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N-Methyl-N'-Nitro-N-Nitrosoguanidine Induced Dominant and Recessive Mutations in Zea Mays

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N-METHYL-N'-NITRO-N-NITROSOGUANIDINE INDUCED DOMINANT AND RECESSIVE MUTATIONS IN *Zea mays*

by

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INTRODUCTION

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is a drastic mutagen in bacteria, fungi, and higher organisms (4,8,16,21,26,27,37,46,54,68). The purpose of this study was to determine if this agent was also mutagenic in Zea mays.

Pollen and seeds of a stock homozygous for three closely linked dominant genes were treated with MNNG and crossed to a tester stock homozygous for the corresponding recessive genes. A sample of F_1 plants from this cross were grown and observed for dominant mutations and were selfed. The resulting F_2 seedlings were observed for recessive seedling mutations. The mutagenicity was measured in terms of descrete genetic changes and chromosome aberrations.

Following pollen treatment, it was found that MNNG produced primarily chromosome aberrations and few single gene mutations (45). Following seed treatment, no mutagenic effects were observed.

LITERATURE REVIEW

Since its introduction as a bacterial mutagen (39) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has been shown to be a powerful mutagen for many different organisms (4,8,16,21,26,37,46,54,68). Much is known about the interaction between MNNG and biologically active macromolecules yet the exact mechanism by which MNNG mutates genetic material is unknown. Evidence suggest that MNNG may act through more than one mutagenic mechanism.

Reactions of MNNG with Proteins and Nucleic Acids

N-methyl-N'-nitro-N-nitrosoguanidine (CH_3-N-C-N-NO_2)

N N H

O H

reacts chemically with proteins and nucleic acids. MNNG reacts with the amino or alkyl amino moieties of proteins to form products containing nitroguanidine (-C-N-NO_2) groups and to release methyl

N H

H

nitrosamino (CH_3-N-NO) groups (42,44). The methyl nitrosamino groups act, directly or indirectly, on amino containing compounds to form methylated products (24).

When MNNG reacts with either DNA or RNA *in vitro* or *in vivo*, its primary effect is the methylation of nitrogenous bases (14,34,54). In reactions with either nucleic acid the major product is 7-methyl guanine. Small amounts of adenine and cytosine are also methylated yielding 3-methyl adenine and 3-methyl cytosine. Small amounts of guanidino containing bases are also formed when MNNG reacts with

nucleic acids (41).

MNNG is chemically stable at pH 5.0 but at more acidic or alkaline conditions it decomposes to nitrous acid and diazomethane (44). Nitrous acid (HNO_2) and diazomethane ($H_2C^- - N^+ \equiv N$) are chemically reactive with nucleic acid bases. Nitrous acid deaminates the amino bases adenine, guanine, and cytosine forming hypoxanthane, xanthane, and uracil, respectively (51). Reactions of diazomethane with DNA yield 7-methyl guanine and 3-methyl adenine plus some phosphortriester linkages (29). Diazomethane can methylate directly by formation of diazocarbonium ions ($CH_3 - N^+ \equiv N$) or indirectly through the release of a carbonium ion (CH_3^+) (18).

Mutation through Methylation

Methylation of nitrogen in nucleic acids is thought to cause base pair errors during DNA replication, resulting in transition mutation (33). MNNG has been reported to induce GC → AT and AT → GC transitions in bacteriophage and bacteria (5,16).

MNNG methylation of guanine and adenine can also result in slow depurination of DNA (36). If a methylated base is removed from the DNA, any base can be incorporated opposite the gap during the next round of DNA replication. Such events can lead to transversion type mutations (20).

If the methylated nucleotide is removed, a base pair deletion may result (20). Depurination by MNNG could be responsible for the deletion of guanine-cytosine base pairs which revert plus frameshift

mutations in Salmonella (28,49,65,66).

Depurination may also cause single-strand breaks in the Escherichia coli chromosome. Methylation of the phosphate moiety of the DNA backbone, resulting in liable phosphate triesters could also be responsible for these single-strand breaks (50).

Since, in the presence of cellular levels of thiol MNNG can methylate both the N-7 and O-6 positions of guanine; MNNG mutagenesis maybe also due to methylation of nucleic acid bases at other than the nitrogen sites (34).

Factors Influencing Methylation Type Mutagenicity

If methylation of the nitrogenous bases is a major mechanism for MNNG mutagenicity, the question arises whether MNNG or one of its decomposition products or both, methylates the nucleic acid bases. Many factors influence the mutagenicity and decomposition of MNNG.

Composition of solvent

Decomposition and mutagenic activity of MNNG varies with the composition of the solvent or treatment media. Beach and Dean (6) treated Aerobacter (Klebsiella) aerogenes with MNNG in a variety of simple media (saline, phosphate buffer, salt solution, tris-maleic (TM) buffer, TM-buffer plus tryptophane, and TM-buffer plus glutamic acid) at pH 6.0. Mutagenicity was greatest in TM-buffer. Subsequent work with Streptomyces coelicolor has shown the rate of MNNG degradation, probably to diazomethane, is higher in TM-buffer than in phosphate buffer (15). These observations suggest that the muta-

genicity of MNNG increases in a solvent that increases its decomposition to diazomethane.

