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INFLUENCE OF CHROMOSOME NUMBER ON CAFFEINE INHIBITION OF DNA REPAIR I. MUTATION FREQUENCY

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ABSTRACT

Dormant seeds of sorghum with 2N=20; mungbean, 2N=22; peanut, 2N=40; and tobacco, 2N=48, were treated with sub-lethal doses of gamma radiation, post-soaked in caffeine solution and grown in field plots to maturity Untreated seeds and seeds treated only with radiation or caffeine were used as control.

At 30 Krads, the species with high chromosome numbers gave a bigger increase in somatic mutation frequency over the radiation control than the species with low chromosome numbers. Similarly, the species with high chromosome numbers gave a bigger increase in frequency of M_2 seedling mutation over the control than the species with low chromosome numbers. The possible influence of chromosome number on caffeine inhibition of DNA repair leading to a higher probability of induced mutation is discussed briefly.

Introduction

It is now universally recognized that living cells are normally equipped with certain enzyme mechanisms that restore damaged DNA segments to their original state. Discovery of DNA repair in the early 1950's has more or less revolutionized present concepts on chromosomal aberration and gene mutation with their important applications in plant and animal breeding. Interest in the modification of DNA repair also lies in the increasing exposure of plants to mutagenic systems in the environment occurring as industrial wastes, pesticide residues, gaseous pollutants and others which could increase spontaneous mutation rates.

Enhancement of DNA lesions and consequently increased rates of mutation reportedly result when the gap-filling process in replicating DNA is inhibited by caffeine (Kihlman *et al.*, 1974; Kihlman and Kornborg, 1972) either by competing with thymine dimers for excision enzyme (Sideropoulos and Shankel, 1968), interacting with DNA rather than with repair enzymes directly (Witte and Bohme, 1972), binding with double-stranded DNA bearing short single-stranded regions (T'so and Lu, 1962; Domon and Rauth, 1969) or other related mechanisms.

A number of factors have been reported to have influenced the enhancing action of caffeine on mutagen-treated material although the mechanisms involved remain to be described. For instance, lowering the temperature of treatment

(Kihlman *et al.*, 1974) has reportedly decreased the synergistic effects of caffeine with various chemical and physical mutagens. The presence of oxygen has been found to inhibit the effects of caffeine on the repair process when applied as a post-treatment (Novick, 1956). The frequency of chromatid aberrations markedly increased when barley seeds were post-soaked in caffeine solution at the G_2-early prophase stage following radiation treatment (Yamamoto and Yamaguchi, 1969).

Materials and Methods

Dormant seeds of sorghum (*Sorghum vulgare* Pers.) with 2N=20; mungbean (*Vigna radiata* (L.) Wilczek.), 2N=22; peanut (*Arachis hypogea* L.) 2N=40; and tobacco (*Nicotiana tabacum* L.), 2N=48, were individually selected for absence of deformities, uniform color and size. They were stored in a moist dessicator for seven days to equalize the seed moisture content to 14% and set in a thin layer in plastic envelope for irradiation. Radiation treatment was made at the Gamma Cell Facility of the Philippine Atomic Research Center in Diliman, Quezon City at total doses of 15 to 60 Krads.

Soon after radiation exposure, the seeds were soaked in 0.10% solution of caffeine (1, 3, 7 Trimethyl 6 xanthine, Nutritional Biochemicals, Cleveland) for four hours at a constant temperature of 28°C. Untreated seeds from the same sources and seeds soaked only in water or caffeine solution as well as seeds treated with gamma radiation were used as control. Some of the seeds were sown on moist tissue paper in a petri dish for measurement of seedling height and the rest of the seeds were planted in field rows in a treatment-to-row plan at the Botany Experimental Garden for scoring of plants with chimeral leaves at the four to six-leaf stage.

The ripe pods or panicles, as the case may be, were harvested by the row, dried at room temperature and after a few weeks of dormancy were sown in nursery rows for determination of the frequency of M_2 seedling mutations. The influence of chromosome number on mutation frequency was determined only at the dose of 30 Krads due to the high degree of lethality at the higher radiation doses and treatment combinations.

Results and Discussion

M_1 somatic mutation frequency. The frequency of M_1 plants with chimeral leaves after caffeine post-treatment is shown in Table 1. While caffeine post-treatment resulted in a marked increase in somatic mutation frequency in the species with high chromosome numbers over the radiation control, there was practically no increase in mutation rate in the species with low chromosome numbers. The increase in mutation rates in the species with high chromosome numbers of 16 and 17 plants per 100 M_1 plants over the control were higher than those of the species with low chromosome numbers by a factor of 4.0 to 8.6.

