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Characterization of X-ray Induced Increase of Mitotic Cross-overs in *Glycine max*

D.A. Evans
Center for Somatic Cell Genetics and Biochemistry and Department of Biological Sciences, State University of New York, Binghamton, N.Y. (USA)

E.F. Paddock
Department of Genetics, Ohio State University, Columbus, O. (USA)

Summary. Three types of spots can be identified on the leaves of heterozygous light green, Y/y, *Glycine max* (L.) Merrill: dark green (D) and aurea (A) single spots (resembling the phenotypes of the homozygotes) and double (Db) spots consisting of adjacent D and A tissue. X-irradiation increased the frequency of each type of spot on simple and first compound leaves. The Db spots, indicative of mitotic crossing-over (MCO), increase linearly with increasing dosage. Moisture content of the seeds was independent of the rate of spot increase. At high dosages morphological alterations were observed, including spots on homozygotes, leaf area reduction, smaller seedlings, and abnormal leaf shapes. The frequency of light green spots on normal dark green, Y/Y, seedlings was tabulated and, as with all other spot types, increased with increasing X-ray dosage. Dormant soybean seeds contain leaf primordia of both simple and first compound leaves. Mature simple leaves contained more spots, reflecting a larger primordial cell number, while first compound leaves had larger spots, since each affected cell underwent more mitoses prior to leaf maturation. Within first compound leaves, the terminal leaflets developed asynchronously in relation to the lateral leaflets. Terminal leaflets were shown to be initiated first, have a larger percentage of the leaflet area covered with spots, and have larger mature leaflet area. The spontaneous rate of MCO, 3.39×10^{-5} MCO events per mitosis, was increased 282-fold by 1600 R. We also ascertained that Mitomycin C is more specific for Db spot induction than X-rays. These results are compared with our similar irradiation experiments on tobacco shoot apices.

Key words: *Glycine max* – Mitotic cross-over – Somatic crossing-over – X-irradiation

1 Introduction

Self fertilization of Y_{11}/y_{11} plants results in a ratio of 1 dark green (Y/Y): 2 light green (Y/y): 1 lethal aurea (y/y). Aurea seedlings usually do not develop beyond the simple leaf stage. Three types of spots are observed on Y/y leaves; dark green (D) or aurea (A) single spots, or double (Db) spots, which consist of adjacent areas, approximately equal in size. All three types occur more frequently on the upper leaf surface than on the lower. Carlson (1974) demonstrated, by progeny testing plants regenerated from tissue cultures of comparable Db spots of Su/su tobacco, that the spots were primarily due to mitotic crossing-over (MCO), while the origin of single spots remained undefined. Spontaneous Db spots have been observed also in other higher plant species, including *Lycopersicon esculentum* (Ross and Holm 1959), *Nicotiana tabacum* (Evans and Paddock 1976), *Antirrhinum majus* (Harrison and Carpenter 1977), and *Tradescantia hirsuticaulis* (Christianson 1975). In most species spot frequency can be increased by either chemical or physical treatment (Evans and Paddock 1979). X-rays cause chromosome breaks in higher plants (Revell 1959). X-irradiation increased the frequency of Db spots, the result of putative reciprocal breakage and exchange of DNA, in *Drosophila* (Becker 1957; Merriam and Fyffe 1972) and *Musca domesticus* (Nöthiger and Dubendorfer 1971). We have attempted to characterize the spot phenomenon on leaves of *G. max* following X-ray treatment of dormant seeds.

