MUTAGENESIS IN LOTUS CORNICULATUS L.

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Montreal

August 1981

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ABSTRACT

Ph.D. MARIO C. THERRIEN Biology

MUTAGENESIS IN *LOTUS CORNICULATUS* L.

The cytological and mutagenic effects of seed treatment of *Lotus corniculatus* cv. 'Mirabel' with X-rays (3,000-12,000 Rads), ethylmethanesulfonate, 8-ethoxycaffeine, N-hydroxyurea, and 2-aminopurine (0.001 to 1.0% solutions) were assessed on 93 selfed, partially inbred, and open-pollinated lines (22,000 plants) over four generations. Mutagenic effects were assessed on germination, seedling survival, meiotic aberrations, pollen abortion, seed and forage yields, pod dehiscence, winter hardiness, brown floral keel tip color, and cyanoglycoside (HCA) content in the leaves. Dose effects were largely confined to the M_1 generation. Germination and seedling survival could not be used to predict mutation rates. Higher levels of forage and seed yields and lower levels of HCA content were induced. Pod dehiscence, winter hardiness and brown keel tip frequency were not affected. Three qualitative mutants, *chlorotica*, *vestigial floret*, and *dwarf*, were tetrasomic recessives. Only tetrasomically inherited characters were found. X-rays and EMS were the most effective mutagens.

SOMMAIRE

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LA MUTAGENESE CHEZ LOTUS CORNICULATUS L.

7,500 graines de Lotus corniculatus, cultivar Mirabel, ont été soumises à divers traitements avec les mutagènes suivants: Rayons-X (non-filtrés, 3,000-12,000 rads), méthane sulfonique d'ethyle, 8-éthoxycaffeine, n-hydroxyurea, et purine aminée-2 (solutions de 0.001 à 1.0%). En tout, 93 lignes comprennant 22,000 plantes ont été engendrées durant quatres générations successives, ceci par entre-croisements et fécondations consanguines des plantes traitées auparavant. Les effets des mutagènes ont été évalués en analysant les resultats obtenus sur la germination, la mortalité des semis, les anomalies méiotiques, l'avortement du pollen, le rendement en grain et fourrage, l'éclatement des gousses, la résistance au froid, le contenu de cyanure au feuilles, et la fréquence du caractère floral «carène brune.» Les effets des diverses doses de mutagènes ont été observés principallement à la génération M_1. Le taux de germination et de mortalité des semis n'on pu être utilisés comme indication de la fréquence des mutations. De meilleurs rendements en grain et fourrage ainsi qu'une diminution du contenu de cyanure ont été induits. Les divers traitements de mutagènes n'ont pas affecté les caractèristiques agronomiques suivantes: l'éclatement des gousses, la resistance au froid et la fréquence du caractère floral «carène brune.» Trois mutants qualitatifs ont été induits; chlorotica (ct), fleur vestigiale (fv), et nain(n), ceux-ci étant de type tétrasomique récessif. Seul des mutants transmis de façon récessive tétrasomique ont été observés. Selon cette étude, les Rayons-X et le méthane sulfonique d'ethyle apparaissent comme étant les mutagènes les plus éfficaces chez le Lotus corniculatus L.

CLAIM TO ORIGINAL RESEARCH

The study reported in this thesis constitutes the original work of the author, and the following items are considered the most important contributions to the knowledge of mutagenesis in Lotus corniculatus:

1. To the author's knowledge, this is the first study on induced mutagenesis in L. corniculatus.
2. This is the first report of quantitative variation having been induced directly in a higher plant by the mutagens 8-ethoxycaffeine, n-hydroxyurea, and 2-aminopurine.
3. Qualitative mutants were induced and their frequencies significantly enhanced for the first time in Lotus. Mutants were tetrasomic recessives and no disomically-inherited characteristics were found in a fairly large population, providing further evidence for the largely tetrasomic nature of this species.
4. It has been shown that qualitative mutants and induced quantitative changes cannot be predicted solely by the frequency of meiotic aberrations, pollen abortion, seed germination or seedling lethality rates.
5. The author has shown that the techniques developed and utilized in this study allow for the recovery of qualitative mutants and potentially useful lines for quantitative characters within four generations from mutagenic treatment in this species.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude and thanks to his Thesis Director, Dr. W. F. Grant, Macdonald College, Department of Plant Science, McGill University, for suggesting the topic of this study and for his patience, support, and help throughout every phase of this work. Financial support of this study by the Natural Sciences and Engineering Research Council, Ottawa, is very gratefully acknowledged.

The author also wishes to pay special thanks to Mrs. May Couture for typing the final form of this thesis.

The author wishes to acknowledge the following individuals for providing technical information, equipment, and personnel: M. Serge Lussier, who provided equipment and personnel in the field plantings and maintenance; Mr. Stuart Willox, for providing technical information on field maintenance of Lotus corniculatus; and Mr. Elwood Quinn, for providing technical information and assistance in preparing and maintaining experimentation in the greenhouse. M. Lussier is the former supervisor of the Emile A. Lods Research Centre, Macdonald College, and Messieurs Willox and Quinn are technical staff members of the Department of Plant Science, Macdonald College, McGill University.

The help of the following individuals in assisting in the maintaining and harvesting of field material, as well as assistance in

data collecting, proved indispensable, and is very gratefully acknowledged. These individuals include summer students Mr. John Argall, Mlle. Jocelyne Lessard, and Mr. Bruce Schneider; technicians Mlle. Marie Gourley and Mr. Guy Rimmer; and Graduate Students Alina Russell and Don Zura.

Dr. N. C. Lawson was most helpful in providing the history of the cultivar «Mirabel» used in this study, along with information and suggestions for use in the breeding of Lotus corniculatus.

Mlle. Louise Donahue assisted in the translation of the Abstract into French, and her help is gratefully acknowledged. The author also wishes to thank Professional Color Services Ltd., Montreal, Quebec, for the printing of the color plate in this thesis, and special thanks to Ms. Sara Dunton for designing and preparing all the graphs in this thesis.

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INTRODUCTION

Lotus corniculatus L., commonly known as birdsfoot trefoil, is a tetraploid ($2n = 4x = 24$) forage legume used worldwide as a pasture grazing and silage crop in temperate regions (Seaney and Henson, 1970). The improvement of agronomic characters in birdsfoot trefoil has been limited due to a number of undesirable characteristics inherent in this species. Elimination or modification of both desirable and undesirable characteristics has been difficult owing mainly to two factors. The first is that this species displays tetrasomic inheritance for qualitative characters and demonstrates a high level of variability and complex inheritance of quantitative characters, resulting from its tetraploid nature. As a result of being a tetraploid, Lotus corniculatus has required extensive selection before any improvement in agronomic characters could be realized (MacDonald, 1946; Seaney and Henson, 1970). The tetrasomic nature of birdsfoot trefoil has also hampered qualitative genetic studies as there is a paucity of genetic markers in this species, and those characters that have been used in genetic studies have indicated tetrasomic inheritance with modifier genes often being involved (Chamberlain, 1961; Buzzell and Wilsie, 1963; Bubar and Miri, 1965). A second factor found to encumber genetic studies and the improvement of agronomic characters is the allogamous nature of this species. Birdsfoot trefoil has been found to contain an S-allele incompatibility system and, in some cases, displays self-sterility. This effectively has prevented the establishment of homozygous lines (Giles, 1949; Miri and Bubar, 1966; Seaney and Henson, 1970).

This study deals with induced mutagenesis and mutation breeding in Lotus corniculatus, with some parallel experimentation utilizing a closely related diploid species, Lotus tenuis Waldst. et Kit. (2n = 2x = 12). Mutagenesis and mutation breeding have proven useful in understanding the basic genetics of a species, providing de novo mutations and novel germplasm in genetic studies and gene mapping studies in a number of diploid and polyploid species (Briggs, 1970b). Induced variation of quantitative characters is another aspect of mutagenesis and mutation breeding which has been shown to be successful in creating modifications and improvements in agronomic and related quantitative characteristics where such modifications and improvements were either not previously present in the species, or where the need for extensive selection for a desired trait, particularly in polyploids, has proven prohibitive (Gregory, 1965; Gardner, 1969; Singleton, 1969; Sigurbjörnsson, 1970). Such efforts have led to the development of a number of superior synthetic varieties in both diploid and polyploid crop species (Sigurbjörnsson and Micke, 1974).

Mutagenesis and mutation breeding have not been attempted previously in this species. This thesis, therefore, presents a comprehensive study on the pre- and post-mutational effects of mutagens from five major categories of mutagens, the frequency and spectrum of qualitative and quantitative mutants obtained, and the genetic information obtained by selectively crossing and breeding these mutants.

I. A REVIEW OF THE LITERATURE ON *LOTUS CORNICULATUS*

A. History

1. Origin

Lotus corniculatus L. obtained its present name from Linneaus in 1753 when he described it in his «Species Plantarum.» Earlier, this species had received the name *Logopus major* by Bock who described it *circa* 1750 in his «Kreuterbuch» (MacDonald, 1946). The relationship of *L. corniculatus* to the other species in the genus is given by Brand (1898) in the only monograph on the Old World species of the genus *Lotus*. The first verifiable account of the use of *L. corniculatus* was in the eighteenth century in Europe where it was reported as a forage crop (Ellis, 1744). The first commercially harvested seed in North America was reported in 1937 in New York State, and therefore as a North American crop species, *L. corniculatus* is of recent origin (MacDonald, 1946).

2. Description

Lotus corniculatus is considered to have its origin in the central or eastern Mediterranean regions of Europe (Brand, 1898). In its natural distribution, it is found growing from 70 degrees north latitude in northern Europe to about 10 degrees north latitude in North Africa, and from Wales in the British Isles to the Ural Mountains in

Russia. Paradis (1951) states that this species can be found in almost any habitat, from semi-desert to marshland, from semi-tropical to alpine. It has been found from below sea level to an elevation of 3,600 meters in the Swiss Alps.

The species displays considerable morphological variability which was first noted by Bentham (1865) who found many indigenous races in the British Isles and who considered that it was impossible to distinguish clearly between the different races. Larsen and Zertova (1963) found Lotus corniculatus to be the most variable species in a genus containing some 350 taxa. This morphological and physiological variability made it impossible for them to classify any distinct, naturally-occurring races of L. corniculatus. They found the most variable characters to include growth habit, flower color, seed coat color, degree of hirsuteness on stems and leaves, mature pod color, number of pods per umbel and number of seeds per pod.

A description of L. corniculatus has been given by Stebler and Schroeter (1889) with addenda by MacDonald (1946) and is summarized as follows. It is a herbaceous papilonaceous legume of perennial habit turning woody with age. The foliar arrangement consists of heavily branched stems arising from a single crown and ranging in length from 30 cm to 1 meter. Leaves are borne alternately on lateral stems, having two stipules and three leaflets. Flowers are borne terminally on stems, in groups of three to seven, as loose umbels. Each flower is approximately 1.5 cm in length and 1 cm in width, with 5 sepals and 5 petals, usually yellow; a standard, two wing petals and two fused

petals form a keel over the pistil, style and stigma. Style and stigma are coiled within the keel until triggered by insects or strong movement. There are ten anthers, nine of which are fused and are borne terminally surrounding the stigma; the tenth resides near the base of the pistil. The pistil is that of a typical legume, containing 30 to 50 ovaries arranged in pairs forming two parallel lines along a dorsal and ventral suture in the pistil. The pistil develops into green pods up to 9 cm in length which change to a buff to dark-brown color upon ripening. After ripening, the pods spontaneously dehisce along two sutures located dorsi-ventrally, and the two halves coil in helices, releasing the seeds within. Pods contain approximately 8 to 30 seeds and are arranged on the umbel in the form of a «bird's foot,» giving this species its most common name «birdsfoot trefoil.» The seeds are usually ovoid with a visible hilum; the seed coat color is quite variable, ranging from tan to dark brown with varying degrees of speckling or mottling. Seed dimensions range from approximately 0.5 X 1.0 mm to 1.0 X 3.0 mm.

Lotus corniculatus is a long-day flowering plant (McKee, 1963) requiring a minimum of 14-15 hours of light for flowering to be initiated. It has a central tap root which grows to one meter in length when mature with many fibrous extensions (MacDonald, 1946). The root system is symbiotically associated with several species of bacteria, namely Rhizobium and Azotobacter, with the capacity to fix free nitrogen. The bacteria are found in nodules of approximately 1 to 5 mm diameter in the roots (Wilson and Westgate, 1942; Currier and

Strobel, 1977). The species is also known to contain at least 50 secondary phenolics in its shoots, leaves, and flowers (Harney and Grant, 1963) and a number of hydrocyanoglycosides in the stems and leaves (Ogilvie, 1970).