The presence of thiol at or about the concentration found in mammalian cells enhances the in vitro methylation of DNA by MNNG at pH 7.0 (34). In cultured mammalian cells methylation of nucleic acids by MNNG was increased with increased thiol content of the cells and as methylation increased the thiol content of the cells decreased. MNNG is known to react with certain thiols (i.e., cysteine) to yield diazomethane (41). It remains to be shown, however, if the in vitro MNNG-thiol reaction that produces diazomethane is also an in vivo mutagenic reaction.

Concentration of solvent

Concentration of the solvent or treatment media also influences the decomposition and mutagenicity of MNNG. The initial rate of MNNG decomposition to diazomethane and the initial rate of mutation both increase with increased molarity of solvent (15).

Concentration of MNNG

Mutagenicity and lethality increase proportionally with MNNG concentration until a mutagenic optimum is reached (1,15). This increase occurred over a range from pH 6.0 to pH 9.0 (15) and could be attributed either to increased MNNG concentration or to increased diazomethane concentration. In many organisms, MNNG is highly mutagenic at concentrations which cause less than 50 percent lethality in the treated material (1,16,69).

Temperature

The alkaline decomposition of MNNG increases as temperature increases. During long periods of treatment at different temperatures the initial rate of mutagenesis increases directly with temperature, but the maximum rate of mutation is observed at lower temperatures where MNNG decomposition is slower. This indicates that maximum mutagenesis maybe dependent on an optimal diazomethane concentration (15). In Triticale and Vica faba increased temperatures increased both the lethal and chromosome breaking effect of MNNG (26).

Visible light

MNNG has been observed to decompose to nitrous acid after irradiation with visible light. This decomposition was accompanied by a proportional decrease in mutagenicity in Arabidopsis seeds (63).

Conformation of nucleic acids

The reaction of MNNG with nucleic acids appears to be dependent on the conformation of the nucleic acid at the time of treatment. Treatment of TMV-RNA in aqueous solutions favors base stacking within the nucleic acid and results in methylation of nitrogenous bases. Treatment in the dispersing agent formamide results in spreading out of the RNA with no methylation of bases (52,53,54).

MNNG acts on replicating DNA in phage and bacteria with the majority of mutations occurring at the replication point (5,7,13). During replication, the DNA double helix is open at the replication point and this conformation may enhance the susceptibility of the

nucleic acid to mutagenesis by MNNG (13).

The observation that MNNG can activate and mutate intercellular, but not free coliphage T2 (68) also supports the hypothesis that MNNG mutagenesis maybe dependent on nucleic acid conformation.

Hydrogen ion concentration

Adelberg, et al. (1) reported that under fixed conditions of concentration and time the optimum pH value for the induction of mutations in E. coli was pH 6.0. Maximum MNNG mutagenicity at pH values around 6.0 has also been observed in other organisms (23,26, 54,60,64). Cerda-Olmedo and Hanawalt (12) and Delic et al. (15), however, have obtained maximum mutagenesis in bacteria at pH values above 7.5 with very little mutagenicity between pH 5.0 and 6.5. Since numerous factors effect the decomposition and mutagenesis of MNNG it could be expected that under different treatment conditions different pH optimums could be observed.

Under treatment conditions favoring the alkaline decomposition of MNNG in solution, mutagenicity through methylation of nitrogenous bases appears to be primarily dependent on the concentration of a decomposition product, diazomethane. Under treatment conditions where MNNG is relatively stable in solution, it is possible that MNNG is taken up by treated cells unaltered. Within a cell, MNNG may react with amino containing compounds (proteins and/or nucleic acid bases) to form guanidino-compounds and methyl nitrosamine. The methyl nitrosamine could then react with DNA to methylate bases (14). It is also possible that under conditions favoring the decomposition

of MNNG to diazomethane in the treatment solution, both diazomethane and MNNG enter the cells of the treated material. In this case, both diazomethane and the MNNG intercellular decomposition product, methyl nitrosamine, could be responsible for DNA methylation.

Mutation through Deamination

MNNG may also induce mutations through deamination of certain bases. Deamination of adenine, for example, can result in base pairing errors during DNA replication and lead to the transition mutation AT → GC (51). Deamination maybe a mechanism in the MNNG induced mutagenesis of TMV-RNA. In this case, there exists an anti-correlation between methylation of nitrogenous bases and mutagenicity. However, prolonged MNNG treatment does result in some deamination of adenine and guanine (54,55,56). Deamination may also be responsible for the mutagenicity observed when bacteria and virus are treated with MNNG under acidic conditions (12,15).

If deamination is a mechanism of MNNG mutagenic activity it is not known whether MNNG or a decomposition product (i.e., nitrous acid) is the deaminating agent.

Chromosome Aberrations

MNNG induces aberrations in many types of organisms. In bacteria, MNNG treatment induces single-strand breaks, base pair deletions and possibly multi-base deletions (31). Treatment of V. faba roots and Triticale seeds induced visible chromosome aberrations in the plant cells (23,26). Chromosome aberrations as indicated by the production of sterile pollen in the mature plants,

also occurs when seeds of Arabidopsis thaliana are treated (22). Treatment of maize pollen may also cause chromosome aberrations (46). Injection of MNNG into young Drosophila males can result in the deletion of gene markers, the loss of the entire Y-chromosome and translocations between chromosomes in meiotic and post-meiotic cells (8). MNNG also produced chromosome exchanges in cultured mammalian cells (25).