2 Material and Methods

In each experiment, seed was obtained from selfed heterozygous Y_{11}/y_{11} plants raised in a garden in 1975. Since chemical treat-

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ment of dormant *G. max* seed produced spots on mature leaves (Vig 1975), dormant seed, rather than shoot apices, were irradiated in these experiments. One hundred two year old seeds were treated at each dosage. The seeds were treated at 185 R/min (20 cm from source) using a Norelco 250 kV/10mA constant potential X-ray unit. Radiation exposure was measured using a Victoreen Radocon placed in the field in such a way as to monitor the total X-ray exposure reaching the seeds. In Experiment 1, seeds were treated with 0 (Control), 500, 1000, 2000, 4000, and 8000 R. In Experiment 1, two moisture contents were used: (1) dry pretreatment (6-8% moisture content) in which seeds were preincubated for 6 days in a dessicator with 75% glycerin and 25% water (Zeleny 1961) and (2) a moist pretreatment (55% moisture content) in which dessicator preincubated seeds were soaked overnight in distilled water. In Experiment 2, seeds soaked overnight received lower dosages: 0 (Control), 50, 100, 200, 400, 600, 800, 1600 R. Spots were counted 30 days after sowing on the two simple and the first compound leaves, respectively. Spots were recorded only on the upper surface, the lower surface having fewer spots (Vig and Paddock 1968). The smallest detectable D, A, or Db spots were equal to 0.25 mm^2. Spot area was measured using a dissecting microscope under fluorescent illumination.

3 Results

3.1 Dosage Response

In Experiment 1, for simple leaves, the frequency of each type of spot (measured as spots per leaf) increased in frequency with exposure dose. Early research has suggested that response to radiation varies with seed moisture content (Bacq and Alexander 1961). At 1000 R, the frequency of Db spots per leaf on simple leaves was increased over controls by 23.64-fold for dry seeds and by 12.47-fold for moist seeds (Table 1). On the other hand, the frequency of Db spots per leaf on first compounds was increased at 1000 R over controls by only 1.81 for dry seeds versus 5.43-fold for moist seeds. The rate of spot increase is not significantly greater for moist seeds versus dry seeds on either kind of leaf, suggesting that X-ray induced spot formation was independent of moisture content. On first compound leaves a much greater frequency increase was obtained from seeds treated with higher dosages (greater than or equal to 2000 R), but these seedlings were marked by morphological alterations. Because the resulting frequency of spots increased with increasing X-ray target area of cells (Evans and Paddock 1977), and since simple leaf primordia are more developed than first compound leaf primordia in dormant soybean seeds, it follows that the frequency of spots on simple leaves could be expected to be greater than on first compound leaves. All first compound progenitor cells present in a dormant seed undergo more mitoses prior to maturity.

With lower X-ray dosages and a single moisture pretreatment, in Experiment 2, dose response curves were analysed. Variances increased proportionally to increased mean spot number, resulting in non-homogeneous variances, as verified by Bartlett's (1937) test. Spot data were therefore log transformed. In simple leaves the rate of increase in spot frequency appeared linear only for Db spots, based on analysis of variance (Table 2), but has a non-linear component for both A and D spots (significant quadratic component). The regression equation for each spot type is $A = 0.334 + 0.043R - 0.0007R^2$; $D = 0.056 + 0.036R - 0.0004R^2$; $Db = 0.006 + 0.008R$. Db spots are indicative of MCO, but as discussed earlier (Evans and Paddock 1979), single spots could arise via MCO, but may also originate via other methods. These additional mechanisms, rather than MCO, may account for the significant quadratic component.

Homogeneity tests for regression coefficients (Fig. 1) indicate for simple leaves that A spots were preferentially increased over D or Db spots, and D spots are, in turn, significantly increased over the rate of increase of Db spots. So that, as in tobacco, both types of single spots were preferentially increased over Db spots, but unlike tobacco, the rate of increase of A spots was also greater than the rate of D spot increase, implying an additional or alternate mechanism of A spot induction in soybean.

3.2 Morphological Alterations

In Experiment 1, morphological alterations including smaller seedlings and leaves, reduced internode distances,

Table 1. Double spot frequency in Experiment 1

	Moist pretreatment		Dry pretreatment	
	Simple	First compound	Simple	First compound
Control	0.326 ± 0.070	0.118 ± 0.057	0.138 ± 0.053	0.389 ± 0.093
500 R	1.059 ± 0.126	0.333 ± 0.084	0.839 ± 0.115	0.545 ± 0.285
1000 R	4.066 ± 0.483	0.638 ± 0.124	3.250 ± 0.189	0.704 ± 0.238
1000 R / Control	12.47 X	5.43 X	23.64 X	1.81 X

Table 2. Dose response of log spots per leaf of simple leaves of Y_{11}/y_{11} *Glycine max* treated in Experiment 2 with 0, 50, 100, 200, 400 and 800 R.