B. The Genetics and Breeding of Lotus corniculatus

1. History

The first mention of Lotus corniculatus L. as an agronomic species was by Worlidge in 1669, who described its use as a forage crop; however, the species description was vague. It was not until Ellis, in 1744, reported a species which was clearly identified as the present-day Lotus corniculatus as being used as forage and for breeding purposes, that L. corniculatus can be dated with certainty as a forage species. Ellis referred to this species as «Lady-finger Grass.»

From the time of Ellis to about 1920 the breeding of Lotus corniculatus (birdsfoot trefoil) was confined to selection of desirable types from large plantings and the growing out of seed from the selected types. Work of a genetic nature had not begun (MacDonald, 1946). The individual credited with the first set of experiments designed to create a synthetic variety of birdsfoot trefoil was M. Shribaux who conducted field trials and experimentation from 1920 to 1934 (see Lesourd, 1928; MacDonald, 1946). Some promising varieties were found, but no recognized, certifiable cultivated variety was developed. It was not until the 1940's that experiments leading to the development of

the first cultivar of birdsfoot trefoil in North America were undertaken by H. A. MacDonald. He described the basic agronomic characteristics of Lotus corniculatus using genotypes from diverse sources. From these studies eventually came the first American cultivar licensed for release, namely "Empire," developed from material that had been introduced into New York State about 100 years previously and which had become established and naturalized in the central and northern regions of the state. "Empire" cultivar was selected for erect growth habit and resistance to cold stress.

By the late 1940's, birdsfoot trefoil was in widespread use throughout many temperate regions of the world as animal fodder and forage. Hughes (1951) reported that L. corniculatus was being harvested throughout Europe, in North Africa, Western Asia, North and South America, India, Japan, Australia and New Zealand. By 1973 Seaney reported that birdsfoot trefoil was being cultivated on 0.5 million hectares of land in the north-eastern United States and eastern Canada, making it the second largest forage crop in that region. Its food value as fodder for dairy and beef cattle, sheep and other ruminants has been shown to be equal in value to alfalfa (Medicago sativa L.) and superior to all other forages (Marten and Jordan, 1979).

2. Cytology and origin of Lotus corniculatus

The first report on the chromosome number of Lotus corniculatus was by Senn in 1938, who found that it was a tetraploid with a somatic chromosome complement of $2n = 4x = 24$. The basic chromosome number of $x = 6$ placed this species in the Eulotus section of the genus. Dawson (1941) confirmed its tetraploid nature by demonstrating tetrasomic inheritance for a number of morphological characters. In addition, Dawson observed multivalent chromosome associations of 0.25 to 0.30 quadrivalents per cell. From this information, Stebbins (1950) postulated that L. corniculatus was a segmental allotetraploid. The classification of this species as a segmental allopolyploid indicated that the chromosome complement of L. corniculatus consisted of chromosomes with both homologous and homeologous segments. Stebbins suggested that L. corniculatus arose as a hybrid from two closely related ancestral diploid taxa. This prompted a cytological and breeding study into the origin of this species by a number of individuals.

Interspecific hybridization was carried out in order to try to resolve the origin of L. corniculatus. Dawson (1941) postulated L. tenuis as a progenitor of L. corniculatus. The naturally occurring diploid form of L. tenuis ($2n = 2x = 12$) was crossed with L. corniculatus by Elliot (1946) who reported obtaining viable seed and McKee (1949) also stated that he obtained viable seed by crossing these two species. However, no further data on these crosses were reported by these authors. Tome and Johnson (1945) produced a colchicine-induced

autotetraploid L. tenuis (4x), but were unsuccessful in crossing with L. corniculatus. Keim (1952) first successfully obtained a hybrid between 4x L. tenuis and L. corniculatus. The hybrid had 24 somatic chromosomes and did not produce any progeny, but the relative interfertility of the two parent species implicated L. tenuis as a possible ancestor of L. corniculatus. Mears (1955) was unable to obtain any viable hybrids from a 4x L. tenuis X L. corniculatus cross, but Erbe (1955) obtained eight hybrids from the same cross, lending support to Keim's findings. This provided further evidence which suggested L. tenuis to be a diploid progenitor of L. corniculatus. Bent (1958) repeated the experiments of Keim, Mears and Erbe with the additional factor of incorporating a genetic marker into the cross between 4x L. tenuis X L. corniculatus. Using a plant of L. tenuis containing hydrocyanic acid and an acyanogenic form of L. corniculatus, Bent was able to recover hybrids in the F_1 that were all positive for the dominant hydrocyanic acid marker in the leaves. This indicated that a true hybrid form had been obtained from the L. tenuis X L. corniculatus matings. This was a further indication that L. tenuis was a diploid progenitor of L. corniculatus. Wernsman et al. (1964) obtained viable, fertile hybrids of 4x L. tenuis X L. corniculatus which possessed 12 bivalents at metaphase I, but they were unable to confirm these findings using genetic markers or by other means of identifying the hybrids. Since emasculation of the female parent was not carried out by Wernsmann et al., there is the possibility that the hybrids may have been autoselfs of L. corniculatus. Brandenburg (1961) indicated that

L. corniculatus is not completely self-sterile and may set up to 49% selfed seed.

Other reports indicated that various other species of Lotus may have contributed to the genome of L. corniculatus. Titz (1964) from a study of meiosis of both L. corniculatus and diploid L. borbazii Pit. & Pit. (Syn. L. corniculatus var. slovacus Zert. 2n = 2x = 12) found evidence to suggest that L. borbazii may have been a diploid progenitor of L. corniculatus and that L. corniculatus was an autoploid of L. borbazii. These findings were not confirmed by any breeding experiments. Harney and Grant (1963), upon examining chromatograms of secondary phenolics from the leaves of L. corniculatus and a number of related diploid Lotus species, concluded that L. corniculatus was of alloploid origin with L. alpinus, L. japonicus and L. pedunculatus as possible diploid progenitors of L. corniculatus. Their conclusion was based on affinity studies of the secondary phenolics of these species. Grant and Sidhu (1967) were also able to demonstrate from affinity testing of leaf hydrocyanic acid glycosides that L. corniculatus was of alloploid origin with the contributors to the genome of this species still uncertain.

3. Reproductive behavior

In 1873 H. Muller was the first to report that plants of L. corniculatus failed to set seed in the absence of insects. This established the species as an insect-pollinated outcrosser. Silow (1931) observed that seed set was possible in the absence of insects or

other vectors but that seed set was low (about 8.7%). Therefore, he concluded that L. corniculatus was highly self-sterile, but not completely. From histological studies, Giles (1949) considered that the mechanism underlying low self-seed set was a gametophytic, S-type incompatibility system. Bubar (1958) confirmed the presence of an S-type self-incompatibility system in L. corniculatus and obtained self-fertile clones with an average of 49.1% self-fertility. Miri and Bubar (1966) enhanced selfing rates over 20% by utilizing a mixture of self- and cross-pollen. They combined a number of genetic markers to confirm that either selfing or crossing had taken place. Seaney (1967) observed that self-fertile clones of L. corniculatus lost their ability to self after several generations of selfing, as well as to be less vigorous and produce lower seed yields. Thus, a form of inbreeding depression had taken place. Seaney postulated that self-incompatibility in L. corniculatus guarded against an increase in the homozygous condition preventing inbreeding. Miller (1969) found that self-incompatibility in L. corniculatus was not constant. He obtained plants that ranged from completely self-sterile to ones that were over 85% self-fertile. Schaaf and Hill (1979) also showed the existence of a cross-incompatibility mechanism in L. corniculatus, allowing for random-mating and assisting in the prevention of the build-up of any deleterious genes in a breeding population.

4. Genetics

a. Qualitative studies

The tetrasomic nature of qualitative gene segregation in Lotus corniculatus was first noted by Dawson (1941) who identified a dominant gene A which controlled the presence of hydrocyanic acid in the leaves in an additive manner. Stebbins (1950) combined the theories of gene segregation in polyploids proposed by Muller (1914), Haldane (1930) and Mather (1936) to predict the ratios that should be obtained for independently assorting and randomly segregating genes in this species. His predictions indicated that both disomic and tetrasomic modes of inheritance should occur in L. corniculatus, as in a segmental alloploid. Donovan (1957) found a single character in L. corniculatus, namely, seed coat mottling, to have both disomic and tetrasomic components of inheritance, that is, the character segregated as a tetrasomically inherited character with disomic modifiers. Donovan also showed that leaf size and floral keel-tip color were inherited tetrasomically. Bubar (1958) in a study of the inheritance of seed coat mottling and floral keel-tip color in L. corniculatus found that brown keel-tip color was dominant to yellow and was inherited tetrasomically. However, Bubar did not find any disomic segregation for the seed coat mottling character as reported by Donovan. Bubar showed that mottled seed coat is dominant over non-mottled and tetrasomically inherited. He also found that the red form of the flower was dominant to yellow and that the character was inherited tetrasomically.

A further study was made on the brown keel-tip floral character in L. corniculatus by Hart and Wilsie (1959) who confirmed that brown keel-tip was inherited tetrasomically by a dominant gene, which they termed B, and that this character was modified by another gene C which in the homozygous recessive condition with gene B (bbbbcccc) caused zygotic lethality. All other gene combinations gave various intensities of color to the keel-tip from dark brown with the homozygous dominant genotype (BBBB CCCC) to a light yellow color with the genotype BbbbCccc. Their findings came about as a result of obtaining dissimilar ratios to those found by Bubar for the segregation of this character. Buzzell and Wilsie (1963) found not one, but at least two modifier genes, which influenced the gene B for floral brown keel-tip color and that these genes appeared to segregate in a dominant, tetrasomic fashion.

Amongst other segregating characters studied in Lotus corniculatus is a naturally-occurring, chlorophyll-deficient mutant, the character for which segregated as a tetrasomic recessive (Pootschi and MacDonald, 1961). Chamberlain (1961), in looking for other possible types of macromutants of L. corniculatus, screened large populations but did not find any mutant types other than those previously discovered. Subsequently, Donovan and McLennan (1964) found that the leaf-size character, originally studied by Donovan (1957), was inherited in a modified tetrasomic fashion. The expected and original segregation ratio for the F_2 of 1:4:1 was actually 1:2:2:1 when a larger sample was tested, indicating that the character was displaying partial dominance for large vs. small leaf size. Bubar and Miri (1965) found that

cyanogenesis in leaves, corollar streaks in flowers, and self-incompatibility in the female gametophyte of L. corniculatus were inherited as tetrasomic dominants, and that pubescence and chlorophyll-deficiencies were characters inherited as tetrasomic recessives. In all, only eight qualitative characters have been studied in this species, and all but one appear to be inherited in a tetrasomic manner. The exact mode of inheritance of seed coat mottling, suspected to be partially disomically inherited, has not been confirmed (Bubar, 1957; Bubar and Miri, 1965).

b. Quantitative studies

It is well established that most characters of agronomic value, such as flowering time, seed and forage yields, and total yields, are inherited in a quantitative fashion (Cockerham, 1956; Allard, 1960). One of the first analyses on quantitative traits in L. corniculatus was by Rachie and Schmid in 1955. They examined winter-hardiness (or cold-resistance) for a number of natural and synthetic varieties and found this character to be inherited quantitatively. The cold-resistance character segregated after repeated cycles of selection which enabled them to select winter-hardy lines. Metcalfe et al. (1957) found that the percentage pod dehiscence varied between eight and 100% for a single clone of cv. «Empire» birdsfoot trefoil when placed under identical light, temperature, and humidity regimes. They concluded that pod dehiscence was quantitatively inherited with a large environmental influence. Peacock and Wilsie (1957) also found that

plants grown from a single clone of birdsfoot trefoil under identical environmental regimes varied considerably in their expression of pod dehiscence. They were able to select for a significant increase in pod indehiscence with clones exhibiting a 17% lower dehiscence after one cycle of selection. Pod dehiscence clearly showed quantitative inheritance with a large genetic component. This work was confirmed by Gershon (1961) who selected for indehiscent lines of birdsfoot trefoil after several cycles of selection and again found inheritance to be quantitative. Hood (1964) found that dehiscence was moderately influenced by humidity and the flowering cycle of the individual plant or clone. He found the flowering cycle was also under quantitative influence.