Other Effects

In addition to its mutagenic effects MNNG is also toxic or at least detrimental to treated material. As with mutagenicity, the exact mechanism of lethality or detriment is not shown and it is possible that they occur through a number of mechanisms.

MNNG has been shown to inhibit cell growth by inhibiting DNA, RNA, and protein synthesis (2,3,30,35). Synthesis of nucleic acids is inhibited immediately after treatment. Since MNNG inhibits DNA polymerase activity in a dose dependent manner (2,3), inhibition of nucleic acid synthesis maybe due to inactivation of nucleic acid polymerases.

MNNG induced the production of some non-functional protein, indicating that it may interact with ribosomes to cause misreading of the DNA code and perhaps complete inhibition of protein synthesis (11).

It is also possible that MNNG may interact with enzymes directly related to DNA replication to cause base pairing errors (11). This maybe another reason why MNNG acts preferentially at the replication

point of bacteria and phage (5,7,13).

MNNG Dark Repair Mechanism

A dark Repair mechanism which repairs DNA damage caused by MNNG has been reported for a number of organisms (11,27,50,57). Resistance and sensitivity to MNNG seems correlated with resistance or sensitivity to ultra-violet light (38,40) and/or x-rays (17). Thus, the repair mechanism which acts on MNNG damage maybe similar to the mechanisms which repair ultra-violet or x-ray damage.

MATERIALS AND METHODS

The Genetic System

The genetic system used in these experiments consisted of a stock carrying the dominant $\underline{A^b}$ - $\underline{Sh_2}$, $\underline{Dt}$ genes (multiple dominant stock) and a stock carrying the genes $\underline{a^m}$ - $\underline{Sh_2}$, $\underline{dt}$ (multiple recessive stock). The $\underline{A^b}$ locus consists of two genes, $\underline{\alpha}$ and $\underline{\beta}$ (32). The genes $\underline{\alpha}$, $\underline{\beta}$, and $\underline{Sh_2}$ are located on the long arm of chromosome three and cover a map distance of less than 0.25 map units (48).

All three genes, $\underline{\alpha}$, $\underline{\beta}$, and $\underline{Sh_2}$, effect the endosperm, producing pale aleurones, purple aleurones, and non-shrunken endosperms; respectively (45). The multiple dominant stock produces purple non-shrunken endosperms and red tipped seedlings and leaves. The multiple recessive stock produces colorless shrunken endosperms and green tipped seedlings.

Crosses between the multiple dominant (as pollen parent) and the multiple recessive (as egg parent) stocks were made. The spontaneous or induced mutation of the middle gene $\underline{\beta}$ produces pale purple (dilute) endosperms and green tipped seedlings in the F_1. The mutation of $\underline{\beta}$ is due to either a single base change or to a multi-base change. These multiple base changes cannot extend beyond the $\underline{\alpha}$ locus proximally and the $\underline{Sh_2}$ locus distally. Therefore, the mutation of $\underline{\beta}$ indicates either a single base change or a small deletion of less than 0.25 map units. These two possibilities cannot be distinguished in this material at this time. The mutation of $\underline{\alpha}$ cannot be determined

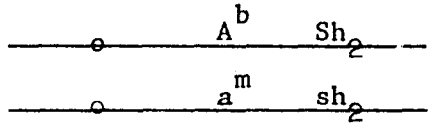
since the $\underline{\beta}$ component alone can produce and anthocyanin in the endosperm that is indistinguishable from the anthocyanin produced by $\underline{\alpha}$ and $\underline{\beta}$ together. The simultaneous loss of $\underline{\alpha}$ and $\underline{\beta}$ will result in a colorless aleurone and green seedlings. The mutation of $\underline{Sh_2}$ is indicated by shrunken endosperms and may result from either single base changes within the $\underline{Sh_2}$ gene or from multi-base changes that extend beyond this gene proximally to $\underline{\beta}$ and distally to the end of the chromosome. Thus, mutations of $\underline{Sh_2}$ to $\underline{sh_2}$ are due either to point mutations of chromosome aberrations. Due to the close association of the three genes, simultaneous mutations of $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$ are taken to indicate the occurrence of chromosome aberrations. The various phenotypes that may result from mutations related to $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$ are summarized in Table I.

A special feature of this genetic system is the presence of the $\underline{a^m}$ allele in the multiple recessive stock and $\underline{Dt}$ in the multiple dominant stock. The alleles $\underline{a^m}$ and $\underline{Dt}$ were included to provide easy recognition of the loss of $\underline{\beta}$ activity. In the presence of $\underline{Dt}$ the $\underline{a^m}$ allele undergoes forward mutation to its dominant allele $\underline{A}$, giving purple anthocyanin producing sectors (dots) in the aleurone. When $\underline{c}$, $\underline{a^m}$, and $\underline{Dt}$ are present together the forward mutation of $\underline{a^m}$ cannot be detected. When $\underline{\beta}$ is mutated the purple dots resulting from $\underline{a^m}$ mutations are visible on the dilute endosperm background. These alleles also help to distinguish colorless shrunken endosperms due to self-contamination of the multiple recessive stock, from colorless shrunken endosperms produced by loss of $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$ activity.