	Analysis of variance			
	Source of variation	d.f.	Sum of squares	F-value
A spots	Total	279	72.482	
	Linear	1	42.780	473.84^{a}
	Quadratic	1	4.461	49.41^{a}
	Remainder	1	0.322	3.57 n.s.
D spots	Total	279	55.845	
	Linear	1	35.015	583.61^{a}
	Quadratic	1	4.183	69.72^{a}
	Remainder	1	0.089	1.48 n.s.
Db spots	Total	279	20.687	
	Linear	1	10.422	283.13^{a}
	Quadratic	1	0.069	1.87 n.s.
	Remainder	1	0.002	0.04 n.s.

^{a} Significant with $p < 0.01$

and abnormal leaf shapes (Fig. 2), were frequent at 4000 and 8000 R. At these high dosages, the X-rays probably (1) interfere with normal cell growth and development and (2) produce additional types of spots which cannot be the result of MCO alone.

Although seed irradiation reduced growth rate (Goodspeed 1929) no growth inhibition was observed in *N. tabacum* when leaf area was assayed following treatment of shoot apices with 300 R X-rays (Evans and Paddock 1977). On the other hand, we used higher dosages on moist soybean seeds. Table 3 is a summary of soybean leaf areas and spot frequencies from seeds treated with 0 or 1600 R in Experiment 2. Soybeans treated with 1600 R have significantly smaller simple leaves ($t = 2.221$, 32 d.f., p less than 0.05). Seed treatment with 1600 R reduced the cellular growth rates in leaves. Since the smaller leaves (1600 R) also have the greater spot frequencies (Table 3), the increase in spot frequency following X-ray treatment was greater per unit area than per leaf. We attribute the unexpectedly large difference between 0.95 and 4.017 mm^2 in mean spot area to small spot sample size. Because frequency per leaf was used for regression analysis, the rate of increase of spots per primordial cell number may be even greater than depicted in Figure 1.

Table 3. Leaf area analysis of simple and first compound leaves following X-irradiation of soybean seeds

	Simple		First compound	
	Control	1600 R	Control	1600 R
Number of leaves	5	39	8	26
Mean leaf area (cm^2)	11.65	8.49	25.36	16.27
Cell density (cells/mm^2)	--	2372	--	2668
Spot number	7	4446	23	219
Spots per cm^2	0.118	13.43	0.114	0.518
Mean spot area (mm^2)	0.25	0.247	0.95	4.017
Total spot area as % of leaf area	0.030	3.32	0.108	2.08

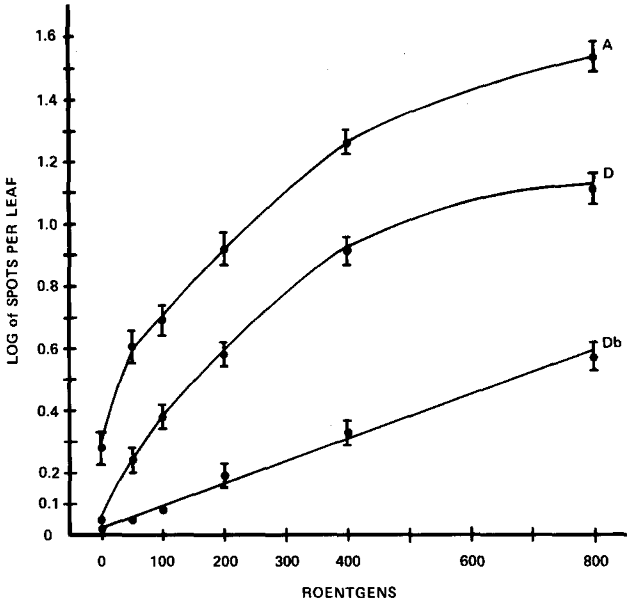

Fig. 1. Regression of the logarithm of spots per leaf for various X-ray treatments. A, D, Db signify aurea, dark green and double spots respectively. Only Db spots are linear for all dosages. Vertical bars represent standard error