Ogilvie (1970) studied the inheritance of hydrocyanic acid glycosides (HCA) in the leaves of birdsfoot trefoil and found this character to be highly variable under changing environmental conditions and, in addition, that the levels of the glycosides varied with the age of the leaves. He concluded that the presence of HCA glycosides is governed, at least in part, by quantitative factors. Urbanska-Worytkiewicz and Wildi (1975) sampled a number of natural populations of L. corniculatus and also found that there was considerable variability for the presence of HCA glycosides in the leaves. They found ratios of HCA+:HCA- ranging from 0:12 to 45:0, indicating that the presence of HCA glycosides may be under quantitative genetic control. Ellis et al. (1977a, 1977b) found a similar situation in a number of other natural populations of birdsfoot trefoil in which the

amount of HCA glycosides varied considerably in the different individuals. These results supported those obtained by Bansal (1971) who showed that the enzyme responsible for the deposition of the HCA glycosides in the leaves of birdsfoot trefoil was inherited in a quantitative fashion.

Among other characteristics that have been studied which display quantitative inheritance are those of flowering time and stem length (Buzzell and Wilsie, 1965). Buzzell and Wilsie found these two characters to be definitely quantitatively inherited and obtained broad-sense heritability estimates of 49 and 73%, respectively, indicating that the former contained a moderate and the latter a large genetic component of inheritance.

Another important character is seed yield. Badcock (1973) showed that seed yield was directly affected by pod dehiscence which in turn was affected by changes in light, temperature, and humidity regimes. Differences in the variability of this character within a single clone of cv. «Leo» birdsfoot trefoil indicated that seed yield was also under quantitative genetic control. Sandha and Twamley (1973) were able to obtain a broad-sense heritability estimate of 61% for seed yield in the cv. «Leo.» Bresciani and Frakes (1973), using clones from 26 varieties of L. corniculatus, obtained a much wider range of heritability estimates for seed yield ranging from 40 to 70%. Their findings indicated that heritability for seed yield is dependent on the specific genome under investigation as well as other genetic and environmental components. Sandha et al. (1977), using 144 polycross

progenies from 100 parental lines of cv. «Leo» birdsfoot trefoil, found that seed yield (broad sense) heritability estimates were 71% for the P_1 generation and 64% for the F_1 generation, demonstrating a high genetic component for the seed yield character. Pankin et al. (1977) were able to demonstrate that plant spacing had a significant effect ($P < 0.05$) on seed yield as well. Khayrallah (1979) obtained a lower heritability estimate for seed yield than did Sandha et al. (53% vs. 64%) in cv. «Leo,» indicating that genetic differences do exist within a variety for a quantitative character, in this case, seed yield.

The main interest in L. corniculatus, with respect to quantitative genetics, is in forage yield and related components, as this species is primarily used for forage. Forage yield studies were conducted as early as the 1920's (Lesourd, 1928), with later studies being continued by MacDonald (1946). Studies of a genetic nature were initiated much later. Miller (1968) grew four clones of birdsfoot trefoil for three years and found that the forage yield components had mean squares for general and specific combining abilities that were highly significant ($P < 0.001$). The evidence indicated that forage yield was a quantitatively inherited trait with a large genetic component of inheritance. Conje and Carlson (1973) found that forage yield components were stable from parent to progeny. In crossing two different lines of birdsfoot trefoil, namely cv. «Leo» and a wild type from the U.S.S.R., they found that forage yield could be significantly increased ($P < 0.05$) in the hybrid; thus heterosis for forage yield

was observed. Keoghan and Tossell (1974) used a large number of diverse sources of L. corniculatus in forage breeding experiments and found the yields of individual lines to vary greatly, ranging in mean value from 11,950 to 18,780 kg/ha dry matter. They determined that forage yield was dependent on the specific genotypic background of the particular line and to be under considerable environmental influence. Sandha et al. (1977) conducted forage yield experiments on cv. «Leo» birdsfoot trefoil and obtained heritability estimates (broad sense) of 76% and 68% for the P_1 and F_1 generations, respectively, indicating that this character had a large genetic component. Khayrallah (1979) using clones of cv. «Leo» and «Mirabel» birdsfoot trefoil, obtained mean squares for general combining ability (GCA) that were significant at $P < 0.05$ for forage yield. A broad sense heritability estimate of 53% was obtained, indicating that forage yield was under moderate genetic influence.

II. MUTAGENESIS AND MUTATION BREEDING

A. History

The first individual to induce mutations artificially was H. J. Muller who in 1927 irradiated male Drosophila with X-rays and obtained recessive lethal mutations in the progeny of the treated flies. The following year, L. J. Stadler succeeded in producing mutations in a higher plant, namely, barley (Hordeum vulgare L.) after irradiation of the pollen with both X-rays and γ-rays. He obtained chlorophyll-deficient mutants in the progeny of plants fertilized with the irradiated pollen. The pioneering work of these two individuals formed the basis for the study of mutagenesis and mutation breeding.

Knowledge on mutagenesis and the mutation process began to expand quickly. Stadler, in 1929, continued his experimentation on the irradiation of higher plants. Working with barley, oats (Avena sativa L.) and wheat (Triticum vulgare L.), he demonstrated that an increase in ploidy level leads to a subsequent decrease in mutation rate per unit dose of radiation, and that the relationship is approximately linear. He termed the phenomenon «buffering capacity» and stated that the buffering capacity in a polyploid was greater than in a diploid owing to the increase in genomes present in the polyploid. This work was confirmed independently by Sapehin (1930) and De Launay (1930).

Effects of irradiation on chromosomes had been studied since the 1920's and 1930's with early studies by Strangeways et al., (1923, 1925) and Marshak (1937) working on choroid tissue of chick embryos and Gasteria, respectively. These workers observed chromosome breakage in cells irradiated with X-rays. The chromosome-breaking properties of X-rays and other radiations were also noted by Sax (1940) in mitotic and meiotic cells of Tradescantia. Gelin (1941), using barley seeds treated with 5 kR X-rays, demonstrated that increased seed moisture content led to an increase in mitotic aberration rate. The cytological effects of radiation on polyploidy level were first shown by Fröier et al. (1941). Irradiating seeds of Triticum monococcum (2x), T. durum (4x), and T. vulgare (6x) with 5 kR X-rays, they found that an increase in ploidy level resulted in an increase in the mitotic aberration rate. They also found that a dosage of 25 kR of X-ray caused massive cell disruptions which were independent of ploidy level.

The types of chromosome and chromatid aberrations as a result of physical and chemical mutagen treatment are well documented and have been illustrated and discussed by several authors (Evans, 1962; Kihlman, 1966; Heddle and Bodycote, 1970; Auerbach, 1976).

Until the early 1940's only ionizing radiations were known to cause mutations in higher organisms. It was not until 1944 that a chemical substance was also shown to be mutagenic, namely, mustard gas, a nerve gas used as a weapon of war during the First and Second World Wars. Its mutagenic properties were first demonstrated by Auerbach and Robson (1944) who treated eggs and larvae of Drosophila melanogaster

with a water-soluble derivative of mustard gas, allyl isothiocyanate, and found sex-linked recessive lethals in the progeny. In a subsequent experiment, Auerbach and Robson (1947) found in the progeny of treated individuals dominant and recessive lethals, visible mutants, as well as numerous chromosomal translocations. Auerbach (1947) further demonstrated that the translocations produced in Drosophila chromosomes by mustard gas and its derivatives persisted to at least the F_4 generation. It was discovered also that these chemicals caused cellular mosaicism. Thus, the mutagens caused related cellular and genetic damage.

While work was progressing on Drosophila mutagenesis, Gustafsson (1947) treated seeds of some ten different agricultural species with various forms of ionizing radiations. He found that the mutation rate in the X_3 (F_2) generation could be predicted by examining the chromosomal aberration rates in the X_1 (P_1) and X_2 (F_1) of X_0 (P_0) treated seed, as well as from the number of lethals obtained in the X_2 (F_1) generation. He found that the mutation rates of the different species at the same ploidy level were not constant, and that each species had its own unique mutation rate for the same dosage of a specific mutagen. Thus, the dosage effect of a particular mutagen was specific to the organism (Gustafsson, 1944).

The first induced mutation involving a reversal of a naturally occurring mutant was demonstrated by Stadler and Roman (1948). Studying the naturally-occurring gene A which controls the deposition of the aleurone pigment in the seed coat of Zea mays, Stadler and Roman

irradiated pollen with 900 to 1100 kR and screened for A in the F_3 and F_4 generations. They obtained segregation ratios other than that expected for the known genotypes, thus showing that the mutagen had reversed a specific trait. D'Amato and Gustafsson (1948) confirmed this observation by producing similar results in barley as that which had been obtained by Stadler and Roman in corn. Thus, it was firmly established that ionizing radiations caused reverse mutational events.

However, the molecular basis for this phenomenon was not known until Scholes et al. (1949) reported the action of ionizing radiations on nucleic acids. They isolated nucleotides from irradiated nucleic acids in vitro which were ionized by the action of the radiations and subsequently lost from the main molecule. It was not until the discovery of the structure of nucleic acids, specifically deoxyribonucleic acid (DNA) by Watson and Crick (1953), that a satisfactory explanation of the relationship of the loss or gain of one or more nucleotides in the process of gene mutation could be explained.

By the time the mechanisms of mutagenesis via ionizing radiations were established in the early 1950's mutagenesis and mutation-breeding were already in widespread use for the improvement of crop species (Torsell, 1954). However, the limits of this approach were quickly ascertained. MacKey (1954) using diploid barley and hexaploid wheat (Triticum aestivum L.) showed that a given species has a maximum inducible mutation rate for a given dose, and that no further mutants will be produced by exceeding the maximum. He also noted that beyond a certain dose level the deleterious effects of the mutagens

became prohibitive to the development of the treated individual. The maximum mutation rate which could be achieved was found to be independent of the ploidy level.

With the discovery by Auerbach and Robson (1947) that a chemical was capable of causing mutations, a new avenue of research was made possible: that of chemical mutagenesis. Amongst the first to demonstrate that an alkylating agent was capable of causing mutations in a higher plant was Gibson et al. (1950) who treated seeds of Zea mays with mustard gas and obtained mutant plants.

In 1958, Loveless demonstrated that ethyl methanesulphonate (EMS), an alkylating agent, produced mutations in bacteriophage T_4. The potential mutagenicity of this compound had been shown previously by Reiner and Zamenhof (1957) from in vitro experiments in which EMS in solution with DNA caused the removal of adenine, thymine and cytosine residues from the DNA molecule as alkyl esters. The process they had observed, termed "alkylation," occurred when an alkyl group was removed from EMS and added to a nucleotide which had been severed from the DNA parent molecule. This reaction was similar to the effect of ionizing radiations on DNA, i.e., the dissimilation and loss of nucleotides from the DNA parent molecule, involving different mechanisms (Gustafsson, 1944; Auerbach, 1976).

Moutschen (1965) showed that EMS-induced meiotic aberrations in barley could persist for several generations, and that the addition of the heavy metallic ions, copper and zinc, enhanced both the cytotoxic and the mutagenic effects of EMS. Kak and Kaul (1973)

achieved similar results after subjecting several species to EMS treatment supplemented with the addition of heavy metallic ions. Tessman et al. (1964) identified the altered base pairs in a small strand of DNA which was mutated by the alkylating agents EMS, hydroxylamine and nitrous acid in an in vitro assay. Their work confirmed the hypothesis of Brenner et al. (1961) who showed that mutations were the result of loss or substitution of bases.

Ramanna and Natarajan (1965) showed that altering treatment conditions changed the effects of a given mutagen, and that different mutagens having a similar mode of action, for example, alkylating agents, produced different mutation rates under identical conditions. From this, they developed the «relative mutagenic efficiency» quotient, defined as the mutation rate per dose of one mutagen divided by the mutation rate per dose of a second mutagen used as a standard. This aided in determining which mutagen is most effective in producing mutations in a given species for a fixed set of treatment parameters and conditions. Nilan et al. (1976) showed that the effects of a mutagen were dependent on the kinetics of the mutagen used, pre- and post-treatment conditions, and the specific genotype of the species. As a result, they found that the same mutagen used on a number of species did not produce the same number or type of mutants in each species. Thus, a mutagen could be classified according to its specific effects on a given species. Nagaraja and Natarajan (1965) demonstrated that EMS-induced meiotic aberrations in barley could be directly correlated to the mutation rates obtained in subsequent progeny, and that these aberrations were stable in successive generations.

Shevchenko (1968) conducted chromosome labelling studies with EMS on several species to determine which regions of the chromosomes were most affected. Using tritiated-labelled EMS, he determined that the adenine-thymine rich regions were most affected. An earlier study by Moutschen-Dahmen et al. (1965), using tritiated-labelled EMS on Vicia faba chromosomes, determined that EMS did localize in certain regions of the chromosomes, but they were unable to identify the specific sectors of the DNA affected.

