

Effects of X-ray dose fractionations with various intervals in inducing somatic mutations in the stamen hairs of *Tradescantia* clone KU 9

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The effects of X-ray dose fractionations in inducing somatic mutations were studied in the stamen hairs of *Tradescantia* clone KU 9 heterozygous for flower color (blue/pink). Young inflorescence-bearing shoots with roots were prepared, and were exposed to X rays acutely. A dose-response regression line with a slope of 1.454 on a log-log graph was obtained for single acute X-ray doses of 0.255 to 1.03 Gy, and it showed that somatic mutation frequency increased curvilinearly with increasing X-ray dose. When 1.00 to 1.15 Gy of X rays were fractionated into two acute doses of about halves given with intervals of 5 to 120 min, decreases in induced mutation frequency were observed. The mutation frequencies induced by the fractionated doses with intervals of 5 and 10 min were not significantly different from those expected for the total single doses. However, the mutation frequency decreased significantly at 5% level with 20- and 30-min intervals, and decreases were highly significant at 0.1% level when the interval was prolonged to 40 to 120 min. The results obtained indicate that the interaction between the first and second doses began to reduce between 10 and 20 min later, and disappeared by 60 min later. That is, the DNA and/or chromosomal breaks induced by the first dose began to be rejoined (repaired) or healed between 10 and 20 min later, and all of them were rejoined or healed by 60 min later, losing their abilities to interact with the DNA and/or chromosomal breaks induced by the second dose.

INTRODUCTION

The stamen-hair system of *Tradescantia* heterozygous for flower color (blue/pink; the blue color being dominant) has proven to be one of the most suitable materials to study the frequency of mutations induced by various ionizing radiations and chemical mutagens, even at low levels, as reviewed earlier (Underbrink et al., 1973; Ichikawa, 1981b, 1992; Schairer and Sautkulis, 1982). The use of this system makes it possible to collect a large number of samples relatively easily and to detect all pink mutant cells occurred without being concealed by other cells (Ichikawa, 1992). Because of these merits, this system has been successfully used for studying the variation of spontaneous mutation frequency (Sparrow and Sparrow, 1976; Takahashi and Ichikawa, 1976; Mericle et al., 1976; Nauman et al., 1978; Ichikawa et al., 1981, 1996a, 1996c; Ichikawa, 1984; Imai et al., 1991; Sanda-Kamigawara et al., 1991), as well as for detecting mutagenic synergisms among several chemical mutagens and X rays (Cebulska-

Wasilewska et al., 1981; Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995).

The data obtained from *Tradescantia* stamen-hair system have suggested that the majority of mutations induced by ionizing radiations are due to DNA strand breaks and/or the resultant chromosomal breaks (Nauman et al., 1975; Ichikawa et al., 1978; Ichikawa, 1981b), and most of them are deletion-type mutations missing small to considerable numbers of base pairs (Ichikawa et al., 1978; Ichikawa, 1981b). Although the somatic mutation frequency in *Tradescantia* stamen hairs has proven to increase linearly with increasing X- or gamma-ray dose when the doses are small (Ichikawa, 1971, 1972b, 1973; Sparrow et al., 1972; Ichikawa and Takahashi, 1977; Ichikawa et al., 1981) or are applied chronically at low dose rates (Nayar and Sparrow, 1967; Ichikawa and Sparrow, 1968; Ichikawa et al., 1978), it has been demonstrated to increase curvilinearly when X- or gamma-ray doses larger than about 100 mGy are delivered acutely at high dose rates, the dose-response relationships showing slopes of about 1.2 to 1.4 on log-log graphs (Sparrow et al.,

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1972; Ichikawa et al., 1978, 1993; Sanda-Kamigawara et al., 1991; Shima and Ichikawa, 1994, 1995; Xiao and Ichikawa, 1995). Such curvilinear increases of mutation frequency with acute radiation dose indicate that some of the induced mutations arise as the results of so-called two-hit events such as interstitial deletions (Ichikawa, 1981b, 1992). The curvilinear dose-response relationships also allow us to hypothesize that fractionations of acute X- or gamma-ray doses will result in reductions in mutation frequency.

