y_{11}y_{11}$ spots on the $Y_{11}y_{11}$ leaves. No effect was noticeable in treatments lasting up to 24 hr independent of the concentration. However, somatic crossing over occurred when the seeds were treated with tritium for 92 hr or longer even with concentrations as low as 0.01 μCi/ml. The data indicate that the soybean system being used is sensitive to very low doses of beta radiation. The damage to the seeds treated with the lowest concentration was not significantly different in quality or quantity from that induced by higher concentrations. This indicates some type of saturation effect to tritium-emitted beta radiation. Analytical results showed that synthesis of organic matter and incorporation of tritium into the organic fraction was delayed until about 96 hr after the imbibition was started. This indicates a relationship between spot induction by tritium and organic synthesis.

Introduction

Glycine max (Soybean) is a rare organism wherein somatic crossing over has been studied by utilizing a single locus system characterized by incomplete dominance. In certain varieties, e.g., L65-1237 and T-219, the gene y_{11}, when in homozygous combination, imparts golden-yellow color to the stem and the leaves of the plant. With an alternate combination, $Y_{11}Y_{11}$, the plant develops a dark-green color; the heterozygous $Y_{11}y_{11}$ plants are light-green. The two simple leaves and the first compound leaf of the $Y_{11}y_{11}$ plants are dotted with dark-green, yellow and composite spots. The first two types of spots are called single spots. The composite spots (also referred to as twin or double spots) are composed of a dark-green component present next to and usually as a mirror image of, the yellow spot. The homozygous plants are devoid of any spots on their leaves except a rare light-green spot on the $y_{11}y_{11}$ leaves. These criteria and many others discussed elsewhere (e.g., Vig, 1973a) permit the conclusion that double spots originate from a process involving complementary exchange of homologous chromosomes in the somatic cells, viz., somatic crossing over. Some of the single spots originate from a failure in development of the yellow or dark-green component. Other factors such as non-disjunction, gross numerical chromosomal abnormalities, segmental losses or point mutations could also contribute to the production of single spots. However, the proportions of single spots arising from different mechanisms remain undetermined.

The frequency of all three types of spots has been increased by treating the seeds and the plumules of developing plants with different chemicals. These chemicals, however, affect the numerical frequencies of different types of spots differently, thereby permitting some general conclusions about their primary effect(s) on chromosomes (see Vig, 1975). Mitomycin C (Vig and Paddock, 1968), colchicine (Vig, 1971), and caffeine (Vig, 1973a), for example, increase the frequency of double spots at least as much as those of singles, while puromycin und FUdR favor the induction of single spots (Vig, 1973a). Deoxyribose cytidine is the only chemical so far found to suppress induction of somatic crossing over (Vig, 1972). This paper deals with the effects of low doses of beta radiation, administered as tritiated water, on this very sensitive index of genetic damage.

Material and Methods

Seeds of soybean varieties L65-1237 and T-219 (used only in one experiment) were immersed in tritiated

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water (HTO) of varying concentrations and for different time periods. The seeds were obtained through the courtesy of Dr. R.L. Bernard, USDA Soybean Research Laboratoy in Urbana, Il. After treating the seeds in sterilized petri dishes without filter paper, they were sown in coarse sand, a material with no nutritive value, in plastic (PVC) lined metallic flats. Plants were watered with tap water as needed. After about five weeks the leaves were analyzed for the frequencies of different types of spots on the two simple and the first compound leaves, separately. The details of treatments and analysis are given along with appropriate tables in the following section.

For purposes of calculations, each trifoliate compound leaf was considered equivalent to three simple leaves. This gave 5-leaf-equivalents per plant analyzed. Frequencies for double and total spots in the control versus treated materials were compared using the chi-square test.

Individual epicotyl weights were determined in connection with the study of organic synthesis, to within $\pm.01$ mg on a Cahn electrobalance (Model GR) equipped with a strip chart recorder. In the interval of 30 seconds required to dissect the epicotyl from the embryo and transfer it to the balance pan, some water loss occurred. This loss was estimated by extrapolating the linear weight loss curve determined for about two minutes back through the origin (time of dissection). Dry weights were determined after drying at 75 °C for 24 hr.