While new types of mutagens were being discovered, work continued on the basic principles of mutagenesis from treatments with ionizing radiations. Russell et al. (1958) showed that mutation frequency was directly proportional to the dose of the mutagen, indicating that there existed a «dose-effect» for the mutation rate of treated individuals. Using mice as the test organism, they found that the mutation rate remained linear to the dose of radiation applied, independent of the animal used, the sex of the animal, or the source of the radiation and whether the source was acute (X-rays), or chronic radiation (γ-rays from a cesium source). Alexander and Stacey (1958) demonstrated that the actions of ionizing radiations and alkylating agents, which now included EMS and its derivatives, and mustard gas and its derivatives, were essentially the same: namely, the removal of nucleotides from the DNA molecule caused the same types of mutations in exposed organisms. Freese (1959) then showed that the loss of nucleotides in the DNA molecule, resulting from exposure to mutagens, caused errors in the genetic code by elimination or improper substitution of a nucleotide which in turn was manifested as a mutation.

The mosaic effect of chemical mutagens, first demonstrated in Drosophila by Auerbach in 1947, was found to occur in plants also. Heslot (1959), working with Hordeum vulgare, found some mutant chlorophyll-deficient mosaics. He also found that the segments of the plants affected produced chlorophyll-deficient offspring. This was evidence that a somatic mutation can cause mutational events in germplasm derived from the mutant somatoplasm.

From the initial discovery of X-rays as a mutagenic agent to the discovery of EMS and related compounds, it was found that all mutagenic agents studied during this period acted essentially in the same fashion, that is, by removal of part of the DNA molecule via the severing or alteration of chemical bonds (Brenner et al., 1961). With the knowledge of the basic physical and chemical structure of DNA came the knowledge that certain chemicals closely related to the nucleotides, or DNA bases, could be incorporated into the DNA molecule without having the chemical or biological activity of the DNA bases for which it had substituted. Thus, substituted bases could cause errors in the genetic coding sequence of the affected DNA strand which could lead to mutational events. These compounds were termed DNA base analogs. Amongst the first of these to be discovered was 2-aminopurine (AP), analogous to 6-aminopurine or adenine. Rudner (1960) found that the addition of 2-aminopurine caused mutations in Salmonella typhimurium by creating errors in base pairing in the 2-aminopurine substituted regions of the DNA. Danilov et al. (1967) found that AP caused mutations in micro-organisms by substitution of the DNA base 6-aminopurine

(adenine). Khan and Alderson (1968) found similar results in Drosophila melanogaster by recovering sex-linked recessive lethals and mosaics in the progeny of AP-treated flies.

The comutagenic properties of AP were confirmed by Degraeve (1971) who demonstrated that organisms pretreated with AP would show a marked increase in chromosome aberration rates after exposure to EMS versus treatment with EMS alone. Mutation induction in viruses by AP was confirmed by Marcovich and Vigier (1972). They used AP-treated Escherichia coli as the mediator agent and found mutants in the bacteriophage T4, which had infected the host E. coli. Tarasenko (1974) demonstrated that post-treatment of X-irradiated barley seed with AP enhanced the mutation rate in the progeny. Rackwitz and Scheit (1975) detected mutants in AP-treated cell cultures of calf thymus cells. The first confirmation of the mutagenic properties of AP without involvement as a co-mutagen in a higher plant was by Inoue et al. (1976) who obtained morphological and chlorophyll-deficient mutants in the progeny from AP-treated rice (Oryza sativa) seeds.

Further studies with AP demonstrated its potent mutagenic properties in bacteria (Janion, 1977, 1978). She recovered a large number of error-prone type mutants in AP-treated Salmonella typhimurium and Escherichia coli. De Serres et al. (1979) obtained excision-repair deficient mutants in Neurospora crassa after treatment with AP and actinomycin D. The mutagenic properties of AP were summed up by Ronen (1980) who stated that AP was a DNA base analog, substituting for sites occupied by 6-aminopurine or adenine and being non-functional, caused

errors in DNA replication and transcription. The effects were seen as error-prone mutations in lower organisms and as single and complex loci mutants in higher organisms.

In 1960 Bautz and Freese found a new group of substances with the ability to alter DNA by base-pair removal in a manner similar to EMS, namely, «amines.» Freese et al. (1961) found that one of these compounds, hydroxylamine, removed nucleotides from nucleic acids in vitro and caused mutations in several organisms. The process was referred to as «deamination.» A second group of mutagenic compounds, the acridines, was also found to be mutagenic and reactive with DNA (Lerman, 1961). Lerman showed that the addition of acridines in vitro to DNA strands caused the loss of nucleotides from the parent molecule causing frameshift mutations, and that these compounds were capable of inducing mutations in several organisms.

In an effort to further increase the knowledge of the processes of induced mutagenesis, work on mutation induction and mutation breeding continued in new directions. Blixt (1961) showed that not only could qualitative mutants be obtained by artificial mutagenesis, but that quantitative variation could be induced as well. Working with the genus Pisum and using X-rays, γ-rays and EMS, Blixt induced quantitative variants for chlorophyll in various lines of peas. Zacharias and Ehrenberg (1962) demonstrated short and long range toxic effects of mutagenic substances by the induction of leaf spots in Vicia faba and Trifolium pratense L. They found both immediate and delayed reactions to high levels of X-rays and EMS as regions of dead

cells in leaf tissue with an increase in the area affected with increased dosage. The concentration of a chemical mutagen as well as the treatment temperature were shown to affect the mutation rate as was first demonstrated in Hordeum vulgare by Froese-Gertzen (1963). Higher temperature and concentration generally increased the mutation rate in the progeny for all but lethal doses.

In 1963 Bendich et al. found a group of compounds which demonstrated DNA synthesis inhibiting properties in in vitro DNA experiments as well as chromosome aberrations in several organisms. Kihlman et al. (1968) found N-hydroxyurea (HU) to be an effective arresting agent of Vicia faba chromosomes at mitotic metaphase. Upon further investigation, they found that HU in addition to being a C-mitotic agent, caused abnormal cell division, chromosome aberrations, and DNA replication errors, all of which inhibited plant growth and development and made this compound a suspected mutagen. The plant growth inhibitory properties of HU were confirmed by Plyler (1970), and its inhibition of DNA synthesis was confirmed by Karon and Benedict (1970), working on human leucocytes. The cytological effects of HU were further demonstrated by Hussey and Turner (1970), who found abnormal cell division and chromosome breaks in treated shoot apices of Lycopersicon esculentum L. In 1971 the mutagenic properties of HU were confirmed by Zimmermann, who induced mitotic gene conversion in HU-treated Saccharomyces cerevisiae. Kihlman (1971) confirmed the chromosome altering properties of HU, which were first noted by Bendich et al. (1963). Kihlman found

evidence of chromosome breaks in HU-treated root-tips of Vicia faba and Allium cepa. Masafi et al. (1972) also demonstrated that the DNA inhibitory properties of HU were potentially irreversible, causing possible long-range damage to the genetic integrity of the organism exposed to the chemical. Kaul and Kak (1973) found that HU could reduce the number of chromosome aberrations induced by X-rays in barley plants by post-irradiation treatment with HU. Khan and Veeraswamy (1974), however, found that post-treatment of colchicine-treated Tradescantia plants with HU enhanced chromatid aberrations in the pollen tubes. Wyrobek and Bruce (1975) and Bruce and Heddle (1979) found HU to be mutagenic in the sperm abnormality test in mice (Mus musculus), but to be non-mutagenic in the Ames Salmonella test. The latter results were confirmed by Jenssen and Ramel (1980). The biochemical nature of HU was deliniated by Timson (1975) who stated that HU acts to suppress DNA synthetic enzymes. It was further noted that HU had more than one effect depending on which phase of DNA synthesis and the cell cycle the chemical was applied, and whether or not DNA damage was already present. Reddy and Miller (1977) found that HU could increase the amount of chromosome and chromatid aberrations in organisms exposed to X-rays, via pre-treatment with HU demonstrating its co-mutagenic properties. Adam and Warr (1979) found HU to be mutagenic causing error-prone type mutations in HU-treated Chlamydamonas reinhardii. Raudaskoski and Lu (1980) recovered hyphal and spore mutations after treating Coprinus species with HU. However, the mutagenic properties of HU in higher plants is, as yet, undetermined.

In 1966 the newest type of mutagen to be added to the list of those already known was the intercalating agent. Intercalating agents were first studied by Natarajan and Ramanna (1966) who observed that these chemicals induced breaks in the chromosomes of a number of species. They determined that the mechanism causing chromosome breakage was the chemicals' close binding affinity with the DNA molecule which caused stress points at the binding site eventually leading to breaks in the structural integrity of the DNA molecule. One of these compounds, 8-ethoxycaffeine (EC) a methylated oxypurine analogous to guanine, was mutagenic in several species and caused both forward and reverse mutations (Natarajan and Ramanna, 1966). The mutagenic properties of EC were confirmed by Heinz (1973) from treating stem plugs of Saccharum species and by Kihlman (1975) after treating seeds and shoots of Vicia faba. EC also enhanced the mutation rate in X-ray treated barley (Kihlman and Sturelid, 1975). Malling and Wassom (1977) reported EC acted mainly as a DNA base analog, in a manner similar to 2-aminopurine (AP).

Investigations on the reactivity and kinetics of chemical mutagens were continued by Lawley (1966) who studied in vitro reactions of EMS and related compounds with DNA. His findings indicated that chemical mutagens did not react immediately or completely with DNA, that the reaction was gradual and temperature-dependent, and that the rate of reaction was specific to each compound. For example, he demonstrated that it took 150 hours at 37°C for one-half of the EMS present to be completely methylated in an in vitro reaction with DNA. This was

termed the «half-life» of the compound at a specific temperature. It was further demonstrated that the half-life of a particular chemical mutagen would have to be considered in the calculation of the exposure time of that particular mutagen for a given organism.

It had been known for some time that treatment conditions, by either physical or chemical mutagens, would affect the response of an organism, as well as the mutation rate in its progeny (Sparrow et al., 1958; Smith, 1969). Other factors found to influence the outcome of mutagenic treatment included pH and the physical condition of the treated material. Mikaelsen et al. (1968) found that the pH of the treatment solution significantly altered the mutation rate in the progeny of Hordeum vulgare plants. They treated barley seeds with EMS and found that a pH above 9 or below 4 greatly increased the mutation rate. Their findings showed that the mutation rate was also affected if the seed was germinating vs. dormant at the time of treatment: germinating seeds were more sensitive to toxic responses from mutagenic treatment than dormant ones. Wickman et al. (1969) confirmed the findings of Mikaelsen et al. by obtaining similar results from the same type of experiment. Grant et al. (1969), working with several chemical mutagens, studied the effectiveness of the mutagens during various phases of the cell cycle of barley. Their findings show that the chemicals were most clastogenic (causing chromosome aberrations; Shaw, 1970) during the S, or DNA synthesis phase, of the cell cycle. They were thus able to demonstrate that most of the damage caused by chemical mutagens on DNA was during replication. Mikälsen (1969) also

found the maximum effectiveness of mutagenic treatment occurred during the S_1 phase when he treated barley seedlings with EMS.

In 1969, Kawai classified mutagens according to their effect on DNA and this classification scheme is most widely used today. According to this scheme, mutagens are of five basic types: (1) physical mutagens, including thermal neutrons, alpha and beta particles, ultra-violet, gamma and X radiations; (2) alkylating agents, including EMS and all other chemicals containing one or more alkyl groups capable of bonding to DNA; (3) clastogenic agents, a broad category encompassing all compounds capable of causing chromosome and chromatid breaks, including such chemicals as acridines and actinomycin D; (4) mitotic poisons and DNA inhibitors; compounds that disrupt the normal cell cycle resulting in aneuploidy, errors in DNA replication and inter- and intra-chromosomal exchanges; and (5) DNA base analogs, which cause missense and nonsense type mutations by substituting for the native nucleotides in DNA.

Some of the cytological and related effects of chemical mutagens have been studied. Sharma (1968) investigated the delayed effects of physical and chemical mutagens in barley (Hordeum vulgare) and found that the genetic effects of mutagens may not be evident until around the F_3 (X_4 or M_4) generation, since some degree of gene segregation was required before mutagenic effects became evident.