TABLE I

NORMAL AND MUTANT GENEOTYPES AND PHENOTYPES OF THE $\underline{A^b}$ - $\underline{Sh_2}$ REGION

F_1 Chromosome Composition:



Mutational change	Putative genotype	Endosperm phenotype	Seedling phenotype
No mutation	$\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$	Purple, normal	Red tipped
Mutation of $\underline{\alpha}$	(_) - $\underline{\beta}$ - $\underline{Sh_2}$	Purple, normal	Red tipped
Mutation of $\underline{\beta}$	$\underline{\alpha}$ - (_) - $\underline{Sh_2}$	Dilute, normal	Green
Mutation of $\underline{Sh_2}$	$\underline{\alpha}$ - $\underline{\beta}$ - (_)	Purple, shrunken	Red tipped
Mutation of $\underline{\alpha}$ and $\underline{\beta}$	(_) - (_) - $\underline{Sh_2}$	Colorless, normal	Green
Mutation of $\underline{\beta}$ and $\underline{Sh_2}$	$\underline{\alpha}$ - (_) - (_)	Dilute, shrunken	Green
Mutation of $\underline{\alpha}$ and $\underline{Sh_2}$	(_) - $\underline{\beta}$ - (_)	Purple, shrunken	Red tipped
Mutation of $\underline{\alpha}$, $\underline{\beta}$, and $\underline{Sh_2}$	(_) - (_) - (_)	Colorless, shrunken	Green

Fertilization of $\underline{a}^{m}$ - $\underline{sh_2}$,$\underline{dt}$ eggs by $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$, $\underline{Dt}$ pollen lacking $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$ activity produces colorless shrunken endosperms with purple dots. Self-contamination of the $\underline{a}^{m}$ - $\underline{sh_2}$ - $\underline{dt}$ stock produces colorless shrunken endosperms without dots.

Methods of Mutagenic Treatment

Seed Treatment

$\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$, $\underline{Dt}$ seeds were pregerminated in water for 24 hours and treated by a 24 hour immersion in phosphate buffer solutions (pH 6.0) containing various concentrations (0.0000, 0.0060, 0.0025, and 0.0010 M) of N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Inc.). At the end of the treatment, excess MNNG was removed from the seeds by a 45 minute rinse in running water. During pregermination, treatment and rinsing the temperature was maintained at 29° C. After rinsing, the seeds were planted in flats and observed for emergence. The seedlings were later transplanted to the field and observed for abnormal phenotypes. Mature plants were crossed on and by the $\underline{a}^{m}$ - $\underline{sh_2}$,$\underline{dt}$ tester stock and the resulting seeds were scored for endosperm mutations of $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$.

Pollen Treatment

Pollen of the $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$,$\underline{Dt}$ stock were treated by immersion in the supernatent of a 0.04 percent MNNG-mineral oil suspension (10). Mineral oil was used because pollen can survive in mineral oil while they don't survive well in any other known buffer or salt solution.

Both, the chemically treated and the control pollen were used to fertilize ears of the multiple recessive tester. Pollen was collected into vials which contained either MNNG-mineral oil supernatant or mineral oil, by use of a funnel and a cheese cloth sieve. A small brush was used to transfer the pollen suspension to the silks of the female parent. No attempt was made to shade the MNNG suspension. The resulting F_1 seeds were handled as shown below.

Scoring Mutants in the F_1 and F_2 Generations

F_1 seeds from both pollen and seed treatments were visually examined for endosperm mutations involving the dominant genes $\underline{\alpha}$ - $\underline{Sh_2}$. The endosperm mutants were either whole endosperm mutations or fractional endosperm mutations. Whole endosperm mutations involved the entire endosperm and arose from mutational events that occurred prior to the first cleavage division of the endosperm. Fractional endosperm mutations involved only part of the endosperm and resulted from mutational events that occurred during the first or later cleavage division of the endosperm. Twin fractional endosperm mutations containing two or more mutant sectors in the same endosperm were also observed.

A sample of the MNNG pollen treated F_1 and control F_1 seeds were planted in sand flats and observed for the occurrence of seedlings mutants. Loss of $\underline{\beta}$ or $\underline{\alpha}$ - $\underline{\beta}$ in the F_1 embryos prevents production of anthocyanin. These losses are indicated by green tipped seedlings. MNNG pollen treated F_1 and control F_1 seedlings were measured for

linear growth over a seven week period. The same sample of seedlings were transplanted to the field for further morphologic examination. At anthesis pollen were examined with a hand microscope Normal pollen have a round yellowish appearance; defective pollen have a clear appearance and become shriveled within a short time in the anthers (9). Plants producing more than 10 to 15 percent defective pollen were scored as semi-sterile. F_1 plants were selfed to produce the F_2 generation. The F_2 seeds were scored for endosperm mutants. A sample of these seeds were planted and scored for germination and seedlings mutations.

Statistical Analysis

The number of mutant endosperm or seedlings (n) out of a total population of endosperm or seedlings (N) were compared in treated and untreated material. The statistical significance was determined assuming that mutational events follow a Poison rather than a normal distribution. The analyses were done in accordance with the table and reasoning presented by Stevens (58). Values of $P < 0.01$ were considered as highly significant, between 0.01 and 0.05 as significant, 0.05 and 0.10 as near significant, and > 0.10 not significant.