In order to correlate the tritium content with spot production, the following studies were carried out. A determination of the total activity was made by soaking 100 seeds in 50 ml of water containing a tritium concentration of 194 μCi/ml, and removing samples after 24, 48, 72, 96, 120 and 144 hr. The seeds were washed, and embryos and cotyledons separated and prepared for scintillation counting by extracting water in a benzene azeotrope (Moghissi, et al., 1973). A Beckman LS-200 counter was used employing the technique of Lieberman and Moghissi (1970). The specific activity of the samples was determined by the method of internal standard with identical system properties (Moghissi and Carter, 1968). The study was also extended to include the analysis of tritium in the embryo and cotyledons after the seeds were soaked in HTO for 96 hr, washed and then kept in moist sand (same procedure as used in study of spots) for up to 144 hr. The organically bound tritium was determined by acid digestion of the epicotyls and by analyzing the digest before and after adding an internal standard (Moghissi and Bretthauer, 1973).

Results

(a) Study of Spot Frequency

The first experiment was performed using 11 g of seed of L65-1237 soaked in HTO (20 ml) for 6, 12, and 24 hr at concentrations of 0.1, 1, 10 and 25 μCi/ml, for each treatment interval. The frequency of spots calculated for any of the treatments was not statistically higher than for the controls. In view of these results, the data need not be reproduced here.

The second experiment was performed using 0.01, 0.1, 5 and 50 μCi of HTO/ml. A sample of 12 g of L65-1237 seed was soaked in 20 ml solution for 92 hr. The data (Table 1) indicate a rise in the frequency of all three kinds of spots in all the treated materials compared to the control. It must be pointed out that even the lowest concentration of tritium, viz., 0.01 μCi/ml, was effective in significantly increasing the frequency of spots. There was an average of only 0.25 dark-green spots per leaf in the control compared to 1.04 spots in the treated material. Similar increases were noticeable for the yellow and double spots. Other treatments also differed significantly from the control. This is not only true for total spots but also for double spots -- which can be the true measure of somatic crossing over's having occurred. It is interesting to note that the increase in frequency of total spots was of a similar magnitude in all the materials treated in spite of a 5,000-fold difference in HTO concentration.

The relative frequency of different types of spots was another point of investigation in these experiments. The last four columns of Table 1 record relative proportions of different types of spots in various treatments. A parallelism in increased spot frequency is seen among different types of spots in all treated ma-

Table 1. Spot frequency on $Y_{11}y_{11}$ leaves from the L65-1237 seed treated with various concentrations of HTO for 92 hr

Concentration (μCi/ml)	Leaves analyzed	Spot frequency per leaf				Proportions of different types of spots			
		DG*	Yl	Db	T	DG/Db	Yl/Db	DG/Yl	T/Db
0.00-Control	150	0.25	0.88	0.74	1.86	0.34	1.19	0.28	2.51
0.01	110	1.04	2.43	2.25†	5.74†	0.46	1.08	0.43	2.55
0.10	150	0.61	2.38	1.27†	4.26†	0.48	1.87	0.26	3.35
5.00[a]	150	0.68	2.25	1.23†	4.17†	0.55	1.83	0.30	3.39
50.00[a]	105	1.69	2.14	2.23†	6.06†	0.76	0.96	0.79	2.72

*DG = dark green, Yl = yellow, Db = double and T = total spots

† = significantly different from the control (χ^2, $p < 0.05$)

[a] = These data were used for a recent paper (Vig, 1974) and have been reproduced here to emphasize the point regarding saturation effect discussed in the text.

terials. The ratio between yellows and doubles, for example, is always around 1 in the control as well as the treated materials. The general picture that emerges about relationships between total versus double spots is that approximately one-third of all spots are double. The treatments with various concentrations of HTO did not alter these ratios except in a few isolated cases, e.g., a disproportionate increase in the frequency of dark-green spots in the material treated with 50 μCi/ml.