The effects of dose fractionation in inducing mutations have been studied in several organisms, but not yet in *Tradescantia* stamen hairs. In the present study, therefore, the effects of fractionations of acute X-ray doses were examined in *Tradescantia* stamen hairs, choosing the young inflorescence-bearing shoots with roots of clone KU 9 as new testers. The young inflorescence-bearing shoots with roots of clone BNL 4430 as described earlier (Shima and Ichikawa, 1994; Ichikawa et al., 1995) have been used as the most ideal mutagenicity testers in our recent studies of synergistic effects between different mutagens (Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995). For choosing the young inflorescence-bearing shoots with roots of clone KU 9, there were two reasons: (1) The spontaneous (background) mutation frequency in the stamen hairs of potted plants is slightly lower in clone KU 9 than in clone BNL 4430 (Ichikawa, 1992); and (2) the spontaneous mutation frequency could be lowered significantly in clone BNL 4430 by using the young inflorescence-bearing shoots with roots (Shima and Ichikawa, 1994), allowing us to expect the same result with clone KU 9.

MATERIALS AND METHODS

Materials used. The young inflorescence-bearing shoots with roots of *Tradescantia* clone KU 9 were used for the first time. This clone is a triploid hybrid ($3x = 18$) between a pink-flowered tetraploid clone of *T. ohiensis* Raf. and a blue-flowered diploid clone of *T. paludosa* And. et Woods. (Ichikawa, 1972a), and shows a relatively stable spontaneous mutation frequency (Takahashi and Ichikawa, 1976; Ichikawa et al., 1981, 1996a; Ichikawa, 1984, 1992). The normal blue- and mutant pink-color pigments of this clone have been confirmed microspectrophotometrically to be identical to those in clone BNL 02 (Sanda-Kamigawara and Ichikawa, 1993), one of the clones most often used in studies of mutations in stamen hairs (Underbrink et al., 1973; Ichikawa, 1992).

The young inflorescence-bearing shoots with roots of clone KU 9 were prepared following the procedures as described below, which were entirely different from the method of simply dividing potted plants as used for clone BNL 4430 (Shima and Ichikawa, 1994; Ichikawa et al., 1995). Namely, comparing with the characteristics of

clone BNL 4430, i.e., its short height and many new shoots constantly emerging from the basal nodes one after another (Shima and Ichikawa, 1994; Ichikawa et al., 1995), the main stems of clone KU 9 grow much taller, and the production of new shoots from the basal nodes is much slower. Therefore, the following three steps were needed to prepare the young inflorescence-bearing shoots with roots: (1) The main stems (having three lower nodes) with roots taken from the plants grown outdoors were cultured using a nutrient solution circulating (NSC) facility set in the growth room (see below) until new shoots from the basal nodes grew to about 100 mm in height; (2) these young shoots with roots were further grown using the same NSC facility, the older main stem of each of them being cut back short at immediately above the second lowest node, until new main stems started to grow; and (3) they were then transferred to another NSC facility set in the Conviron E8 growth chamber (see below) until young inflorescences were formed at the tips of the new main stems. All these procedures took about two to three months.

Growing conditions. The environmental conditions in the growth room used for culturing the main stems with roots and the young shoots with roots were $23.0 \pm 0.5^\circ\text{C}$ (constant), 50% humidity, and a 16-h day length with a light intensity of 6 klx from Toshiba DR400/T(L) metal-halide sunlamps plus white fluorescent tubes. In the Conviron E8 used for growing the shoots with roots until young inflorescences were formed and also for cultivating the young inflorescence-bearing shoots with roots for experiments, the temperature was controlled to change gradually with a sine curve between $21.0 \pm 0.5^\circ\text{C}$ (at 2 pm) and $19.0 \pm 0.5^\circ\text{C}$ (at 2 am), keeping 50% humidity, and the day length was 17 h with the maximum light intensity of 15 klx for 13 h and 40 min, changing the light intensity from Sylvania VHO cool white fluorescent tubes and incandescent bulbs also gradually.

The nutrient solution used for the NSC facilities was a 1/3000 Hyponex solution, and the solution was exchanged every two weeks.

X-ray treatments. X-ray treatments of young inflorescences were performed using a Hitachi MBR-1505R X-ray generator. The treatments were conducted acutely at 150 kVp and 4 mA with a 0.5 mm Al + 0.1 mm Cu filter at $23.0 \pm 0.5^\circ\text{C}$. The target distance was 600 mm. The exposures were measured simultaneously with thermoluminescence dosimeter (TLD) elements (National UD-170L) set at the same target distance as the inflorescences treated, and with a thermoluminescence reader (National UD-502B). The exposure data obtained in R were then converted into absorbed doses in Gy with a converting factor of 9.57×10^{-3} (i.e., $1 \text{ R} = 9.57 \text{ mGy}$).