With continued use of mutagenesis in basic research, greater knowledge developed on the basic processes and relationships in mutagenesis and the mutation process. Abrahamson et al. (1973), in

looking at a wide range of species from microorganisms to man, showed that a linear relationship existed between the DNA content per cell and the forward mutation rate per locus per unit dose of mutagen, and that this relationship was independent of the species tested. Khalatkar and Bhatia (1975), using Hordeum vulgare as a test species, showed that one mutagen was capable of enhancing the effect of another. They found that post-treatment of X-rayed barley plants with EMS significantly increased the mutation rate, and that this rate was greater than either of the two mutagens used singly. The effects of mutagenesis on ploidy levels was also studied. Zutshi and Kaul (1975), using EMS and other alkylating agents, did not find any positive relationship between ploidy level and sensitivity to the alkylating mutagens from treatment of diploid, tetraploid, and hexaploid species of the genus Solanum.

In 1978, Lockhart and Shankel showed that substances not mutagenic themselves could significantly enhance the effects of a known mutagen. They found that when caffeine was used to pretreat barley seeds prior to EMS treatment, the mutation rate was greatly enhanced compared with EMS alone, and that caffeine alone did not cause mutations. Thus, caffeine was comutagenic to EMS and was found to bind to the DNA at specific sites allowing for greater access of the mutagen EMS to the DNA.

The accumulated knowledge on artificial mutagenesis in higher plants has resulted not only in an increased knowledge of the basic genetics of higher plants, but has also contributed substantially to the development of plants of superior qualities for use in crop

improvement. From 1929 to 1971 over 93 crop plant varieties have resulted from induced mutagenesis and mutation breeding. As well, the production of over 8,000 mutants in the 93 species has made a substantial contribution to the understanding of the basic genetics of these species (Sigurbjörnsson and Micke, 1974).

III. ARTIFICIAL MUTAGENESIS AND MUTATION BREEDING IN THE LEGUMINOSAE (FABACEAE)

In Sweden in the 1920's, Nilssen-Ehle and his colleagues began experimenting with ionizing radiations for the induction of mutations in agriculturally useful plants including some pulse and grain legumes. By 1933 Herbert Lamprecht, a colleague of Nilssen-Ehle, was successful in recovering an artificially-induced mutation in a legume. Using X-rays, he found a unifoliate leaf-type mutant in Pisum sativum L., which segregated as a disomic recessive. Work in legumes was nearly abandoned from around 1934 to 1950, with the majority of the work on artificial mutagenesis and breeding being concentrated heavily on other crops, particularly on the cereals, and mostly on Hordeum vulgare (Gustafsson, 1954).

Work on legumes resumed again in 1950 with Stubbe, who selected X-ray induced mutants of agronomic importance in Pisum. Most were high seed-yielding mutants with changes in stem or reproductive structures that allowed for higher yields. Similar results were obtained in Glycine max L., by Humphrey (1951) who used thermal neutrons as a mutagen source. The mutants were morphological types which produced higher seed yields.

Artificially-induced mutants were not confined to high seed yielding types, however. Despande and Jewswani (1952) produced a pigeon pea Cajanus cajan (L.) Millsp. mutant with a prostrate growth

habit by means of X-rays. The mutant was a disomic recessive. Tedin and Hagberg (1952) obtained a number of X-ray induced mutants in Lupinus luteus L., an allotetraploid species. The mutants were morphological and chlorophyll-deficient types which were inherited as tetrasomic recessives. These mutants were the first to have been successfully induced in a polyploid legume. Induced mutants were obtained also in another polyploid legume, namely, tetraploid alfalfa, Medicago sativa L., by Torsell et al. (1953). A number of morphological, tetrasomically-inherited recessive mutants, as well as a number of quantitatively inherited mutants from X-ray treatments, were obtained.

Amongst the first chemically-induced mutants in a legume was a chlorophyll-deficient mutant of Melilotus alba L., obtained by Scheibe and Hülsmann (1958) from ethyl methanesulphonate (EMS) treatment of seeds. However, most successful work on artificial mutagenesis in legumes was largely confined to X-ray induced mutants. Scheibe and Bruns (1953) were the first to successfully produce a dwarf-type mutant in the legume Trifolium pratense L. using X-rays. The first induced cultivated variety of a legume was the cultivar «Stål,» a garden pea (Pisum sativum) produced by Gelin (1954) using X-rays.

Commercial legume cultivars were also obtained from artificially-induced mutants of polyploids. Gregory (1955) first successfully produced a synthetic variety from induced mutant types of the tetraploid peanut Arachis hypogaea L. He found a «bunchy» high-seed yielding mutant after three cycles of selection from a large population of X-irradiated seed. This mutant was released in 1960 (Gregory, 1960).

The initial success by Gelin and Gregory led to the development of a number of synthetic varieties from induced mutant stocks in a number of species. Several agriculturally useful mutants were successfully obtained by Lamberts (1955) who obtained high forage yielding mutants of yellow sweet lupine (Lupinus luteus L.) using X-irradiation. Kress and Zachow (1956) obtained similar mutants in Lupinus using both X-rays and γ-rays. Down and Anderson (1956) obtained high seed-yielding mutants of Phaseolus vulgaris L. with X-irradiation, while Zacharias (1956) obtained high seed-yielding mutants of a similar nature in soybean, Glycine max L., also using X-rays. Thus, by 1960 a number of agriculturally useful mutants was obtained in several leguminous species via the use of physical mutagens.

The mechanisms involved in the induction of mutants in leguminous species were found to parallel those known for other species. Micke (1958) found that X-ray induced mutations in sweet clover, Melilotus alba L., were induced and inherited in a fashion similar to that in other diploids. Most induced mutants were single-locus recessives. Gladstones (1958) found that mutation induction in polyploid legumes was essentially the same as in other polyploid species. Using X-irradiated Lupinus digitatus Forsk., Gladstones found that most of the mutants resulting from X-irradiation treatment were tetrasomically inherited recessives, concurring with previous findings. Extensive work by Blixt and colleagues with Pisum species from 1960 to 1975 (Blixt and Gottschalk, 1975) produced over 2000 mutant types from radiations, and chemical mutagens. Their findings on the nature

of induced mutants in species of Pisum were essentially the same as that found in other species, i.e., that most mutations affected a single locus, that the mutant alleles produced were largely recessives, and that dose-response patterns for both physical and chemical mutagens were of a similar nature as found for barley (Hordeum vulgare) and other diploids.

In the 1950's, mutation breeders were using induced mutants in legume breeding with the same frequency and success as in other commercial crop species (Blixt and Gottschalk, 1975). In 1960, Heslot reported that some 12 leguminous pulse and forage species were being used in various mutation breeding programs worldwide. New mutant germplasm was being generated utilizing a wide range of mutagens in a number of species. Zachow (1960), working on tetraploid Lupinus luteus L., obtained a number of recessive mutants useful in breeding for increased total yields. Cooper and Gregory (1960) bred for disease resistance in the tetraploid pulse legume, the peanut (Arachis hypogaea), and obtained strains resistant to leaf spot after several selection cycles from X-irradiated seed. Bilquez and Martin (1961), also working with the peanut, demonstrated that the response of this species to X-irradiation was genome specific as they found significant differences in treatment sensitivity between various synthetic varieties. Van der Walt (1962) was unsuccessful in obtaining disease resistant peanuts after seed X-irradiation, but Bilquez (1962) reported that he was successful. Bilquez found that the success of induction of disease resistance was highly dependent on the specific

germplasm used; some varieties could easily be induced to produce disease resistance to a number of pathogens, whereas other varieties failed to respond. On the diploid level, Wellensiek (1961) found that thermal neutrons induced the same frequency and types of mutants in species of Pisum as reported in other species previously, and Sjödin (1962) observed that the X_1 sterility and X_2 mutation rates of X-irradiated Vicia faba were comparable in response to other diploid species.

By 1965, there were 242 new mutants of Lupinus and some 11,000 of Arachis (Gustafsson and Gadd, 1965a, 1965b). Amongst the most useful mutants up to this time were those of Lupinus showing low or zero levels of stem and leaf alkaloids, and the «bunchy top» highly productive mutant of the peanut Arachis hypogaea (Gustafsson and Gadd, 1965a, 1965b). Ashri and Goldin (1965) found that diethyl sulfate (DES) was a particularly effective mutagen for leguminous species since a number of quantitative mutants in the peanut were induced of high agronomic value. Sharma and Rapoport (1965) were the first to produce successfully a dominant mutation of agronomic value in a legume. Using X-irradiated peas (Pisum sativum), they obtained a stem modification which gave altered seed yields. Their findings indicated that dominant mutations of a useful nature could be obtained in some leguminous species, thus bypassing the need to obtain progeny over several generations in order to recover segregating recessive mutants.

Studies on induced quantitative variation in legumes continued with Fierlinger and Vlk (1966) who studied quantitative variants in Pisum and other legumes utilizing a number of physical and chemical mutagens. Their findings indicated that a large variety of mutants could be induced with altered flowering times and seed yields. Blixt et al. (1966) studying induced quantitative variance in Pisum developed a method for obtaining optimum levels of induced variability. Mutant lines demonstrating superior seed yields in the peanut were successfully obtained by Patil (1966). High forage yielding mutants were obtained in Trifolium and Melilotus species, and high seed yielding mutants in Pisum and Phaseolus species (Scheibe and Micke, 1967). Other induced mutant types of agronomic value included high forage yield mutants of Lupinus albus (Porsche, 1967), and of Lupinus and Melilotus species (Jashovsky and Golovchenko, 1967) and early flowering mutants of Glycine max (Kawai, 1967; Matsuo and Yamaguchi, 1967).

The differential effects of various types of physical and chemical mutagens were studied in depth in Pisum sativum by Blixt and Mossberg (1967). They found that equivalent doses of the mutagens tested did not result in similar responses, even for mutagens of a chemically or physically similar nature. Responses to treatment were mutagen and dose specific; they devised a general method for determining the optimal dose for the most effective mutagen. Gottschalk (1968a), also working with Pisum, found that closely-linked genes could undergo simultaneous induced mutation after exposure to physical and chemical mutagens. Genes controlling meiotic pairing and

other meiotic processes could also be altered in Pisum via mutation induction (Gottschalk, 1968b). Further work on the processes of mutagenesis in Pisum continued with Gelin (1968) who demonstrated that the pH of the treatment solution as well as the duration of treatment could significantly affect the mutation rate in the progeny of treated plants. This work was confirmed by Sidorova (1968) in a similar experiment with the same species. The effects of X-rays and EMS on Pisum were studied with respect to type and frequency of mutations induced in the progeny of plants treated as seed by Tageeva et al. (1968). They found that the most frequent mutations induced by both mutagenic agents were various chlorophyll-deficient types. These mutants displayed both nuclear and cytoplasmic inheritance independently, and tended to be inherited in a recessive manner.

Effects from mutagenic treatments similar to those found in Pisum were also confirmed in other leguminous species. Kleinhofs et al. (1968) recovered six different types of chlorophyll-deficient mutants in Melilotus alba treating seeds with EMS. Chopde (1969) also recovered a high frequency of chlorophyll-deficient mutants in X-irradiated Cajanus cajan L., and Sharma (1969) obtained similar results after treatment of Vigna sinensis (L.) Savi. seeds with EMS. Along with chlorophyll-deficient mutants, Sharma (1969) recovered plants of Vigna which contained a wide range of mutations which demonstrated polymeric genetic properties for total yields. This polymeric genetic property was induced in a number of other leguminous species with various mutagens. A mutant type affecting seed yield was

produced in the progeny of seeds of Phaseolus aureus Roxb. treated with a number of physical and chemical mutagens (Santos, 1969). From these same experiments, Santos obtained a number of morphological and chlorophyll-deficient mutants. Moh (1969) successfully obtained a large number of mutants for a single character, namely, seed coat color, in the common bean Phaseolus vulgaris. These mutants were induced by treating seed with EMS.

Sigurbjörnsson and Micke (1974) in reviewing mutation breeding in legumes and other crops up to 1971 found that mutants of agronomic value were successfully recovered in the following genera: Pisum, Phaseolus, Medicago, Melilotus, Lupinus, Vigna, Vicia and Arachis, of which 21 induced mutant varieties were catalogued for these genera. Sidorova et al. (1969) have listed 14 agriculturally useful mutant types in a single genus, namely, Pisum.