The effectiveness of very low concentrations of tritium, viz., 0.01 and 0.1 μCi/ml, warrants concern and needs further study. With this point in view, as well as to confirm that there were no real differences in the induced spot frequency over a large range of tritium concentrations, another experiment was conducted. Seed samples (11 g) (L65-1237) were soaked for 94 hr in 25 ml of H_2O containing 0.01, 0.1, 5, 10, and 25 μCi of HTO/ml. The data (Table 2) demonstrate the effectiveness of all concentrations tested in increasing the frequency of spots. The spot frequency in the seeds treated with non-tritiated distilled water (control) was rather high in this case; however, environmental factors, primarily temperature fluctuations during growth, are though to be responsible. Such factors, or some experimental error, may be responsible for lack of increase in double spots observed in a few cases in this experiment. Nevertheless, when compared to the control, all seeds treated with HTO had significantly higher total spot frequencies. Again it was observed that an increase in the frequency of total spots in all treatments was almost the same. This confirmed earlier observations (Table 1) that increase in the frequency of spots was not dose dependent. Also the relationships of any two types of spots appeared to be similar in almost all treatments. In Table 2 the relationship for either type of single spots to the doubles is approximately 1 : 1. Therefore the total spot population, in any treatment, was about three times the population of double spots.

The equivalency in the total increase in spots for treatments with widely varying concentrations of HTO for 92 (Table 1) and 94 hr (Table 2) was further investigated by increasing the period of treatment. Seeds (12 g samples) were treated with 0.05, 0.1, and 1 μCi of tritium/ml, in 25 ml of H_2O. Sixty hr after imbibition started, an additional 25 ml of HTO was added. The total treatment period was 142 hr in 50 ml of HTO at the above concentrations. The analyzed data given in Table 3 confirmed the observations made previously. A twentyfold increase in concentration (0.05 to 1 μCi/ml), doubling of the amount of HTO, and an almost 50% increase in treatment period, did not suggest a linear increase in the frequency of spots. The relationships of different types of spots remained comparable to those calculated for the control.

To determine if this saturation effect on spot frequency by tritium was a unique property of the variety

Table 2. Type and frequency of spots on the $Y_{11}y_{11}$ leaves of L65-1237 plants obtained from the seed treated with 0.01, 0.1, 1, 5, 10 and 25 μCi/ml for 94 hr

Concentration (μCi/ml)	Leaves analyzed	Spot frequency per leaf				Relative frequencies of spots			
		DG	Yl	Db	T	DG/Db	Yl/Db	DG/Yl	T/Db
0.00-Control	130	1.50 (0.33)*	1.38 (0.25)	1.32 (0.18)	3.85 (1.76)	1.14	1.05	1.09	2.92
0.01	140	2.00 (0.31)	1.85 (0.33)	1.30 (0.17)	5.13† (0.81)	1.54	1.42	1.08	3.95
0.10	140	2.14 (0.30)	2.16 (0.24)	1.55 (0.19)	5.84† (0.73)	1.38	1.39	0.99	3.77
1.00	130	1.39 (0.41)	2.16 (0.36)	2.13† (0.31)	5.69† (1.08)	0.65	1.01	0.64	2.67
5.00[a]	150	1.61 (0.36)	2.24 (0.46)	1.76† (0.27)	5.61† (1.09)	0.92	1.27	0.72	3.19
10.00[a]	100	1.73 (0.83)	2.87 (1.01)	1.68† (0.65)	6.28† (2.59)	1.03	1.71	0.60	3.74
25.00[a]	130	1.29 (0.66)	2.30 (0.59)	1.49 (0.31)	5.08† (1.56)	0.87	1.54	0.56	3.41

* = Figures in parenthesis refer to spot frequencies per leaflet of the compound leaf
† = Significantly different from the control (χ^2, $p < 0.05$)
[a] = See footnote to Table 1.

Table 3. Types and frequencies of spots on the $Y_{11}y_{11}$ leaves of L65-1237 plants obtained from the seed treated with 0.05, 0.1 and 1.0 μCi/ml for 142 hr

Tritium concentration (μCi/ml)	Number of leaves analyzed	Type and frequency of spots/leaf				Relative frequencies of spots			
		DG	Yl	Db	T	DG/Db	Yl/Db	DG/Yl	T/Db
0.00-Control	100	1.08	1.68	1.93	4.69 (2.0)*	0.56	0.87	0.64	2.79
0.05	85	1.36	2.64	3.26	7.26† (6.0)	0.42	0.81	0.52	2.23
0.10	100	2.69	3.54	4.68†	10.96† (5.7)	0.57	0.76	0.76	2.34
1.00	110	2.80	3.02	2.89†	8.71† (3.7)	1.00	1.05	0.93	2.70