For determining the standard dose-response curve for

acute X-ray dose, four single doses of 0.255 to 1.03 Gy were applied. In dose fractionation experiments, on the other hand, X rays of 1.00 to 1.15 Gy in total were delivered fractionating into about two halves with intervals of 5, 10, 20, 30, 40, 60 and 120 min.

Scoring mutations. The methods used for scoring pink mutations in the stamen hairs in the present study were identical to those described earlier in detail (Ichikawa, 1992). Briefly, the numbers of stamen hairs and of pink mutant events (PMEs) were scored on each of six stamens, and the number of hair cells was also counted on 10 representative hairs each of two oppositely located stamens per flower to estimate the average number of cells per hair for calculating mutation frequency per hair-cell divisions (Ichikawa and Takahashi, 1977, 1978; Ichikawa and Ishii, 1991; Ichikawa, 1992). A PME represents the result of a single mutation as defined earlier (Ichikawa, 1981a, 1992). Somatic mutation frequency was expressed as the number of PMEs per 10⁴ hair-cell divisions (rather than that per 103 hairs), since the average number of cells per hair may differ after different treatments (Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995; Xiao and Ichikawa, 1995). The data were pooled for the 4-day peak period for each treatment as in earlier studies (Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995).

Statistical examinations. Somatic mutation frequencies induced by fractionated doses were compared with the expected mutation frequencies for the total single doses. Since it was often difficult to apply exactly the same dose of X rays as the first and second doses and to maintain the same total dose in each of the dose fractionation experiments, the expected mutation frequency for each total single dose was calculated using the equation expressing the standard dose-response curve on a log-log graph determined in the present study (see Results). The differences

between the observed and expected mutation frequencies were examined by chi-square tests.

RESULTS

Spontaneous mutation frequency. It was necessary to confirm the spontaneous pink mutation frequency in the stamen hairs, since the young inflorescence-bearing shoots with roots of clone KU 9 were used for the first time. The scoring of spontaneous mutation frequency was continued for two full years (1994 and 1995). The total numbers of stamen hairs observed and PMEs detected were 926,351 and 1,268, respectively, and the average cell number per hair was 20.74. Although some fluctuations were observed among 24 monthly spontaneous mutation frequencies (but mostly 0.6 to 0.8 PMEs per 10⁴ hair-cell divisions), a pooled spontaneous mutation frequency of 0.693 ± 0.019 PMEs per 10⁴ hair-cell divisions was obtained.

Dose-response curve. The data collected to construct the standard dose-response curve for acute X rays are presented in Table 1. Acute single doses of 0.255, 0.610, 0.740 and 1.03 Gy were applied, setting the respective control for each treatment. When the induced mutation frequencies after subtracting each control frequency are plotted against X-ray doses on a log-log graph, a dose-response regression line as shown in Fig. 1 is obtained, showing that there is practically no fluctuation. The regression line is expressed by the equation of $y = 39.900 x^{1.454}$, where y is the number of PMEs per 10⁴ hair-cell divisions, and x is the X-ray dose in Gy. This equation shows that induced mutation frequency increased curvilinearly with X-rays dose, i.e., with the slope of 1.454 on the log-log graph, and was used to calculate expected mutation frequencies for the total doses in dose fractionation experiments.

Effects of dose fractionations. The data obtained from seven experiments of dose fractionations are pre-

Table 1. Somatic pink mutation frequencies induced by acute single doses of X rays in the stamen hairs of clones KU 9

X-ray dose (Gy)	No. of hairs observed	No. of PMEs ^a scored	Average no. of cells /hair	No. of PMEs /10 ⁴ cell divisions (\pm SE)	Minus control (\pm SE)
0	31,908	50	21.02	0.783 \pm 0.111	
0.255	12,989	163	21.03	6.27 \pm 0.49	5.48 \pm 0.50
0	48,938	85	20.83	0.876 \pm 0.099	
0.610	11,377	430	19.54	20.4 \pm 1.0	19.5 \pm 1.0
0	38,767	61	20.66	0.800 \pm 0.102	
0.740	17,139	858	20.05	26.3 \pm 0.9	25.5 \pm 0.9
0	48,268	92	20.98	0.954 \pm 0.099	
1.03	9,992	769	18.96	42.9 \pm 1.5	41.9 \pm 1.5

^a Pink mutant events.