3.3 Spot Formation on Homozygotes

At dosages greater than 200 R, spots were prevalent on both kinds of homozygotes. The spots included light green (L) on the simple leaves of y/y seedlings and also on Y/Y seedlings (Fig. 2). These spots can not be the result of MCO. Light green spots on y/y leaves have been observed in untreated seedlings (Evans, unpublished) and after treatment with caffeine (Vig 1973a), gamma rays, beta-particles (Vig 1974), alkylating sulfonates (Vig et al. 1976), diepoxybutane, and trenimon (Vig and Zimmerman 1977). L spots have also been seen on Y/Y leaves following sodium azide (Vig 1973b), and gamma-ray (Vig 1974) treatments. Vig (1975) has suggested that L spots on y/y leaves arise via point mutations. Evans and Paddock (1979) have proposed as additional possible mechanisms, homoeologous crossing-over (translocation) or nondisjunction, particularly since *G. max* is basically tetraploid and translocational exchanges have resulted in spot formation in other such species (Barrow et al. 1973;

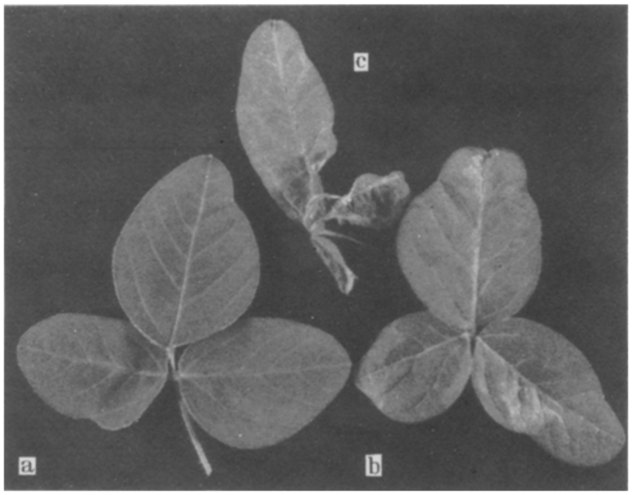

Fig. 2a-c. Representative first compound leaves of Y_{11}/Y_{11} soybean treated with **a** O R, **b** 2000 R, **c** 8000 R

Dulieu 1975). It has been suggested that L spots on Y/Y leaves may arise via non-disjunction, deletion, or point mutation (Vig 1975). We did not count spots on y/y seedlings, as all of them died prior to maturation of the first compound leaves. The frequency of L spots on Y/Y leaves (Table 4) increase with increased dosage of X-rays on both simple and first compound leaves. Dark dark-green (DDG) spots as well as Db spots, composed of adjacent L and DDG tissue, were noted at 800 and 1600 R, but were not recorded since the DDG phenotype was barely distinguishable from the Y/Y background phenotype. No comparable spots were observed on y/y leaves. The rare Db spots on Y/Y leaves can be attributed to non-disjunction. They have been observed more distinctly on leaves of xa-2/xa-2 tomato, following X-ray treatment of shoot apices (Evans 1977). The semidominant nature of the soybean mutant, as well as the appearance of the alternative spot types (L on both Y/Y and y/y leaves, and DDG on Y/Y leaves), suggest that chloroplast development as governed by the y_{11} locus is a gene dosage phenomenon. Therefore, at X-ray treatments greater than 2000 R, it is evident that separate spots appear which can simultaneously be ascribed to MCO (Db spots on Y/y leaves), non-disjunction (Db spots on Y/Y leaves), as well as other possible mechanisms (Vig 1975) including mutation, gene conversion and translocation to account for L spots on Y/Y leaves, L spots on y/y leaves, and single D or A spots on Y/y leaves.