* = % total spots on the first compound leaf
† = Significantly different from the control (χ^2, $p < 0.05$)

Table 4. Types and frequencies of spots on the $Y_{11}y_{11}$ leaves of variety T219 from the seed soaked in various concentrations of HTO for 92 hr

Concentration (μCi/ml) and treatment time	Number of leaves analyzed	Type and frequency of spots per leaf				Proportions among different types of spots			
		DG	Yl	Db	T	DG/Db	Yl/Db	DG/Yl	T/Db
0.00-Control	150	1.27	1.15	1.35	3.77	0.94	0.85	1.10	2.79
0.005 - 92 hr	90	1.68	1.01	1.89†	4.58	0.89	0.53	1.66	2.42
0.01 - 92 hr	150	2.37	1.57	2.02†	5.96†	1.17	0.78	1.51	2.95
0.10 - 92 hr	160	1.43	1.27	1.30	4.00†	1.10	0.98	1.13	3.08
1.00 - 92 hr	120	1.88	1.77	2.06†	5.71†	0.91	0.86	1.06	2.51

† = Significantly different from the control (χ^2, $p < 0.05$)

L65-1237, seeds of variety T-219 raised at Columbus, OH, during 1971 (kindly supplied by Dr. E.F. Paddock) were soaked in 0.005, 0.01, 0.10 and 1.00 μCi/ml of tritium for 92 hr. Statistical comparison of control spot frequency with the observed frequency on the treated plants revealed significant differences for total spot frequency at all tritium concentrations tested, and in all but one case when double spots were considered for such calculations. Also, it is of particular interest to note that the lowest concentration, 0.005 μCi/ml, used only in this experiment was also effective in increasing spot frequency. As in the previous experiments, the increase in all these cases was not proportional to the concentration. Thus both varieties, viz., L65-1237 and T-219, responded similarly regarding low dose saturation. The relative frequencies of spot types, as indicated by the right half of Table 4, were similar in the control versus the treated materials. A last set of two experiments was conducted to make sure that the so-called saturation effect is real. Seed samples were soaked in HTO of two different concentrations (1 and 10 μCi/ml). The data presented in Table 5 agree with previous data that whereas the control population had significantly lower frequency of spots compared to the two tritium treatments, there were no real differences in spot frequency between 1 and 10 μCi/ml treatments. This was true for both sets of data.

(b) Dosimetry

Water content of developing embryos increased rapidly after imbibition of water was initiated. The fact that no isotopic discrimination occurred in imbibed water was verified by analyzing the imbibed water and comparing its specific activity with that of the treatment solution. Rapid exchange of water in the developing embryo resulted in negating the dilution effect of water present in the "dry" seed prior to imbibition. Thus the water in the embryonic cells had the same specific activity as the treatment solution after several hours of soaking.

Dry embryos were found to contain about 10% moisture which rapidly increased upon soaking to about 55% within 12 hr, to about 75% within 24 hr, and equilibrated just above 80% three days after soaking started (Figure 1).

Table 5. Spot frequency of the leaves of $Y_{11}y_{11}$ plants (var. L65-1237) treated with two different doses of tritium. Eighty seeds were soaked in 25 ml of given concentrations of HTO and 48 hr later another 15 ml of the solution of the same concentration of HTO was added. The seeds were washed in distilled water at the end of 93 hr and sown

		Type and frequency of spots per leaf			
^{3}H Concentration (μCi/ml)	Number of leaves analyzed	DG	Yl	Db	T
Set 1:					
.0-Control	140	0.91	1.29	1.39	3.59
1	70	1.94	2.13	1.37	5.44†
10	110	1.95	2.28	1.75†	5.98†
Set 2:					
.0-Control	80	1.08	1.39	1.08	3.55
1	50	1.52	2.46	2.16†	6.14†
10	130	1.62	1.88	1.50†	5.00†

† = Significantly different from the control (χ^2, $p < 0.05$)

In spite of imbibition of large amounts of water, the dry weight of the embryos did not increase significantly until about 96 hr after soaking (Figure 1). Thereafter embryo weight increased sharply throughout the duration of the experiment.