Studies on the effects of induced mutagenesis in legumes continued with Blixt (1969) who, while working with Pisum, found that responses to irradiation in individual plants arising from treated seed were specific to the genomic background of each individual plant. Mutation rates were found to differ significantly between individuals of different genomes that were completely interfertile. Gottschalk and Müller (1970) found that induced mutants from a single locus for seed coat protein in Pisum gave multiple protein types and patterns. Using X-rays as the mutagen, and seed as treated material, they found that the progeny displayed mutant forms that were pleiotropic for the deposition and conformation of seed coat protein. The non-mutant form of the

character had been established as being controlled by a single locus. Gottschalk (1970) also found that a heterotic effect could be induced for seed yield by means of X-rays in completely self-fertile clones of Pisum which previously lacked this characteristic. A similar heterotic effect was obtained after X-irradiation by Shumnyi et al. (1970) and independently by Rod and Vagnerova (1970).

Many other mutant types were successfully obtained in other leguminous species. Boreyko (1970) obtained high-yielding, morphological and chlorophyll-deficient mutants in the soybean (Glycine max) using various physical and chemical mutagens. Malchenko (1970) and Gengenbach et al. (1970) obtained similar mutants in Glycine using EMS which behaved as monogenic recessives. Similar chlorophyll-deficient mutants were induced by Hackbarth (1970) in Lupinus angusifolius L. using EMS and X-rays. Kaicker and Swarup (1971) obtained similar results in Melilotus alba from EMS treatment. De la Flor et al. (1971) found that the production of chlorophyll-deficient mutants could be altered by applying the mutagen at different stages of germination when they treated seeds of Phaseolus vulgaris.

Other mutants included a number of early-flowering types obtained in Medicago polymorpha var. polymorpha by Brock et al. (1971) from γ-ray treatment. Blixt (1972) induced similar mutants in Pisum using γ-rays and EMS. Mutants of a biochemical and quantitative nature were also obtained for a number of Pisum mutants with altered seed protein content. Mehanjiev (1974) obtained similar mutant types in Pisum, and Khan and Veeraswamy (1974) using various mutagenic agents obtained a

large number of qualitative and quantitative mutants for seed yield and related characters in gram (Cajanus cajan (L.) Millsp.). One particularly useful mutant was fasciata obtained in Pisum by Gottschalk and Hussein (1975). This mutant which was found in the progeny of X-ray treated plants displayed a quantitative mode of inheritance. Fasciata increases branching of the lateral stems of a plant thereby increasing the number of flowers and subsequently the number of pods each plant bears. The fasciata character was most useful in transforming low seed yielding lines of Pisum into higher yielding ones. Sidorova et al. (1975) found that by treating a fasciata mutant of Pisum with EMS the progeny of the treated mutants would have the fasciata character enhanced.

By the late 1970's many different types of mutants were successfully induced in a large number of leguminous species from a wide range of physical and chemical mutagens. The most frequent types were chlorophyll-deficiencies which a number of authors obtained for various leguminous species of agronomic value (Ronnenkamp et al. (1975) in EMS-treated progeny of Melilotus alba; Kiang and Halloran (1977) in EMS-treated Glycine max progeny; Nerkar (1976) in Lathyrus sativus progeny treated with various physical and chemical mutagens). All mutants induced were monogenic recessives, and in the case of Glycine max, the mutants arose independent of dose effects.

Aside from chlorophyll-deficient mutants, a number of other mutant types were induced with relatively high frequency in leguminous species. Many of these could be classified as morphological mutants inherited either in a qualitative or quantitative fashion, and a number

were of agronomic value. These included an EMS-induced multicarpalate condition in Cajanus cajan (Venkateswarlu et al., 1976), seed-coat color variations in Phaseolus induced by a number of physical and chemical mutagens (Moh, 1976), and a number of high seed yielding mutants in Glycine max induced by X-rays, γ-rays, and EMS (Kotvics, 1976). High seed yielding mutants were also obtained from seed in the progeny of EMS-treated Vicia faba (Ismail et al., 1976) and a high seed yielding mutant of Pisum was also obtained from γ-irradiated plants (Jaranowski, 1976).

Mutants demonstrating detrimental effects on yields and other agronomic components were also produced. A number of these mutants were studied in detail; these included an X-ray induced plasmon variant of Arachis hypogaea obtained by Ashri and Levy (1977) which was inherited in a quantitative fashion. This mutant retarded growth rate, thereby decreasing the overall seed yield. Kiang and Halloran (1977) found a somewhat similar mutant in Glycine max which exhibited slow growth and dwarf characteristics, as well as early flowering. Unlike the Arachis mutant of Ashri and Levy (1977), the dwarf mutant of Glycine was inherited as a monogenic recessive and was linked to the early flowering character.

A number of superior seed yielding mutants were produced also at this time in several leguminous species. Amongst these was an X-ray induced Arachis hypogaea mutant which showed an altered stem growth habit (Patil and Mouli, 1978). The stems of these plants were heavily branched, had shortened internodes, and were termed «bunchy top.» The

altered stem morphology allowed for seed yields that greatly surpassed those obtained from untreated plants of the same variety and pedigree. Sharma and Sharma (1978) reported X-ray induced mutants of the lentil Lens culinaris Medic. The mutants demonstrated a quantitatively inherited alteration of pod and seed size, with greater seed number and seed and pod size than untreated plants resulting in superior seed and pod yields.

One specific type of morphological abnormality induced in several leguminous species was the floral mutant. Male-sterile mutant lines have been produced in Glycine max by Chaudhari and Davis (1977), in Vigna sinensis by Reddy et al. (1978), and a shrivelled floret type mutant of Glycine max by Singh and Jha (1978).

Cytological abnormalities accompanying mutational events were also noted in a number of leguminous species. Gottschalk and Baquar (1971) obtained a mutant in Pisum sativum after X-irradiation in which desynapsis was observed at metaphase I. The condition was inherited over several generations and appeared to be a recessive character. Multivalent associations, translocations and bridges were observed in meiotic cells of the progeny of the mutant plants.

Cytological irregularities resulting from X-irradiation were noted in a number of other species. Shaikh and Godward (1972) observed numerous meiotic irregularities after X- and γ-irradiation of Lathyrus sativus and Vicia ervila. These included micronuclei, fragments and bridges; they persisted over several generations and were proportional to the mutation rates obtained. Similar effects have been observed for

a wide range of leguminous and non-leguminous species (Shevchenko, 1968).

Other cytological and cytomolecular phenomena were observed in several leguminous species accompanying mutagenic treatment and subsequent mutational events. Nagy-Porpaczy (1974), after treating seed of Pisum sativum with various physical and chemical mutagens, found a linear relationship between mitotic and meiotic aberration rates. Reiger et al. (1975) noted that specific regions of the chromosomes of Vicia faba were affected by the action of X-rays, EMS, and other chemical mutagens. This indicated that the chromosomes of this species had regions which were more sensitive to clastogenes. Schubert and Reiger (1977) showed that where breakage of the chromosomes occurred they were near regions which contained mutant loci. Following microbial geneticists, they termed these affected regions «hot spots» and demonstrated that, for this species, clastogenic effects from exposure to mutagens was the cytological expression of a mutational event. This work was confirmed by Rekhmatulla and Gostimskii (1976), who obtained similar results in Pisum sativum from EMS treatment. Narsinghani and Kumar (1976) found that the application of the plant hormone gibberellic acid (GA) after exposure of actively dividing plant tissue to the mutagen EMS, effectively reversed the clastogenic effects of the mutagen. They believed the nullification of the clastogenic effects of EMS was caused by the alteration of the cell cycle, via prolongation of the cycle by GA.

The greatest number of induced mutants resulting from exposure to various physical and chemical mutagens has been visible mutants with altered morphology (Sigurbjörnsson and Micke, 1968; Brock, 1969). In leguminous species, some 1733 mutants affecting morphological characters have been induced in six species between the period 1930 and 1974 (Blixt and Gottschalk, 1975). Since that time, further studies on induced mutagenesis in legumes have produced additional mutant types in a number of species, including some novel mutants, which has helped to further the knowledge on mutagenesis in legumes. Narsinghani (1976) produced a de nova leaf mutation in Pisum, utilizing EMS and MMS (methyl methanesulfonate) as mutagens. The mutant displayed fused leaflets and the character was inherited as a monogenic recessive. Motto et al. (1975) found chimaeric stem formation in the M_1 of EMS-treated Phaseolus vulgaris. The abnormal stems produced seed that gave rise to progeny with a high mutation rate. The greatest number of mutants were chlorophyll-deficiencies. None of the progeny displayed the chimaeral stem characteristics of the parents. Similar results were obtained by Kaul and Matta (1976) in Pisum from EMS seed treatment in which the progeny of morphological variants in the M_1 had a higher mutation rate than progeny obtained from avariant types. Tai et al. (1977) found that three different types of chlorophyll-deficient mutants, produced in the progeny of EMS-treated Arachis hypogaea, were on the same linkage group. Linked mutant genes with similar phenotypic expressions were also obtained in EMS-treated Pisum sativum by Kleinhofs et al. (1968) and Gottschalk (1979). These results were

similar to those obtained by Prasad and Prasad (1977) in EMS-treated Phaseolus species. Yankulov et al. (1979) found that the expressivity of induced, linked mutant genes in Phaseolus species was dependent on the specific genome of the plants involved. They found that a substantial difference existed for different varieties of Phaseolus vulgaris, the different varieties producing dissimilar numbers of linked mutant characters.

The knowledge on induced mutagenesis in leguminous species is fairly extensive, particularly in diploid taxa (Blixt and Gottschalk, 1975). Extensive work on mutation genetics and breeding of leguminous species has been accomplished in the genera Pisum (Blixt, 1972), Lupinus (Gustafsson and Gadd, 1965a), Glycine (Zacharias, 1967), Vigna (Sharma and Bansal, 1972), Melilotus (Micke, 1958), Trifolium (Scheibe and Micke, 1967) and Arachis (Gustafsson and Gadd, 1965b). Of these, the most successful and extensive work has been conducted on the genus Pisum, primarily by Blixt and Gottschalk (1975). They obtained some 1040 qualitative mutants of Pisum from a long-range study using a large number of physical and chemical mutagens, and from which they were able to construct a fairly detailed gene map of the species by selective breeding techniques. They also obtained some 200 mutants of agronomic value, whose inheritance was largely quantitative. A fairly large number of genetically and agronomically useful mutants have been obtained in a number of other legumes. They include some 690 mutants in Melilotus alba (Scheibe and Micke, 1967); 650 mutants in Glycine max (Zacharias, 1967); 164 mutants in Lupinus albus (Porsche, 1967);

132 in Lupinus digitatus (Gladstones, 1958); and 97 mutants in Trifolium subterraneum (Carpenter, 1958). These mutants were largely morphological and were inherited disomically or as monogenic recessives, or displayed indeterminate quantitative modes of inheritance. The exceptions were mutants for Lupinus albus and L. digitatus, which were either tetrasomically inherited recessives or displayed quantitative inheritance. In addition to the two Lupinus species, two other polyploid species have been studied fairly extensively, namely the peanut Arachis hypogaea and alfalfa Medicago sativa, both of which are tetraploid. Most of the induced mutants obtained in these two species segregated in a complex fashion, indicating a quantitative mode of inheritance; the few qualitatively inherited characters were largely recessive (Gregory, 1955, 1960; Ashri and Goldin, 1965; Gustafsson and Gadd, 1965a, 1965b; Brock et al., 1971).

IV. MATERIALS AND METHODS

A. Mutagenic treatments

Fresh seed was obtained from the 1975 collection of cultivar «Mirabel» birdsfoot trefoil (Lotus corniculatus L.) from a single line licensed for cultivar breeder seed under the name «Apollo»; license no.1688 and certified May 6, 1976 under Dr. N. C. Lawson (Macdonald College). Due to the previous use of «Apollo» as a name for a forage cultivar, the name «Apollo» was changed to «Mirabel» in February, 1978. The seed was certified as Breeder seed of cultivar «Mirabel» by Dr. N. C. Lawson and the Department of Agronomy, Macdonald College, Quebec. (I am grateful to Dr. N. C. Lawson for providing the history of this cultivar.) From a 500 gram sample, lots of 100 seeds were placed in 5 x 10 cm paper envelopes; 75 envelopes were prepared. The envelopes were placed in a desiccator at 30°C until ready for use.

A second species was also studied, namely, Lotus tenuis Waldst. et Kit. One kilogram of L. tenuis (2n = 12) seed was obtained from Dr. R. R. Seaney (Cornell University) from plants which had undergone two cycles of selection in varietal trials at the University of California Field Experimental Station, Davis, California. From this accession, 15 samples of 100 seeds were taken at random, and stored in the same manner as given for L. corniculatus.