Table 4. Light green spots on Y_{11}/Y_{11} leaves of *Glycine max*

		Simple		First compound	
Treatment	Plants	Leaves	Spots/Leaf	Leaves	Spots/Leaf
0 R	13	25	0.0	18	0.06
50 R	15	30	0.03	19	0.11
100 R	23	46	0.14	31	0.13
200 R	17	25	1.56	21	0.43
600 R	18	31	3.03	27	0.81
800 R	22	40	8.58	27	1.19
1600 R	14	24	35.58	17	1.53

3.4 Leaf Development

The simple leaf primordia were more mature in the dormant seed resulting in smaller mean area of spots than on comparable first compound leaves following X-ray treatment (Table 3). On leaves treated with 1600 R, maximum area of a spot on simple leaves was 0.35 mm^2, while maximum area of a spot on first compounds was 144 mm^2. On the other hand, the simple leaf primordium contains a larger cell number thereby resulting in a greater spot frequency per cm^2. The total spot area expressed as a percentage of the leaf blade area in X-ray treated seedlings was greater in simple leaves than in first compound leaves. Since cell density was nearly equal (as indicated by preliminary observations in Table 3), we conclude simple leaf primordia were more sensitive than first compound primordia to the X-ray treatment of dormant seeds. Variable X-ray sensitivity of cells in different development stages has also been reported in *Drosophila* (Abbadessa and Burdick 1963).

3.5 Leaflet Development

The lateral leaflets of first compound leaves both develop from the terminal leaflet primordium of soybean after it has reached a length of 80 μm (Decker and Postlethwait 1960). In Table 5, we have partitioned the spot frequency of first compound leaves per component leaflets. Because both lateral leaflets were initiated at the same time (Sun 1957), have the same mean leaflet area, and the same cell density, they were developmentally synchronous. Differences observed between lateral and terminal leaflets in primordium initiation time (Sun 1957), mean mature leaflet area, and cell density, imply asynchronous development. Also, since mean size of spots on a leaf was proportional to developmental age (Evans and Paddock 1977), the large mean spot area of terminal leaflets implies that the terminal leaflet primordium was relatively young at the time of X-irradiation (i.e., cells present must undergo numerous cell divisions prior to leaflet maturation). From Table 5, it can be concluded that the mean number of spots was proportional to cell target area that lateral and terminal leaflets in the dormant seeds were composed of approximately the same number of cells at the time of irradiation, but that each cell present in terminal leaflet

Table 5. Analysis of leaflets of first compound leaves of *Glycine max* following X-ray treatment of seeds

	Mean leaflet area[b]	Cell density	Mean area of a spot[a]	Total number of spots
Lateral				
Left[a]	7.1 cm^2	2630 $\frac{\text{cells}}{mm^2}$	0.6148 mm^2	61
Right	6.9	2680	0.3173	52
Terminal	9.3	2700	7.8730	53
Test of significance	$P \leq 0.025$	n.s.	$P \leq 0.025$	n.s.
Of lateral	$t = 2.655$	$X^2 = 0.4673$	$t = 2.3906$	$X^2 = 0.1119$
Versus terminal	40 d.f.	1 d.f.	184 d.f.	1 d.f.

[a] when viewing adaxial surface of leaf
[b] random sample of 14 first compound leaves (including controls and 1600 R)
[c] from sample of 18 morphological normal leaves treated with 1600 R

primordia underwent more mitoses resulting in larger spots. Terminal leaflets were therefore initiated earlier, had approximately the same target area in dormant seeds (unless leaflets vary in sensitivity to X-rays), underwent more mitoses following germination, and resulted in more final cells (and larger leaflets) than comparable lateral leaflet primordia.

3.6 Mitotic Crossing Over per Cell

Using data from Experiment 2, the frequency of MCO per cell based on the frequency of double spots, was calculated as by Evans and Paddock (1976), using Db spots on Y/y leaves. The spontaneous frequency of MCO among controls, 3.39×10^{-5}, is comparable to both our earlier estimate of 5.74×10^{-5} MCO per mitosis for *G. max* (Evans and Paddock 1976) and the spontaneous frequency of other higher plant species (Evans and Paddock 1979). For 1600 R, the frequency of MCO on simple leaves is 9.55×10^{-3}, representing a 282-fold increase over the control frequency.