RESULTS

Results of Pollen Treatment

F_1 endosperm mutations

F_1 seeds from the cross of $\underline{a}^m$ - $\underline{sh}_2$ egg parents with treated and control pollen from $\underline{\alpha}$ - $\underline{8}$ - $\underline{Sh}_2$ plants were scored for endosperm phenotypes (see materials and methods for various phenotypes). Mutation frequencies per 10^4 endosperms were calculated. MNNG significantly increased the occurrence of all but one observed mutant type, the whole seed $\underline{8}$ loss. The majority of mutational events involved the simultaneous mutation of two or more genes and occurred as fractional endosperm mutations rather than whole endosperm mutations (Table II).

Three types of twin sectored fractional endosperm mutants were observed: 1) forty-five containing identical mutant sectors: 2) two containing two different types of mutant sectors: and 3) one containing three different types of mutant sectors.

Germination and growth of the F_1

A sample of F_1 seeds that were observed for endosperm mutations (above) were planted in sand flats to determine percent of germination. Of 1033 control seeds planted, 87.5% germinated and of 992 MNNG F_1 seeds, 39.1% germinated. MNNG F_1 seeds germinate less than half as well as control F_1 seeds.

Another sample of F_1 seeds that have been tested for endo-

TABLE II

ENDOSPERM MUTATIONS PER 10^4 POLLEN FOR THREE CLOSELY LINKED GENES ($\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$)

Treatment	Population	Whole Seed Cases			Fractional Seed Cases		
		2-3 gene loss	$\underline{Sh_2}$ loss	$\underline{\beta}$ loss	2-3 gene loss	$\underline{Sh_2}$ loss	$\underline{\beta}$ loss
MNNG	8,140	20*	5*	2	519*	60*	4*
Control	25,759	1	0	5	54	0	0

(* $P < 0.01$)

sperm mutations and percent germination were planted in soil containing flats to observe seedling growth (seedling growth was measured as height from the soil level to the top of the longest leaf). A portion of the seedlings in the flats were transferred into pots where they were further measured for growth. A comparison of MNNG F_1 and control F_1 seedling growth over a seven week period (Fig. 1), indicated that MNNG pollen treatment resulted in lower growth of MNNG F_1 seedlings.

The occurrence of phenotypic mutants among F_1 seedlings

A sample of F_1 seedlings including both MNNG F_1 and control F_1 material was observed in the greenhouse and field for the occurrence of mutant seedlings (Table III). The occurrence of mutant seedlings was significantly increased in the MNNG F_1 material. A variety of seedling mutants were observed. Among the whole seedling mutants (mutants of the entire seedling) these phenotypes were found: 1) green seedlings; 2) yellow-green seedlings; and 3) dwarf seedlings. Among the sectored mutant seedlings (the mutant phenotype covers only parts of the seedling) yellow-green sectored, necrotic sectored and a yellow-green seedling with green stripes were observed.

Mutant and non-mutant seedling from both MNNG and control F_1 were grown to maturity (Table III). Of 17 plants grown from MNNG F_1 mutant seedlings, 53% exhibited reduced height. Reduced plant height was exhibited by green and yellow-green whole seedling mutants and by the yellow-green seedling with green stripes. Of 18 mature