One mutagen, ethyl methanesulfonate, was used in the treatment of seeds of both Lotus corniculatus and L. tenuis, based on this

mutagen's known effects, potency, and widespread use in treating legumes (Blixt and Gottschalk, 1975) and other crop species (Sigurbjörnsson and Micke, 1974). Four other mutagens were selected for treatment of seed of L. corniculatus, based on their effectiveness and representing four major classes of mutagens, according to the classifications of Briggs (1970) and Heslot (1970). They included unfiltered X-rays, 8-ethoxycaffeine, n-hydroxyurea, and 2-aminopurine. Treatment of seed by each mutagen was carried out as follows.

1. X-rays

Fifteen envelopes, each containing 100 seeds, were removed at random from a desiccator and placed in five lots of three envelopes. Each envelope within each lot was labelled replicate 1, 2, and 3, respectively. The first set of three envelopes was labelled «0» and served as controls. Seeds in the second through fifth sets of three envelopes were subjected to X-rays at 15 cm from an X-ray unit. Acute X-rays were used (unfiltered) at 490 rads/min, until dosages of 3, 6, 9, and 12 Kilorads* were obtained. Seed treatment was carried out by Dr. Beatrice L. Murray, Research Branch, Central Experimental Farm, Ottawa, Ontario. Each lot of 100 seeds was planted according to treatment and replicate in 50 x 75 cm flats filled to a depth of 4 cm with a peat: perlite:humus mixture in a 1:1:2 ratio. Individual flats were labelled according to treatment and replicate, and placed on standard 10 x 2 meter benches in a randomized complete block design (Steel and Torrie, 1960). Seeded flats were maintained in the greenhouse at a temperature of 12°C night and 25°C day and a 12-hour photoperiod until emerging seedlings were at the four-leaf stage.

*Kilorad = 1 x 10^5 ergs/g absorbed dose; the equivalent transmitted dose in Kiloroentgens (kR) = 9.8 x 10^5 ergs/g for hard X-rays (Bacq and Alexander, 1961).

2. Ethyl methanesulfonate

Ethyl methanesulfonate (EMS) was obtained in liquid form from the Eastman Kodak Company as a pure reagent. Ten ml of EMS were placed in 90 ml of glass-distilled water, forming a 10% (volume:volume) solution. The solution was adjusted to a pH of 6.0, using 1 N sodium hydroxide. The solution was kept in a brown glass-stoppered bottle at 20°C, for no more than one-half hour from initial mixing to final use. From this initial solution, 1 ml was extracted with a 1 ml graduated pipette and placed in a 20 ml square culture jar with a plastic screw-cap. To this was added 9 ml of glass-distilled water. The procedure was repeated three times to complete the entire seed treatment. Using the original 10% solution, lots of 1 ml were extracted with a graduated 1 ml pipette and placed in 100 ml and 1,000 ml volumetric flasks; the flasks were then filled to volume with glass-distilled water. Each solution was adjusted to a pH of 6.0, using 1 N sodium hydroxide. Ten ml were then extracted from the 1,000 ml solution, using a graduated 10 ml pipette, and placed in a 100 ml volumetric flask. The flask was then filled to volume with glass-distilled water and the pH adjusted to 6.0. Ten ml aliquots were extracted from each volumetric flask and placed in 20 ml square culture bottles with screw caps. The procedure was repeated three times. Thus there were 12 bottles in lots of three, containing solutions with concentrations of 1.0, 0.1, 0.01, and 0.001% EMS (volume:volume), respectively. Three bottles were filled with 10 ml of glass-distilled water, adjusted to a pH of 6.0, and served as controls. Each of the three bottles for each concentration was labelled

replicate 1, 2 and 3, respectively. Fifteen envelopes were removed from the desiccator and 100 seeds from each envelope were placed in one of 15 bottles. The bottles were capped and placed in the dark at 30°C for six hours. The bottles were then removed, the solutions extracted, and the seeds rinsed in continuously running tap water for two periods lasting 15 minutes each. The tap water was adjusted to a temperature of 20°C ± 3°C. Seeds were then removed from the bottles and placed in individually labelled 10 cm petri dishes containing 9 cm no.3 Whatman filter paper. After one-half hour of drying at room temperature, seeds were transferred according to treatment and replicate to flats and grown in conditions as given for the X-ray treated seed until the emerging seedlings were at the four-leaf stage.

Treatment of Lotus tenuis seeds proceeded as follows. Nine solutions of ethyl methanesulfonate were prepared in the manner as given earlier for L. corniculatus. The concentrations used were 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, and 2.0% (volume:volume). The solutions were adjusted to a pH of 6.0 using 1 N NaOH; three 10 ml aliquots were extracted from each solution. Each aliquot was then placed in a 20 ml capacity culture bottle and labelled according to concentration and replicate 1, 2 or 3.

A total of 30 bottles was placed for six hours in the dark at 25°C. All bottles were then removed, and the liquid portions extracted. Bottles and contents were rinsed twice for 15 minutes each in running tap water. The seeds from each bottle were placed in 10 cm petri dishes, fitted with 9 cm Whatman no.3 filter paper. Seeds were

air dried for one-half hour and transferred into envelopes labelled with the appropriate treatment and replicate. Seeds were then surface broadcast in flats. Flats were kept under nylon mesh to isolate plants from pollinating insects. Plants were allowed to mature, flower, and produce pods from selfing for a period of six months. During this period, no selfed pods were produced, and the plants were transferred to the field (summer of 1979) for one month until pod production from cross-pollination was established. Mature pods were harvested and stored in envelopes in a dessicant-equipped cabinet at approximately 25°C until ready for use.

3. 8-ethoxycaffeine, n-hydroxyurea, and 2-aminopurine

Solutions of 10% aqueous 8-ethoxycaffeine (EC), n-hydroxyurea (HU), and 2-aminopurine (AP) were prepared by dissolving 10 g of each compound into 100 ml volumetric flasks containing glass-distilled water, which were filled to volume after the addition of each chemical. Aliquots of 10 ml were extracted from each flask and transferred into 100 ml volumetric flasks which were filled to volume with glass-distilled water. This yielded a 1% (weight:volume) solution of each compound. From each flask containing a 1% solution, 10 ml aliquots were removed and individually placed into 100 ml volumetric flasks. Another 10 ml was removed from each of the 1% flasks of EC, HU and AP solutions, and placed into 1000 ml volumetric flasks. Both the 100 and 1000 ml groups of flasks were filled to volume with glass-distilled water to give solutions of 0.1 and 0.01% (weight:volume) for each chemical. From

each of the 1000 ml flasks containing 0.01% solutions, 10 ml aliquots were removed and individually placed into 100 ml volumetric flasks, which were filled to volume, giving solutions of 0.001% (weight:volume) of each chemical. The 10% solutions were discarded, and the remaining solutions of 1.0, 0.1, 0.01, and 0.001% EC, HU, and AP were appropriately labelled and adjusted to a pH of 6.0 For each concentration of each solution, three 20 ml square culture bottles with screw caps, were filled with 10 ml aliquots of the individual solution and labelled with chemical and concentration. As well, each of the three bottles per concentration for each chemical was labelled replicate 1, 2 or 3. For each chemical, an additional three bottles were filled with glass-distilled water (pH 6.0) which served as control replicates. Thus, for each chemical, there were 12 bottles containing solutions of 1.0, 0.1, 0.01, 0.001% (weight:volume), replicated three times per concentration and three bottles containing glass-distilled (pH 6.0) water which served as controls.

The remaining 45 envelopes each containing 100 seeds were removed from the desiccator, the seeds were placed in the bottles of solutions and each bottle was capped. The bottles were then placed in the dark at 30°C and incubated for six hours. After incubation, the individual solutions were removed and the seeds rinsed twice in running tap water at 20° ± 3° for 15 minutes each rinse. The seeds were then removed from the bottles and placed on filter paper in petri dishes. The seeds were air-dried for one-half hour at room temperature, after which each lot of seeds was sown by broadcasting on

individual flats. The germinating seeds were maintained under growing conditions as described for the X-ray treated seeds until the emerging seedlings reached the four-leaf stage.

B. Propagation and breeding of treated material

Flats containing the treated seedlings were screened at the four-leaf stage for the number of seedlings per 100 seeds sown per flat. Values obtained were tabulated according to treatment and replicate and statistically analyzed. The methods used for the statistical analyses are described in Section O.

After determining the number of seedlings in each flat, the seedlings were transplanted, four per pot to standard 15 cm pots containing 2:1:1:1 humus:peat:vermiculite:sand. Each pot was placed in a randomized complete block design on benches in a greenhouse, with a day/night temperature of 25/12°C and a photoperiod of 16 hours. Plants were fertilized regularly at two-week intervals with water-soluble commercial fertilizer with an NPK value of 20-20-20 until mature. At this time, each bench was completely enclosed in nylon netting with a mesh size of 2 mm so that plants could be excluded from pollinating insects. Then an application of fertilizer with an NPK value of 0-30-30 was applied at two-week intervals to assist in initiation and maintenance of flowering. The plants were designated as belonging to the M_1 generation, according to the convention proposed in the Manual on Mutation Breeding (1970) for mutagenically treated parental lines and progeny.

All M_1 plants were allowed to flower under nylon enclosures for six months to allow for self-seed set. All handling of plants was kept at a minimum in order to prevent mechanical selfing. During the six months of flowering, all ripe pods were harvested and placed in 5 x 10 cm paper envelopes labelled with treatment, replicate and plant number. Data were collected on number of pods per plant, number of seeds per pod, and total number of seeds and pods produced per plant per treatment for all treatments for each mutagen and statistically analyzed. Pods and seeds were placed in a desiccator at 30°C and dried for six hours after harvest, and then stored in a cabinet equipped with a desiccant at 20°C until ready for use. Seeds obtained in this fashion were designated M_1S seed and the progenies from this seed were designated M_2S.

1. Selfed progeny

M_1S seed formed the first selfed progeny of the M_1 or the initially treated parental line. Only the control M_1 generation and the EMS-treated lines produced enough viable seed to provide sufficient mature progeny for statistical testing. Therefore, M_1S control and EMS-treated seeds were sown in flats in a manner and under environmental conditions as described for the X-ray treated seed until the seedlings were at the four-leaf stage. Flats were individually screened for the total number of emerging seedlings per 100 seeds sown per flat, and the number of variants for leaf anomaly and chlorophyll deficiency per flat according to treatment and replicate and the data statistically analyzed as described in Section O.

Seedlings of the M_2S generation were transferred in groups of four from flats to 15 cm pots which were placed on benches in the greenhouse under nylon netting in a randomized complete block design. Plants were subjected to a day/night temperature of 25/12°C with a photoperiod of 16 hours to promote flowering. Water soluble fertilizer with an NPK value of 20-20-20 was applied. When plants were mature, the fertilizer was changed to an NPK value of 0-30-0 to stimulate and maintain flowering. Flowering was maintained for six months during which time the mature pods obtained from spontaneous selfing were harvested and placed in 5 x 10 cm paper envelopes. During the period of flowering, manipulation of the plants was minimized in order to avoid obtaining mechanically self-pollinated seed set. Data collected on plants producing pods included number of pods per plant, number of pods per treatment, and number of seeds per pod in each treatment of the EMS-derived M_2S seeds and controls. Data were statistically analyzed as for the M_1S generation.

Pods or seeds of M_2S EMS-derived plants, plus those of their respective controls were placed in 5 x 10 cm envelopes, dried at 30°C for six hours, and then stored at 20°C in a desiccant-equipped cabinet until ready for use. After harvesting was completed, seeds were removed from the pods by hand and separated in lots of 100 seeds according to treatment and replicate. This seed was designated as M_2S generation seed, and the subsequent progeny was designated M_3S. Each lot of 100 seeds was surface broadcast on flats in a manner and under environmental conditions as described for the X-ray treated seed.

When the seedlings reached the four-leaf stage, they were screened for the number of emerging seedlings per 100 seeds sown per flat, and for any morphological or chlorophyll-deficient mutants, and the data were statistically analyzed as for the M_1S generation.

M_3S plants at the four-leaf stage were then transplanted in groups of four into 15 cm pots except those plants suspected of being mutants which were placed in single pots. The pots were labelled according to generation, treatment, replicate and parental type and returned to a bench in the greenhouse, under nylon netting, with temperature, photoperiod and fertilizer treatments as for the earlier generations. Plants were allowed to flower for six months. During this period mature pods were harvested and placed in envelopes labelled with treatment, replicate, generation (M_3S), and a number corresponding to the parent plant. Data collected on pod production included number of pods per plant per treatment and number of seeds per pod. M_3S plants did not produce enough seed to yield an adequate number of progeny that would produce valid statistical comparisons; therefore, experimentation was terminated at the M_3S generation.