After the treatment in HTO, the seeds were sown in sand and watered with tap water. Under these conditions the rapid water exchange rate in the embryo and cotyledons resulted in removing tritium from cellular and intracellular water pools. The depletion rate of HTO under these conditions was examined after leaching the seeds for several different periods. Data revealed a logarithmic loss of HTO which was similar for all periods of imbibition. Twenty-four hr after sowing, the HTO content in both the cotyledons and embryo was about 1% of the original concentration and at 120 hr was less than 0.01% of the original value.

Organic incorporation of tritium in the developing embryo (Figure 2) followed a pattern similar to dry weight increase. Within the first three days the specific activity of tritium in organic molecules was from 3 to 6% that of the imbibing solution but starting after the fourth day, organic synthesis as well as tritium incorporation increased rapidly. Tritium activity in the organic fraction was determined in a way that yielded only μCi/g of dry embryo tissue. Since no analysis was done to determine the C : H : O ratio in the embryos only approximate values are available for the percentage of tritium equilibration. Conversion from activity per gram to a meaningful tritium to hydrogen ratio was based on the assumption that the embryo was about 14% protein, 2% fat and 82% carbohydrate. Using these estimated values and general protein, carbohydrate and fat formula, it was calculated that 0.5 ml of water is recoverable per gram of tissue. Recovery of H_2O from plant samples oxidized in an oxygen pressurized Parr Bomb have yielded 0.45 to 0.50 ml per gram of tissue. Thus, although this is an inaccurate estimate it appears to be an acceptable value and allows an interesting observation. When seeds were imbibed in HTO for 24 hr only 4 μCi/g was observed in the organic fraction. This concentration of tritium is about 4% of the activity of the imbibing solution. The rate of tritium incorporation into organic fraction was not rapid until about 96 hr. Subsequent incorporation of tritium is indicated by the linear slope in Figure 2 and confirmed by the increase in dry weight shown in Figure 1. However, after six days only 20% of the organic hydrogen was derived from organic material of the cotyledons.

Discussion

A study of the biological consequences of radioisotopes located within tissues is important in view of the environmental contamination with man-made sources, such as from fission power reactors. This is particularly true of tritium which may be incorporated into DNA thus raising several practical and theoretical problems (Cleaver, Thomas, Burki, 1972). It has been estimated that exposure to 1 μCi of tritium per g of fresh tissue of soybean is equivalent to 0.3 rad/day (Chorney, Scully and Dutton, 1965). Suppose, referring to Table 1, 12 g of soybean imbibed 20 ml of HTO with 0.01 μCi tritium/ml. Using this value, the calculated dose would be [0.20 μCi ÷ (12 + 20 g) × 0.3

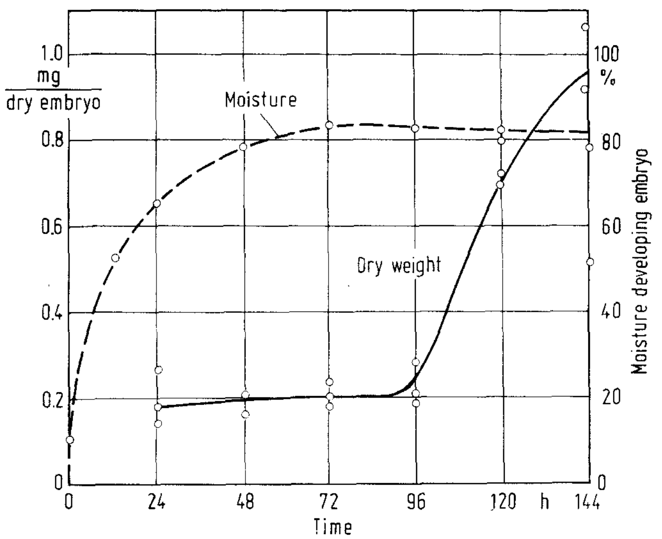

Fig. 1. Percent moisture increase and embryo dry weight changes during imbibition and early growth