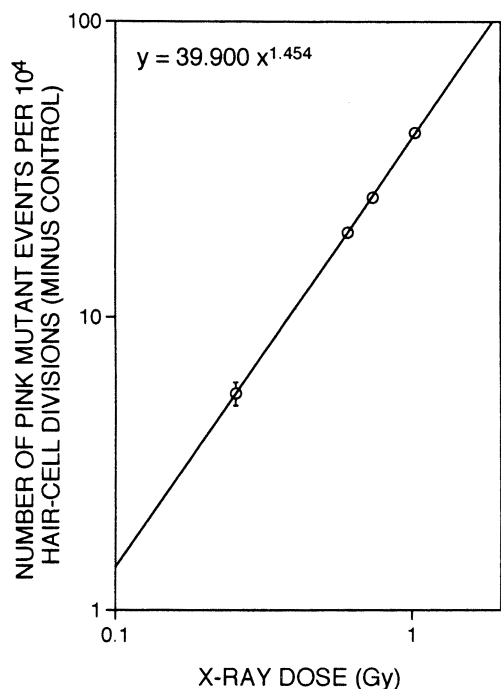


Fig. 1. The dose-response curve of somatic pink mutation frequency in the stamen hairs of clone KU 9 obtained after treatments with acute single doses of X rays. The best-fit regression line determined with the least squares method and the standard errors for the points plotted (excepting those within open circles drawn) are shown. The equation of $y = 39.900 x^{1.454}$ for this curve was used for calculating the expected mutation frequencies for total single doses in dose fractionation experiments.

sented in Table 2. The respective controls were also set in these experiments. The expected mutation frequencies for the total single doses calculated using the above equation in Fig. 1, the ratios of the observed against expected mutation frequencies, and the statistical significances between the observed and expected mutation frequencies are also shown in this table. The mutation frequencies induced by the fractionated doses with intervals of 5 and 10 min were not significantly different from those expected for the total single doses. However, the mutation frequency decreased significantly at 5% level with 20- and 30-min intervals, and it decreased highly significantly at 0.1% level when the interval was prolonged to 40 to 120 min.

The ratios of the observed against expected mutation frequencies in Table 2 are plotted against the interval period of fractionated doses in Fig. 2, for analyzing the relationship between the interval period and the induced mutation frequency. It is seen in this figure that the ratio decreased with prolonged interval period, reaching the lowest level at 60-min interval.

DISCUSSION

Validity of using shoots with roots. A pooled spontaneous mutation frequency of 0.693 ± 0.019 PME's per 10^4 hair-cell divisions was obtained in the present study, through the two-year-long scorings of pink mutations in the stamen hairs, cultivating the shoots with roots of clone

Table 2. Somatic pink mutation frequencies induced by fractionated acute X-ray doses delivered with intervals of 5 to 120 min in the stamen hairs of clone KU 9

Dose I (Gy)	Interval time (min)	Dose II (Gy)	No. of hairs observed	No. of PME's ^a scored	Average no. of cells /hair	No. of PME's / 10^4 cell divisions (\pm SE)	Minus control (\pm SE) (O)	Expected no. of PME's / 10^4 cell divisions ^b (E)	O/E	P
0	—	0	24,337	35	20.49	0.738 ± 0.125				
0.535	5	0.477	5,666	438	18.71	43.6 ± 2.1	42.9 ± 2.1	40.6	1.06	NS ^c
0	—	0	29,320	29	20.42	0.509 ± 0.095				
0.476	10	0.525	10,484	701	18.00	39.3 ± 1.5	38.8 ± 1.5	40.0	0.97	NS
0	—	0	33,164	48	21.27	0.714 ± 0.103				
0.621	20	0.513	8,970	676	18.26	43.7 ± 1.7	42.9 ± 1.7	47.9	0.90	<0.05
0	—	0	21,090	32	20.93	0.761 ± 0.135				
0.577	30	0.555	11,743	907	18.63	43.8 ± 1.5	43.0 ± 1.5	47.8	0.90	<0.05
0	—	0	22,222	27	20.42	0.626 ± 0.120				
0.577	40	0.568	11,353	769	17.99	39.9 ± 1.4	39.2 ± 1.4	48.6	0.81	<0.001
0	—	0	26,848	41	20.86	0.769 ± 0.120				
0.577	60	0.448	13,143	728	19.39	30.1 ± 1.1	29.4 ± 1.1	41.4	0.71	<0.001
0	—	0	25,179	30	20.38	0.615 ± 0.112				
0.520	120	0.518	9,287	540	18.53	33.2 ± 1.4	32.6 ± 1.4	42.1	0.77	<0.001

^a Pink mutant events.