Table 1. Percent increase in frequency of M_1 chimeral plants after post-irradiation caffeine treatment

(30 Krads)

Species	Chrom. No. (2N)	Gamma radiation		Radiation + Caffeine		
		Total M_1 plants	Chimeras per 100 M_1 plants	Total M_1 plants	Mutation per 100 M_1 plants	% increase
Sorghum	20	398	18.34	267	21.72	21.16
Mungbean	22	320	21.56	236	25.85	19.90
Peanut	40	336	8.33	242	25.62	207.56
Tobacco	48	354	6.77	290	23.10	241.21

The low mutagenic response of the species with high chromosome numbers when post-soaked only in water after radiation treatment probably explains in part the very marked increase in frequency of chimeral plants. This radio-tolerance exhibited by species with high numbers of chromosomes has been a subject of active investigation by earlier workers (Sparrow *et al.*, 1961; Swaminathan, 1961) who perceived a protective or "buffering" effect of high chromosome numbers against radiation damage. As somatic mutation in mutagen-treated material is essentially due to chromosomal deletions (Sparrow, 1961), the proportion of chromosomal breaks would likely be smaller in species with higher chromosome numbers than in those with lower chromosome numbers given the same radiation dose.

The data on seedling growth reduction shown in Figure 1 supports the view (Sparrow *et al.*, 1961) that the low mutagenic response of species with high diploid numbers of chromosomes is due to radio-tolerance commonly observed in this type of material. The 50% seedling growth reduction was found at a dose of approximately 27 Krads after caffeine post-treatment in species with low chromosome numbers and about 36 Krads in species with high numbers of chromosomes. A much higher radiation dosage was required to produce the same effect in species with high chromosome numbers than the species with low chromosome numbers.

At a dose of 30 Krads, seedling growth reduction after post-treatment with caffeine caused a lethality increase of only about 8% over the control in species with low chromosome numbers and about 19% over the control in the species with high chromosome numbers. As seedling growth reduction is highly correlated with chromosomal breakage (Conger and Stevenson, 1969), it is assumed that the marked increase in somatic mutation frequency in the species with high chromosome numbers is due to large scale chromosomal breakage.

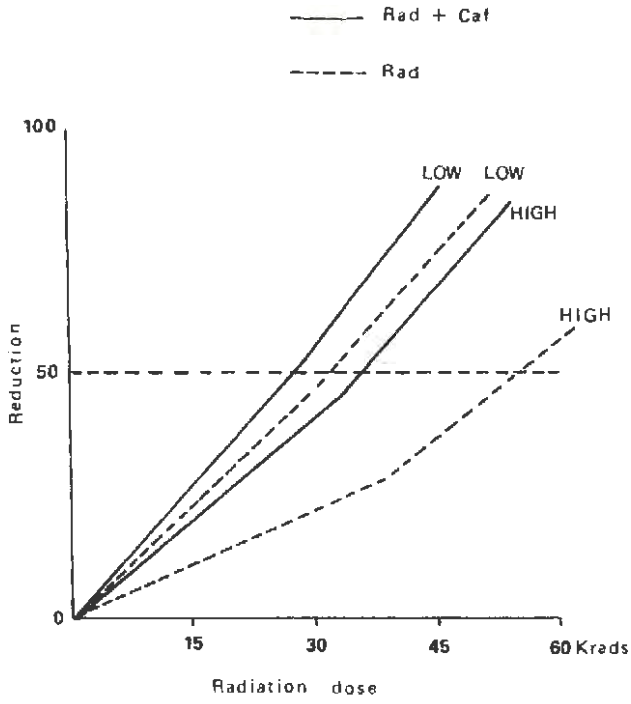

Figure 1. Mean M_1 seeding growth reductions in species with low and high chromosome numbers.

Mutagenic effects of caffeine. When used alone, caffeine did not cause any effect both in the M_1 and M_2 generations. Trial tests using concentrations lower and higher than 0.10%, even when applied alone for long periods of up to 24 hours did not cause any effect on present materials. Some slight leaf damage in M_1 seedling from seeds soaked for long periods at temperatures of 32°C – 35°C were also observed in the water control.