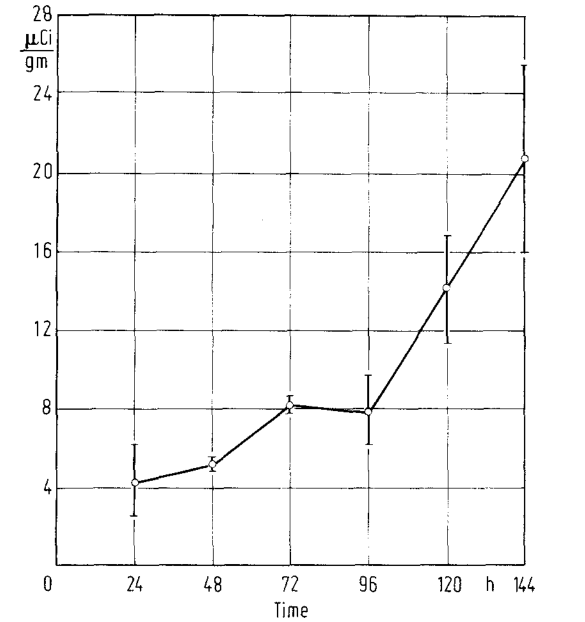

Fig. 2. Tritium incorporated into the organic fraction of developing soybean embryo. Values are expressed as activity per gram of dry embryo. Imbibing solution was 194 μCi/ml of tritiated water

rads/day/μCi] or less than 2 millirads/day/g during the first 94 hr of treatment. This dose would be approximately 10 times higher than the natural background radiation.

There was a close correlation between the synthesis of organic matter in the developing embryo and the incorporation of tritium into organic molecules with the minimum duration of tritium needed for induction of spots. These events required about 96 hr, suggesting that the newly synthesized organic fraction was affected by tritium emitted beta and was eventually responsible for the induction of spots. The data in Figure 1 suggest that the incorporation of tritium into organic fractions during the first 72 hr was due to exchange of tritium on already present molecules, probably at OH sites. The very small quantities so incorporated were apparently too small to create detectable quantitative differences in spot frequency. Alternatively in short treatment periods, imbibition of tritiated water apparently stopped prior to the start of organic synthesis in the embryo. Re-exchange of tritiated sites could occur rapidly and result in genetic material essentially devoid of tritium. Considering the low energy of tritium emitted beta (0.018 MeV), and short effective range in biological systems (approximately 1 μm), it is not surprising to find that tritium did not become effective unless incorporated into organic material and presumably into DNA.

Previous work (Vig, 1973b) showed that small quantities of tritium from exogenously supplied tritiated thymidine (T-TdR) were incorporated in soybean plumular tissue within 36 hr of the initiation of imbibition. Evidence from the present experiments suggests that this early DNA synthesis was either too small to have a detectable impact on spot formation or the early synthesis was derived from already present precursors so that tritium in the water was not appreciably incorporated.

It is interesting that the rise in spot frequency reached a certain maximum with rather low levels of treatment and neither several fold increase in concentration nor an increase in the duration of treatment (92 versus 142 hr) changed it. An explanation of this finding is not possible with the present data. However, a similar observation of a saturation effect by tritium was made by Wolff (1964) regarding the effect of the T-TdR in causing sister-chromatid exchanges in *Vicia faba* (broad bean) chromosome. This was later supported by the work of Martin and Prescott (1963) and Wolff and Heddle (1968). More recently Gibson and Prescott (1972) have shown that Chinese hamster cells fed with as low as 0.005 μCi/ml of T-TdR for as little as 6 hr show a saturation effect. In their experiments, using autoradiography and sister chromatid exchanges as index, they found the maximum effect on chromosomes showing only 0.3 to 0.4 grains per day/chromosome. It is also worth mentioning that the *Glycine max* system does not show saturation effect for genetic damage when treated with up to 750 R of gamma radiation (Vig, 1974).

The potential significance of processes like somatic crossing over is not yet completely understood in evolution and human biology. Zimmermann and associates (Zimmermann, Schwaier and Laer, 1966) have pointed out that some forms of cancer in man may result from the expression of recessive genes in otherwise heterozygous individuals through the process of somatic crossing over. Unfortunately, no report of successful demonstration of the occurrence of somatic crossing over using genetic markers is available from mammalian tissue culture systems. Perhaps a correlation between the induction of somatic chromosome exchanges in human cell cultures demonstrating exchange of corresponding segment involving homologous chromosomes and the induction of twin spots in the present system with a given mutagen can be used at this stage to some advantage. Similar studies also seem possible using mutants of *Nicotiana tabacum* (Deshayes and Dulieu, 1974) which appear to express somatic crossing over.

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