^b Calculated for total single dose using the equation shown in Fig. 1.

^c Not significantly different statistically.

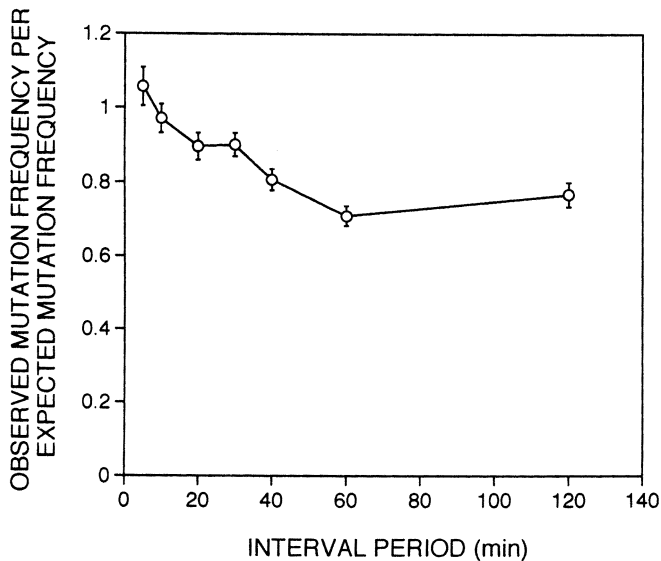


Fig. 2. The relationship between the interval period of fractionated dose and the ratio of the observed against expected mutation frequencies. The standard errors of the values plotted were calculated based on those of the observed mutation frequencies.

KU 9 using the NSC facility set in the Conviron E8. This mutation frequency is significantly lower than those reported earlier for potted plants of this clone grown under controlled environmental conditions, i.e., 1.31 to 1.76 (Ichikawa et al., 1981), 1.58 (Ichikawa and Takahashi, 1977), 1.74 (Ichikawa, 1984) and 1.89 (Ichikawa, 1992) PME per 10^4 hair-cell divisions. Much higher spontaneous mutation frequencies have also been reported for clone KU 9 grown outdoors, i.e., 1.88 to 2.18 (Ichikawa et al., 1996a; excepting the data affected by the Chernobyl accident) and 2.56 (Ichikawa, 1984) PME per 10^4 hair-cell divisions. These comparisons show that the spontaneous mutation frequency is significantly lowered by cultivating the shoots with roots using the NSC facility than by growing potted plants. A similar result of significantly lowered mutation frequency has been observed earlier in the shoots with roots of clone BNL 4430 cultivated in a NSC growth chamber (Shima and Ichikawa, 1994). It is likely that cultivation of the shoots with roots with a fixed nutrient solution in such NSC facilities can reduce the occurrences of spontaneous mutations, probably by stabilizing the growing conditions. The lower spontaneous (background) mutation frequency is favorable especially for detecting the genetic effects of low-level mutagens (Shima and Ichikawa, 1994; Ichikawa et al., 1995).

Reliability of standard dose-response curve. The standard dose-response curve determined for acute X rays in the present study had the slope of 1.454 on the log-log graph (Fig. 1). This slope value indicates that about 55% of mutations induced by X rays were the results of so-called one-hit events, and the remainings were resulted

from two-hit events. The slope value is at least somewhat larger than those of the dose-response curves for acute X rays determined earlier in other clones based on the mutation frequencies in 4-day peak periods, i.e., 1.274 (Ichikawa et al., 1993) and 1.404 (Sanda-Kamigawara et al., 1991) in clone KU 27; 1.237 (Sanda-Kamigawara et al., 1991) in clones KU 27 and BNL 02; and 1.252 (Shima and Ichikawa, 1994), 1.314 (Shima and Ichikawa, 1995) and 1.390 (Xiao and Ichikawa, 1995) in clone BNL 4430. However, the data employed to construct the standard dose-response curve showed practically no fluctuation (Fig. 1), and the relationship is therefore judged to be reliable.