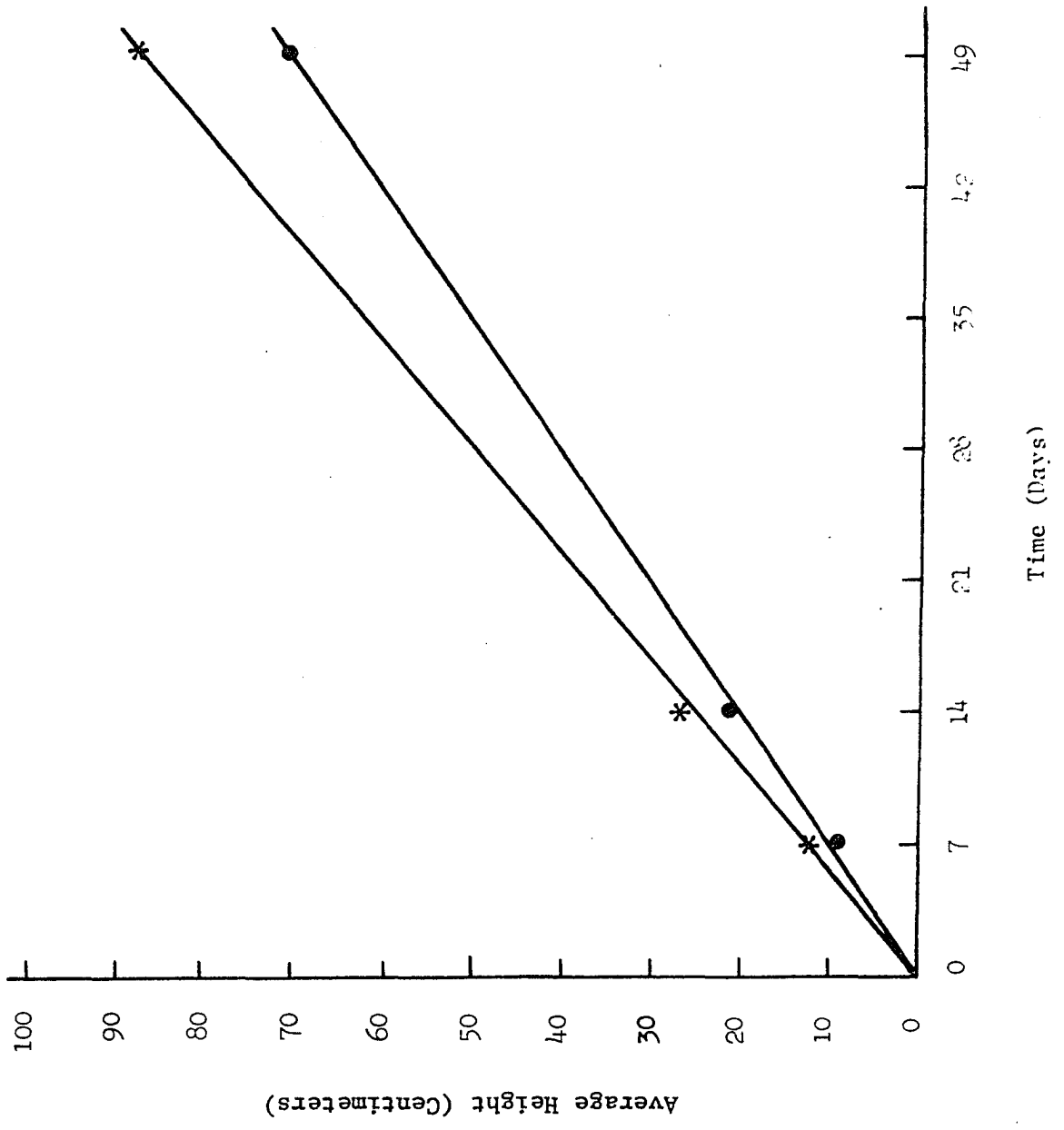


Figure 1. Growth Rates of F_1 Seedlings Resulting from Pollen Treatment.

· , Growth rate of MNNG F_1; * , Growth rate of control F_1. The MNNG F_1 7 day point is the average of 66 measurements; the 14 day point is the average of 68 measurements; the 49 day point is the average of 18 measurements. The control F_1 7 day point is the average of 197 measurements; the 14 day point is the average of 211 measurements; and the 49 day point is the average of 19 measurements.

TABLE III

SEEDLING AND PLANT MUTATIONS IN THE F_1 RESULTING FROM TREATED POLLEN

Treatment	Seedlings		Mature plants			
	Population	No. mutant seedlings	Population	No. plants with reduced height	No. plants exhibiting pollen sterility	No. plants with both pollen sterility and reduced height
MNNG	328	22*	17 mutant plants**	9*	11*	7*
			18 non-mutant plants	1	3*	0
Control	698	8	8 mutant plants**	0	0	0
			46 non-mutant plants	0	0	0

* $P < 0.01$

** Plants grown from mutant seedlings

plants from MNNG F_1 non-mutant seedlings, 6% exhibited reduced height. No reduced plant height was noted in any of the control material.

Pollen sterility in the F_1 plants

A sample of mature MNNG F_1 plants and control plants from $\underline{A}^{b}$ - $\underline{Sh}_2$, $\underline{Dt}$ seeds were observed for pollen sterility, 42.0% were found to be semi-sterile and 4.5% were found to be completely sterile.

Of the 17 mature plants grown from MNNG F_1 mutant seedlings (previous section), 65% exhibited pollen sterility. All types of seedling mutants, with the exception of necrotic sectored mutants, exhibited pollen semi-sterility. In 7 of these, pollen sterility was accompanied by reduced plant height. Of the 18 plants grown from MNNG F_1 non-mutant seedlings, 17% were semi-sterile. Of 167 control plants observed, none were found to exhibit pollen sterility.

Transmissibility of mutants to the F_2

The F_2 generation resulting from selfing of the F_1 plants were scored for endosperm and seedling mutants. Pollen treatment did not induce endosperm mutants in this material. Pollen treatment did significantly increase the occurrence of seedling mutants (Table IV). These mutant phenotypes were observed in the F_2 generation: 1) yellow-green seedlings: 2) yellow-green mutable seedlings: 3) glossy seedlings and 4) albino seedlings.

Results of Seed Treatment

Both treated and control seeds were planted in flats containing soil and observed for percent germination. Germination decreased with increased MNNG concentration (Table V). The seedlings were transplanted to the field and further observed. The growth of the treated seedlings were inversely proportional to the MNNG concentration. Except for this decreased growth rate, the MNNG group appeared normal. Plants from treated $\underline{A}^{b}$ - $\underline{Sh}_2$, $\underline{Dt}$ seeds were crossed with the multiple recessive tester, $\underline{a}^{m}$ - $\underline{sh}_2$, $\underline{dt}$. Seed set of treated plants was inversely proportional to MNNG concentration. When the treated plants were used as male parents, 29,794 seeds were produced with no increase in mutant endosperms for $\underline{\alpha}$ - $\underline{B}$ - $\underline{Sh}_2$. When the treated plants were used as female parents 4,403 seeds were produced with no increase in mutant endosperms (Table VI).