Several reports, however, are available indicating that caffeine when used alone is an inducer of point mutations and producer of chromosomal breaks (Kihlman, 1974; Ostertag and Haake, 1966; Witkin, 1958; Novick, 1956; Kihlman and Levan; 1949; Fries, 1950) in various organisms ranging from bacteriophage, bacteria and yeast to plants, *Drosophila* and mammalian cell cultures. However, Glass and Novick (1959) were able to demonstrate that the mutagenic action of caffeine requires DNA synthesis, an indication that caffeine is indeed specific for DNA (Kihlman, 1974).

It is not surprising, therefore, that the synergistic effects of caffeine with various chemical and physical mutagenic agents has attracted more interest than its use alone as a mutagen. Investigators on the potentiating effects of caffeine as a

Table 2. Percent increase in frequency of M_2 seedling mutations after post-irradiation caffeine treatment

(30 Krads)

Species	*Chrom. No. (2N)*	*Gamma radiation*		*Radiation + Caffeine*		
		Total M_2 seedlings	*Mutation per 1000 seedlings*	*Total M_2 seedlings*	*Mutation per 1000 seedlings*	*% increase*
Sorghum	20	2,256	35.02	1,922	37.98	7.01
Mungbean	22	1,095	38.36	1,338	43.35	13.00
Peanut	40	612	17.97	545	44.04	245.07
Tobacco	48	1,283	14.03	1,004	39.84	183.96

post-treatment in various organisms (Yamaguchi, 1979; Thacker, 1979; Hartley-Asp and Kihlman, 1971; Sideropoulos and Shankel, 1968; Shimada and Takagi, 1967; Clarke, 1967) agree that the enhancing effect of caffeine as a post-treatment results from its inhibitory action on the repair of DNA damage, particularly the post-replication repair even in such materials as barley, bean and onion root-tips among others. This view on the nature of caffeine action has been supported by Chan *et al.*, (1979); Lehman and Kirk-Bell (1974); Buhl *et al.*, (1972) who showed that caffeine indeed inhibits post-replication repair in mutagen-treated material.

Frequency of M_2 seedling mutations. The frequency of seedling mutations in the form of chlorophyll-deficient M_2 seedlings was employed to indicate the induction of point or gene mutations, a method originally proposed by Gaul (1961) and universally adopted by plant radiation workers. As shown in Table 2, the frequency of gene mutations due to radiation alone in species with low chromosome numbers was higher than the mutation rates in the species with high chromosome numbers. This is probably due to the protective or buffering effect of high chromosome numbers previously discussed above. Thus, the increase in mutation frequency after caffeine post-treatment was markedly higher in the species with high chromosome numbers than in the low chromosome group although their mutation rates were, similar.

To induce the same effect on the genetic material, a higher dose will likely be required in cells with high chromosome numbers than in cells with few chromosomes (Sparrow *et al.*, 1961). The proportion of lesions in the genetic material will most probably be lower in cells with more chromosomes than in cells with fewer chromosomes given the same radiation dose. When the repair process is interfered with, however, as when mutagen-treated seeds are post-soaked in a solution of caffeine, the number of unrepaired lesions in cells with more chromosomes could most probably equal the number occurring in cells with low chromosome num-

bers. If each "open" or unrepaired DNA lesion becomes "fixed" as a point mutation (Kihlman *et al.*, 1974; Ahnstrom, 1974), the increase in gene mutation frequency in the species with more chromosomes will be higher than in plants with fewer chromosomes.

Chromosome number is probably related to DNA content per cell and both have been closely correlated with radiosensitivity (Underbrink *et al.*, 1968) and radiation-induced mutation rates (Abrahamson, 1973). Thus the DNA C values for the species with 2N=22 and 2N=48 of 0.60 to 2.20 pg and 10.10 to 12.50 pg per nucleus, respectively, (Bennett *et al.*, 1982) could be a good index of the induced mutation frequency in the four species. The C value of the two other plants in the present work were not available. Now in barley seeds, an average of 0.052 break is reportedly formed per 10^6 daltons of DNA strand per radiation dose of one kilorad (Yamaguchi, 1979). The molecular weight of chromosomes in many higher plants are probably similar to those of human chromosomes of 32×10^9 to 160×10^9. If the quantity of possible DNA breaks is derived for each species and the percentage of seedling growth reduction is employed to estimate the relative quantity of unrepaired DNA lesions, a means of quantifying the protective effect of high chromosome number would have been found.

Summary and Conclusion

The role of chromosome number on the repair-inhibiting action of caffeine on DNA lesions was investigated using species with low and high chromosome numbers.