It should be noted that the mutation frequency in *Tradescantia* stamen hairs has proven to increase linearly with increasing X- or gamma-ray dose, when the doses are small (Ichikawa, 1971, 1972b, 1973; Sparrow et al., 1972; Ichikawa and Takahashi, 1977; Ichikawa et al., 1981) or applied at low dose rates (Nayar and Sparrow, 1967; Ichikawa and Sparrow, 1968; Ichikawa et al., 1978), indicating that mutations are induced as one-hit events in such cases (Ichikawa, 1981b, 1992). The mutations in *Tradescantia* stamen hairs have also proven to be induced as one-hit events with fast (Ichikawa, 1970; Sparrow et al., 1972) and thermal neutrons (Ichikawa, 1997). No effect of dose fractionation will be expected in these cases of solely one-hit events.

Effects of dose fractionations detected. The mutation frequencies induced by two fractionated doses applied with 20-min or longer intervals were found to be significantly lower than those expected for the respective total single doses (Table 2). Also, the ratio of the observed against expected mutation frequencies decreased with prolonged interval period, reaching the lowest level at 60-min interval (Fig. 2). These results indicated that the ability of the first dose to interact with the second dose started to reduce between 10 and 20 min later, and disappeared by 60 min later. That is, rejoinings (repairs) or healings of the DNA and/or chromosomal breaks induced by the first dose began to occur between 10 and 20 min later, and were completed by 60 min later, the breaks induced by the first dose losing their abilities to interact with those induced by the second dose. In fact, the mutation frequency of 29.4 ± 1.1 PME per 10^4 hair-cell divisions induced by the fractionated doses with 60-min interval (Table 2) is very close to 30.4 PME per 10^4 hair-cell divisions which is calculated, using the equation in Fig. 1, as the expected mutation frequency for the merely additive effect of the two fractionated doses.

The present finding agrees well with an earlier report of a significant decrease of somatic mutation frequency in leaves of a diploid *Avena* strain heterozygous for an albino gene by fractionating acute X-ray dose with 20-min interval (Nishiyama et al., 1966). On the contrary, no evident

effect of dose fractionation has been detected in the stamen hairs of *Tradescantia* including clone KU 9, when gamma-ray doses delivered at much lower dose rates were fractionated (Ichikawa et al., 1996b), as expected as mentioned above.

As for rejoinings of X-ray-induced chromosomal breaks, existence of two different types of rejoinings has been demonstrated. Namely, chromosomal breaks rejoining rapidly within 1 min and those rejoining slowly requiring 2 to 4 h in *Vicia* root tips (Wolff and Luippold, 1956), and breaks rejoining within 15 min and those requiring a few hours to rejoin in *Allium* root tips (Cohn, 1958) have been reported. Two types of rejoinings have also been demonstrated in barley root tips, i.e., about 40% of the rejoinings of X-ray-induced chromosomal breaks occurred very rapidly within 20 to 30 sec, but the remainings took 15 to 30 min (Ichikawa et al., 1965).

There have been several reports also on repairs of radiation-induced DNA strand breaks. It has been reported that 50% of single-strand breaks in gamma-rayed wild carrot protoplasts were repaired within 5 min and the remainings within 1 h (Howland et al., 1975). The periods required for repairing 50% of X-ray-induced single-strand breaks in the root-tip cells of *Tradescantia* clones BNL 02 and BNL 4430 have been reported to be 7 to 10 and 20 min, respectively (Velemínsky and Van't Hof, 1984). The latter report seems to agree with the results obtained in the present study.

Some recent reports on mammalian cells also appear to be consistent with the present results. Namely, 50% repairing period of 21.3 ± 3.1 min determined for single-strand breaks induced by X rays in rat splenic lymphocytes (Coogan et al., 1992), and fast rejoining process of gamma-ray-induced double-strand breaks requiring 18.0 ± 1.4 to 36.4 ± 3.2 min in human tumor cell lines (Nuñez et al., 1995) are such examples.

Considering all these earlier evidences, especially findings of the evident effect of X-ray dose fractionation with interval of 20 min on somatic mutation frequency in diploid oats (Nishiyama et al., 1966) and of the 50% repairing time of 7 to 20 min for X-ray-induced DNA single-strand breaks in *Tradescantia* clones (Velemínsky and Van't Hof, 1984), the effects of dose fractionations on somatic mutation frequency found in the present study are considered to agree with earlier findings. The present results also support our hypothesis based on the curvilinear increases of somatic mutation frequency with acute X-ray dose in *Tradescantia* stamen hairs.

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