TABLE IV

SEEDLING MUTATIONS IN THE F_2 RESULTING FROM TREATED POLLEN

Treatment	No. F_1 plants tested for mutation transmission	No. F_1 plants transmitting mutations to F_2 seedlings	P
MNNG	41	18	0.01
Control	44	3	

TABLE V

EFFECT OF CONCENTRATION OF GERMINATION, PLANT HEIGHT, AND SEED PRODUCTION FOLLOWING SEED TREATMENT WITH MNNG

Molar Concentration	% Germination*	Height			Seed Production	
		No. plants observed	% of control (day 19)	% of control (day 26)	% of plants producing 1 ear	Ave. No. kernels per ear
0.0060	38.0	14	66.6	65.0	36.0	109
0.0025	57.0	21	73.4	75.0	43.0	152
0.0012	89.0	33	100.0	95.0	42.0	155
0.0000	84.0	31	100.0	100.0	55.0	164

* 37 seeds were treated per concentration

TABLE VI

ENDOSPERM MUTATIONS 10^4 SEEDS FOR THREE CLOSELY LINKED GENES (α - β - Sh_2) FOLLOWING SEED TREATMENT

Treatment	Population	Whole Seed Cases			Fractional Seed Cases		
		2-3 gene loss	Sh_2 loss	β loss	2-3 gene loss	Sh_2 loss	β loss
Plants used as males							
MNNG	29,794	1	0	3	50	4*	0
Control	25,759	1	0	3	54	0	0
Plants used as females							
MNNG	4,403	0	2	11	9	20*	0
Control	6,825	0	0	9	22	4	0

* $P < 0.01$; The endosperm phenotype reduced by mutations of Sh_2 is sometimes difficult to distinguish from that produced by fungal infection. This increase is attributed to experimental error.

DISCUSSION

Pollen Treatment

Endosperm mutations

MNNG induced primarily fractional endosperm mutations. A few whole endosperm mutations were also induced. Most mutations in both the whole or the fractional endosperm cases resulted from chromosome aberrations.

A significant increase of *a* mutations were observed among the fractional endosperm mutations. *a* loss is due to either single-base changes or small deletions of less than 0.25 map units that include the *a* locus. These two mutational events cannot be distinguished in this material.

When the endosperm mutations induced by MNNG are compared to those induced by ethyl methanesulfonate (EMS), ultra-violet (UV), and x-rays; MNNG exhibits characteristics of all three mutagens. It resembles x-rays by inducing mostly chromosome aberrations and resembles EMS and UV by inducing mostly fractional endosperm mutations (19,47,59). It also resembles EMS and UV by inducing mutations of *a*. All three mutagens, MNNG, EMS, and UV induced different types of *a* mutations (19,47). MNNG induced only fractional *a* loss. EMS induced only fractional *a* loss. EMS induced only whole endosperm *a* loss. UV induced both whole and fractional endosperm *a* loss.

The occurrence of twin sectored fractional endosperm mutations suggests that MNNG induced the initiation of breakage-fusion-bridge

cycle (BFBC) events (43). Chromosome breaks occurring in the pollen may lead to fusion of the sister chromatids after DNA replication. This produces a dicentric chromosome. During mitotic cell division within the endosperm a portion of the dicentric chromosome maybe lost to one of the daughter cells. This can lead to the production of twin sectored fractional endosperm mutations containing two or more identical mutant sectors. This can also cause twin sectored fractional endosperm mutations containing two or more different mutant sectors.

One twin sectored fractional endosperm mutant was of particular interest. This seed had three different mutant sectors. The largest portion of the endosperm was deficient for $\underline{\beta}$. One eighth of the endosperm was deficient for both $\underline{\alpha}$ and $\underline{\beta}$. Two smaller sectors were lacking $\underline{\alpha}$, $\underline{\beta}$, and $\underline{Sh_2}$. The probable sequence of mutational events during development was 1) loss of $\underline{Sh_2}$ activity, 2) loss of $\underline{\beta}$ activity, and 3) loss of $\underline{\alpha}$ activity.

This developmental pattern could be due to BFBC. Here, in addition to the induced or spontaneous initial loss of $\underline{\beta}$, the $\underline{\alpha}$, - $\underline{Sh_2}$ region must be inverted. The broken end distal to the inverted $\underline{\alpha}$ remained unfused until after DNA replication at which time it fused with its sister chromatid.

A second possible mechanism is based on the induced loss of $\underline{\beta}$. The deletion of the $\underline{\beta}$ locus involved both strands of the pollen DNA double helix. The deletion of $\underline{\beta}$ was accompanied by the partial deletion of the $\underline{\alpha}$ locus in one strand of the double helix. Rejoining between the $\underline{\alpha}$ and $\underline{Sh_2}$ portions occurs in both strands. In the strand

containing a partial $\underline{\alpha}$ locus the two portions are improperly rejoined. One strand of the original double helix gives rise to a dilute cell line. The second strand, containing the partial $\underline{\alpha}$ locus, produces a colorless cell line. Due to improper rejoining of the two portions of this second strand, the colorless cell line eventually loses the $\underline{Sh}_2$ gene.