While the species with high chromosome numbers gave a marked increase in frequency of M_1 somatic mutations after caffeine post-treatment over the radiation control, the plants with low chromosome numbers did not respond similarly.

Likewise, the species with high chromosome numbers gave a marked increase in frequency of M_2 gene mutations after caffeine post-irradiation treatment over the radiation control while the low chromosome number species failed to exhibit a similar increase.

These results indicate the significant influence of chromosome number on the action of caffeine. The protective or buffering effect of high chromosome number against radiation damage more or less accounts for the low mutagenic response of plants with high chromosome numbers. The lower proportion of breaks in the genetic material in species with high chromosome number than in plants with low numbers of chromosomes probably explains the radio-tolerance observed in species with large numbers of chromosomes.

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Adoracion T. Arañez, Discussant

Changes produced by gamma rays on the DNA may be due to a direct effect in the form of molecular changes occurring in the molecule where the energy has been absorbed or indirect effects which are brought about by the chemical reactions of free radicals. Effects of ionizing radiation on the DNA of the cell is usually secondary, since most of the energy is deposited in the aqueous phase as cells contain much water. Highly reactive free radicals are formed in the radiolysis of water (Casarett, 1968).

The free radicals may convert the bases to a form with different base-pairing tendencies as the enol form of thymine and imino form of cytosine; cytosine may be converted to uracil (Lim-Sylianco, 1981). The free radicals may react with thymine producing a thymine free radical and two of such free radicals adjacent to each other may produce a dimer. The changes mentioned above may produce point mutations. Dimerization of thymine on two different strands produce interchain dimerization which may result in incomplete unwinding of the two strands during replication. As a result, the DNA strands produced are shorter and with deletions. Single strand breaks and double chain breaks are also produced by ionizing radiation (Casarett, 1968 and Lim-Sylianco, 1981) which may produce chromosomal aberrations.

The extent of charges produced by mutagens depends on the type and amount of DNA damage and the amount of repairs done on the affected DNA.

The potentiation of effects of gamma rays by post-irradiation caffeine treatment may be due to the effects of caffeine on the repair of DNA damage. The repair process affected is probably the post-replicative type rather than the excision repair, since plants lack or have very inefficient mechanisms for excision repair (Kihlman *et al.*, 1974).

The mechanism by which post-irradiation caffeine treatment may increase the mutation frequency may be similar to the action of caffeine on ultraviolet induced pyrimidine dimers given below.

Lehmann (1972) mentioned that there are gaps in daughter DNA strands when dimer-containing DNA is replicated semiconservatively. These gaps are usually filled up by post-replication repair. He presented evidence that in mouse, post-replication repair does not involve recombinational exchanges and that parental DNA does not seem to be involved in the gap-filling process. Recombinational models have been made to account for a similar gap-filling process observed in bacteria. According to Lehmann (1972) the gaps in the daughter strands opposite the pyrimidine dimers are filled in with newly synthesized DNA. It has been observed that caffeine potentiates cell killing and producing of chromosome aberrations if present during DNA synthesis period (Lehmann and Kirck-bell, 1974).

The process of filling up gaps in the daugher DNA strand may be inhibited by caffeine. Lehmann and Kirck-bell (1974) mentioned that methylated xanthines like

caffeine specifically inhibit the filling of the gaps in daughter DNA strands opposite UV-induced pyrimidine dimers in the parental strand.

There is an indication that caffeine binds most effectively to double-stranded DNA with short single-stranded regions (Kihlman, 1974). Although the inhibition produced by caffeine is reversible, the gaps in the daughter DNA strand are sealed slowly after caffeine treatment, gaps persist in the DNA for many hours and could act as focal points for production of chromosomal aberrations (Lehmann and Kirck-bell, 1974) and possibly of point mutations.

The frequency of M_1 somatic mutations and M_2 seedling mutations produced by gamma radiation treatment is more in sorghum and mungbean with chromosome number of 20 and 22 respectively as compared with peanut and tobacco with chromosome number of 40 and 48 respectively. However, in batches given post-irradiation caffeine treatment, the frequency of M_1 somatic and M_2 seedling mutations did not vary very much among the four plants studied. This is an indication that probably peanut and tobacco have better post-replication repair mechanism than sorghum and mungbean and that the post-irradiation caffeine treatment inhibited this repair mechanism. It is interesting to note that peanut and tobacco are polyploids.

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