3.7 X-ray versus Mitomycin C

In contrast to X-rays, which preferentially produce D and A single spots (Fig. 1), Vig (1967) has suggested that Mitomycin C (MMC) preferentially increases the frequency of Db spots versus single spots. Vig and Paddock (1968), though, used the change in ratios of spots with increasing concentration of MMC as the basis for this conclusion. Because MMC preferentially increases Db spot frequency, the effects of X-rays and MMC on spot induction differ. The values obtained using Vig's (1967) ratio method, though, can not be used to assess quantitatively the effectiveness of chemical or physical treatments. Since our experimental protocols are comparable to his, we subjected the original data of Vig (1967, Appendix) to regression analysis. Data were log transformed to produce homogeneous variances. All three types of spots do increase linearly with increasing concentration of MMC using log transformed data. The rate of increase in Db spots is greater than either A or D single spots (Table 6). There is non-homogeneity of regression coefficients when all three types of spots are grouped together, F = 11.73, 2 d.f. This non-homogeneity is specifically due to the preferential increase of Db spots over A spots, F = 16.98, 1 d.f., while D versus A spots was non-significant F = 0.015, 1 d.f. Therefore, the rate of increase of Db spot frequency is significantly greater than A or D single spots. In Table 6, we have compared the regression coefficients for MMC and our X-ray increased frequencies of all three types of spots. In each case data represent log transformed spot number on simple leaves. Our X-ray data are derived from Experiment 1. It is obvious that MMC does preferentially increase the frequency of Db spots. Therefore, although both treatments result in an increase in spots, the mechanism of action of the two treatments is different, reflected by non-homogeneous regression coefficients.

Table 6. Regression coefficients for the rate of increase of each type of spot on simple leaves of *Glycine max*

Spot type	Mitomycin C[a]	X-rays[b]
D	0.01609	0.01700
A	0.01394	0.01673
Db	0.09287	0.00438

[a] log spots per μM MMC
[b] log spots per R in Experiment 1

3.8 Comparison with Nicotiana tabacum

Evans and Paddock (1977) used X-ray treatment of tobacco shoot apices to increase the frequency of spot formation on tobacco leaves. Since shoot primordia are present in dormant seeds, soybean can be compared with *N. tabacum*. The rate of increase in Db spots is linear in both tobacco and soybean. Further, because as the regression coefficient is greater for tobacco, it appears the tobacco shoot apex was more sensitive to X-ray induced MCO than the shoot apex in the dormant soybean seed. Like tobacco, in soybean, large spots appeared on younger leaves, single spots were increased more than Db spots, and only leaves whose primordia were present at the time of irradiation had an increased spot frequency. In soybean, unlike tobacco, A spots were preferentially increased over D spots, leaf area was altered with X-rays and most importantly, soybean leaf primordia present in the dormant seed in various stages were developmentally asynchronous and thereby resulted in different spot frequencies and spot areas.

4 Conclusion

We were able to ascertain that Db spot frequency on light green leaves increased linearly with increasing X-ray dosage applied to Y_{11}/y_{11} *Glycine max* seeds. This response was independent of moisture content. The analyses of alternate spot types, as well as comparisons between Db spot increase with MMC and with X-rays, demonstrate that X-rays, at the dosages examined, were not specific for MCO. Nonetheless, 1600 R of X-rays increased the frequency of MCO per cell by 282-fold. When simple and first compound leaves were compared it was evident that spot number was proportional to primordial cell number while spot size depended upon the number of mitoses from the time of X-irradiation to leaf maturation. Spot area analyses of first compound leaves suggest that terminal and lateral leaflets were developmentally asynchronous.

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Dr. D.A. Evans
The Center for Somatic-Cell Genetics and Biochemistry
Department of Biological Sciences
State University of New York
Science 3, Room 144
Binghamton, N.Y. 13901 (USA)

Dr. Elton F. Paddock
Department of Genetics
1735 Neil Avenue
The Ohio State University
Columbus, Ohio 43210 (USA)