A third possible mechanism is also based on the induced loss of $\underline{\beta}$. Deletion of the $\underline{\beta}$ locus followed by the rejoining between $\underline{\alpha}$ and $\underline{Sh}_2$ brought the two genes closer together. This change in position may have sequentially inactivated $\underline{\alpha}$ and then $\underline{Sh}_2$.

Dominant mutations

MNNG induced some dominant mutagenic efforts among the MNNG F_1 embryos and seedlings. The difference in embryo germination between MNNG F_1 and control F_1 seeds indicates the presence of dominant lethal mutations in the genome of many MNNG F_1 embryos. Dominant lethal mutations may result from dominant single gene mutations or from deficiencies induced in the treated pollen (other types of chromosomal aberrations are not known to exert such effects in the heterozygous condition). A pollen containing defective sperm nuclei, but a normal tube nucleus can accomplish normal fertilization. Thus deficiencies can be passed to the embryo (9).

Seedlings resulting from MNNG treated pollen grew slower than seedlings resulting from untreated pollen. This retardation in growth maybe due to dominant effects resulting from changes that are similar to those that cause lethality (above), but which are less extreme in their effect.

In addition to the above effects MNNG treatment increased significantly the frequency of dominant F_1 seedling phenotypes. One MNNG whole seedling mutant grew into a dwarf plant. This plant was about two feet tall (as compared to untreated siblings that were six feet tall) and had a bushy grass-like appearance. This mutant plant was completely pollen sterile. When fertilized with pollen from a normal plant, it produced a small ear that was covered with anthers and had a very low seed set.

MNNG increased the frequency of yellow-green seedling mutations. Yellow-green seedlings maybe due to either chromosome aberrations or point mutations. Since most of these yellow-green seedlings developed semi-sterile tassels they were probably due to chromosome aberrations (see next section).

MNNG also induced yellow-green sectored seedlings, necrotic sectored seedlings, and a yellow-green seedling with green stripes. Yellow-green and necrotic sectored seedlings result from mutations which occurred after the first cleavage division of the embryo. The yellow-green seedling with green stripes exhibited slow growth and matured into a small sterile plant. This plant may have originated as a unstable dominant yellow-green mutation. During development of the embryo, the yellow-green allele of a few cells may have reverted, giving rise to a normal green cell line. Such a developmental pattern would be most likely to occur if the yellow-green character were due to a point mutation, rather than a chromosome aberration. Since this plant was also small and sterile it is possible that chromosome aberrations were also introduced into the embryo genome.

Green seedlings (Table I) were induced at a significantly increased frequency. Green seedlings can be due to either loss of $\underline{B}$, of $\underline{A}$ and $\underline{B}$, or dominant mutations that suppress the anthocyanin producing genes.

Pollen sterility

When pollen that carry chromosome aberrations are crossed with normal eggs, the resultant embryos are heterozygous for the aberrations. During development of the embryo and the resulting plant, these aberrations are reproduced mitotically. During the first meiosis following treatment, translocation, inversion and deletion heterozygotes will produce some aberrant gametes. These aberrant gametes develop into non-viable pollen and ovules (9). Duplications cause less deleterious effects when in the heterozygous condition.

When both viable and non-viable pollen are present in the same anther, the pollen is called semi-sterile. In general, semi-sterility is regarded as evidence of chromosome aberrations in the treated material (9,61). Following MNNG pollen treatment, 42.0% of the F_1 plants were semi-sterile. This is a further indication that MNNG induces chromosome aberrations in maize.

Recessive mutations

Yellow-green, glossy and albino whole seedling mutants were observed in the MNNG F_2 generation. These seedlings were homozygous for recessive mutations induced in the MNNG treated pollen. Since these mutations survived the meiotic gametophyte screen in both the

tassels and ears; they were probably the result of minute chromosome aberrations or point mutations rather than gross chromosome aberrations (9).

Some yellow-green mutable seedlings were also observed in the F_2. These were yellow-green seedlings which had several green sectors per leaf. In these plants, the yellow-green mutant allele was unstable. During development of the embryo or seedling, the mutable allele of some cells reverted to the normal green allele.

Seed Treatment

Following pollen treatment about 6.0% of the resulting endosperms were mutant for one or more genes in the $\underline{\alpha}$ - $\underline{\beta}$ - $\underline{Sh_2}$ region. When seeds were treated no endosperm mutations were observed. However, plants which developed from treated seeds were affected by the chemical treatment. Both plant height and seed set were inversely proportional to MNNG concentration. It is possible that MNNG seed treatment caused mutations which were not detected. Mutant embryo cells may have been out grown by normal cells or extensively damaged chromosomes may have been screened out during meiosis. In either case, mutations would probably not have been incorporated in the tassels or ears. It is also possible that maize seeds can in some way alter MNNG so that the resulting products are toxic but not mutagenic.

CONCLUSIONS

1. MNNG pollen treatment is mutagenic in maize.

2. MNNG pollen treatment induces primarily chromosome aberrations.

3. MNNG pollen treatment induces fractional endosperm mutations in the F_1.

4. MNNG pollen treatment induces dominant mutations of the F_1.

5. MNNG pollen treatment induces transmissible mutations.

6. MNNG pollen treatment induces stable mutations.

7. MNNG is not mutagenic following seed treatment.

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