Plants from the M_1, M_2S and M_3S generations were grown together under identical conditions in a greenhouse, in a randomized complete block design. Treatments for all three generations were screened for leaf, stem, and floret anomalies, as well as number of plants surviving to maturity vs. initial number of 100 seeds sown, for each replicate and treatment in all three generations. The data were then statistically analyzed.

In the spring of 1978 all plants in the greenhouse were cut back to a height of 15 cm and placed out of doors in 5 x 5 meter cold frames. Temperatures ranged from approximately 7 to 20°C. Plants in the M_2S and M_3S generations suspected of being mutants were maintained in the greenhouse until further experimentation could be conducted. Plants placed in the cold frames were maintained for approximately two weeks until acclimatized. All plants were then transplanted to the field in a heavy clay loam soil. Plants were placed in plots 3 x 6 meters with 30 cm between plants and 60 cm between rows. One hundred plants per plot were replicated for a total of three replicates per treatment. There were five treatments per mutagen per generation, which included three replicates of controls, M_1, M_2S and M_3S plants, all planted in adjacent areas in the field; in each area plants were placed in a randomized complete block design with each replicate labelled according to generation, treatment (or control), and replicate number.

The total number of plants in the field for all three generations, including controls, was 7,500 for the M_1 generation (3 replicates of 100 plants each for 5 dosages and for 5 mutagens), 1,200 for the M_2S generation (3 replicates of 100 plants each for 4 dosages of EMS-derived and control progeny), and 900 for the M_3S generation (3 replicates of 100 plants per replicate for 3 dosages of EMS-derived and control progeny). M_2S and M_3S generations were screened for plants exhibiting chlorophyll deficiencies, and floret and stem anomalies. The number of anomalous plants was recorded for each replicate of each treatment and the data were statistically analyzed.

2. Crossed progeny

a. Lotus corniculatus

M_1, M_2S, and M_3S plants which were transplanted to the field in the spring of 1978 were allowed to grow to maturity over a period of two and one-half months. At the onset of flowering, all plants within each plot were cut back to a height of 30 cm weekly, except for control plots and the plants for one treatment for each generation which were allowed to flower. This procedure was repeated for each treatment, thus allowing only the replicates of each treatment to be in flower simultaneously. No control of insect visitations was attempted. Mature pods were harvested in bulk on a plot basis four weeks after the beginning of flowering in each plot. This time frame was partially based on the time required for pod maturity from initial pollination to the development of mature pods as had been determined for Lotus corniculatus (MacDonald, 1946). Pods were threshed in a circular hand thresher with a 75 cm diameter screen with a pore size of 4 mm. Each lot of threshed material was placed individually in envelopes and each envelope was labelled with the corresponding generation, treatment and replicate. The pods from each envelope were finely threshed on a standard 50 x 50 cm gravity table. Seeds of uniform weight, 1.0 ± 0.05 mg, were returned to their respective envelopes. All other seeds and extraneous matter were discarded. Envelopes containing seeds were dried at 30°C for six hours and then placed in a cabinet equipped with a desiccant at 20°C until ready for use.

Selectively cross-pollinated seeds of the M_1, M_2S, M_3S generations were identified as M_1, M_2S and M_3S seed, respectively, and plants grown from these seeds formed the M_2X, M_3X and M_4X generations, respectively. A total of 100 seeds was selected at random from each replicate of each treatment for each generation, and surface broadcast in flats in a manner and under environmental conditions as described for the X-ray treatment. After two weeks the number of germinated seedlings was counted, the number of surviving seedlings counted again at the four-leaf stage, and the data statistically analyzed.

At the age of six weeks (approximately the eight-leaf stage) all M_2X, M_3X and M_4X plants were cut back to a height of 15 cm and placed in cold frames out of doors for two weeks to allow plants to harden before being transplanted to the field. During this period, the temperature ranged from 5 to 18°C. Plants of all three generations were transplanted to the field in the late spring (May 30) of 1979 in plots adjacent to the M_1, M_2S and M_3S plants already present. Plants of each replicate for each treatment were placed in 3 x 6 meter plots in a randomized complete block design. The plants were placed within each plot in rows 30 cm between plants and 60 cm between rows. A total of 100 plants was initially planted per plot. The M_2X generation had a total of 7,500 plants with three replicates of 100 plants per replicate, for five dosages each of the five mutagens, including controls. The M_3X generation had a total of 900 plants with three replicates of 100 plants per replicate, for three dosages of one mutagen, EMS, including controls. The M_4X generation did not survive to the transplanting stage and, therefore, was discounted.

At the onset of maturity, one month after transplanting, all M_2X and M_3X plants were screened for number of survivors to maturity per plot (from 100 plants initially planted per plot), number of plants per plot with leaf and floret anomalies, chlorophyll deficiencies, and number of plants demonstrating dwarf growth characteristics. All data were statistically analyzed.

At one-week intervals, after the onset of flowering, plots were selectively cut to a height of 30 cm, starting with control M_2X and M_3X plots, allowing one treatment for each mutagen, or control, to go uncut and to flower. All other plots were kept trimmed to a height of 30 cm. Plants in each treatment were allowed to flower successively, starting with the controls and continuing with the lowest to highest dosages of each mutagen in the order of X-rays, EMS, EC, HU and AP derived M_2X and M_3X plants. This allowed only the three replicates within a single treatment to flower simultaneously. Plants were allowed to be openly pollinated by indigenous insects. Mature pods were harvested one month after the onset of flowering. Pods were harvested on a per plot basis, threshed as described for the M_1, M_2S and M_3S generations and seeds of uniform 1.0 ± 0.05 mg weight were selected and stored.

Seeds obtained from selectively open-pollinated M_2X and M_3X plants were labelled according to treatment and plants grown from these seeds were labelled as the M_3O and M_4O generations, respectively. At the same time, non-selectively open-pollinated seeds were obtained from the M_2S and M_3S generations by harvesting pods in bulk on a

per-plot basis, in mid-August of 1979. Mature pods obtained from each plot were hand-threshed as previously described and seeds of uniform weight of 1.0 ± 0.05 mg were retained. Progeny resulting from these seeds formed the M_3I and M_4I generations, respectively.

Ten 1000-seed samples were chosen at random from each of the M_3O, M_4O, M_3I, and M_4I generations and the mean weights calculated for each generation. These weights served as the average 1000-seed weight.

Approximately 1,500 seeds were saved (by weight estimate) from each replicate of each treatment for each of the four generations. Each lot of 1,500 seeds was broadcast-surface seeded in the field, with the aid of a 1 x 1 meter screen with a 4 mm mesh, on 1 x 1 meter plots. The plots were arranged in a randomized complete block design. All plots were artificially irrigated at the rate of approximately one liter per m^2 per minute for three hours daily for three consecutive days after sowing. Seeds were initially sown in June 1980, and by July, seedlings had attained the four-leaf stage. At this stage, the number of emerging seedlings was counted in each flat for each treatment of each generation, as well as the number of chlorophyll-deficient plants, and the data were statistically analyzed.

b. Lotus tenuis

M_2X seeds obtained from crossing M_1 EMS-treated Lotus tenuis (in the same manner as L. corniculatus) were surface broadcast in flats at the rate of 100 seeds per flat. The flats were placed in the greenhouse in a randomized complete block design under temperature,

photoperiod and fertilizer regimes as previously described. At the four-leaf stage, the number of seedlings in each flat was counted and again when the seedlings reached the eight-leaf stage (approximately one month). All seedlings were screened for chlorophyll deficiencies and other anomalies of stem and leaves. The total number of anomalies per replicate per treatment was ascertained. When plants were mature and in full flower, each replicate was screened for number of plants with abnormal floret formation. All data were statistically analyzed.

C. Cytology

1. Lotus corniculatus

From all plots in all parental and filial generations, ten inflorescences were collected at random from ten fully mature plants. Individual inflorescences were selected which contained at least three floral buds with a maximum length of 3 mm. The ten inflorescences for each group were placed in shell vials (5 ml capacity) containing 3 parts 95% ethanol:1 part glacial acetic acid (Carnoy's fixative; Carnoy, 1886). Vials were stoppered with corks and individually labelled according to generation, treatment, and replicate. Florets were fixed for 24 hours at 2-5°C. After the removal of the fixative, the contents of each vial were rinsed under running tap water for two 15-minute periods. Four milliliters of 70% aqueous ethanol were added to each vial which was sealed tightly with cork stoppers. Vials were stored at 2-5°C until ready for use. A total of 309 vials of florets was collected from nine generations comprising 103 treatments.

Inflorescences were prepared for staining by removal of the ethanol and rinsing for 15 minutes under continuous-flowing tap water. After rinsing, 4 ml of 1 N hydrochloric acid (HCl) were added to each vial and placed in a thermostatically-controlled water bath at 60°C for a 10-minute hydrolysis. Each vial was then removed from the water bath, and the hot HCl rapidly evacuated by means of a syringe-fitted suction pump. The inflorescences were rinsed for 15 minutes in continuous-flowing tap water, and then stained with leuco-basic fuchsin according to the procedure first determined by Feulgen (1926) for DNA specific staining.

The stain was prepared by dissolving 5 g leuco-basic fuchsin (Gurr, England) in 500 ml of 1 N HCl at 60°C, using a magnetic stirrer. The solution was maintained at 60°C for 20 minutes, then filtered through a Buchner funnel (10 cm diameter) containing activated charcoal to a depth of approximately 3 cm. The filtered solution was chilled in an ice-water bath for 10 minutes and filtered again into an evacuation flask. The Feulgen reagent was stored in a brown glass bottle with a plastic screw-type cap at 2-5°C until required.

Staining of inflorescences was accomplished by adding 4 ml Feulgen solution to each vial, tightly fitted with cork stoppers, and placing the vials in the dark at 30°C for 30 minutes. The stain was removed from each vial by a syringe-fitted suction pump, and vials and contents were rinsed for 15 minutes twice in continuous-flowing tap water. After rinsing, 70% ethanol (aqueous) was added to each vial and cork stoppered.

For cytological examination, florets were individually placed on a standard microscope slide and covered with two drops of 45% aqueous acetic acid. Anthers were dissected from each floret with the aid of a dissecting microscope, the remains of the florets were discarded, and the anthers were macerated with fine needles. After maceration, one drop of 2% aceto-carmine stain was added and a coverslip placed on each preparation. The aceto-carmine stain was prepared by dissolving 2 g carmine (Gurr, England) in 45% aqueous acetic acid, at 60°C for 15 minutes. The solution after being cooled for five minutes in an ice-water bath, was filtered into an evacuation flask fitted with a Buchner funnel.

Further maceration was carried out by tapping the coverslip with a dissecting needle while holding the coverslip in place with curved flat-tipped forceps. Each preparation was squashed by placing the preparation under a hand-operated spring-type press. Excess fluid was removed from the coverslip and the coverslip was sealed in paraffin. Preparations were individually examined with a Zeiss photomicroscope at a magnification of 650 X with phase-contrast optics. Scanning proceeded from the upper left-hand corner of each coverslip, across to the upper right-hand corner, and continued in a series of parallel lines until the entire field was scanned. The calibrations on the microscope stage were used as a guide. For each slide, the total number of metaphase I (MI), anaphase I (AI), and quartet (Q) cells were scored, along with each cell displaying one or more of the following aberrations: fragments, lagging chromosomes, bridges,

micronuclei, and pairing anomalies. For each of the three replicates for each treatment in each generation, scanning proceeded until 100 cells for each stage had been scored. Data were statistically analyzed on the basis of number of aberrant cells per total number of cells scored.

2. Lotus tenuis

One umbel was removed from each of ten M_1 plants at random from all replicates growing in the field for each treatment. Umbels with florets measuring approximately 3 mm were chosen and fixed individually in 3:1 95% ethanol:glacial acetic acid for 24 hours at 2-5°C. The procedure for staining and examination has been described earlier. Preparations were examined for cells at metaphase I (MI), anaphase I (AI), and quartet (Q) stages, and cells were scored as normal or aberrant for the following: multivalents, laggards, fragments, bridges, and micronuclei. A total of 100 cells was scored for each stage in each replicate, giving a total of 300 cells scored per replicate, 900 cells scored per treatment, and 9,000 cells scored for the 10 treatments in total. The data were expressed as per cent aberrations per replicate per treatment. The statistical procedures were described previously.

D. Pollen stainability studies

Ten plants were randomly selected from each replicate for each treatment in each generation and one mature floret was removed from each plant at random. Mature pollen was extracted from each floret by forcing the pollen through the keel-tip of the floret onto a standard microscope slide. Slides were individually labelled according to generation, treatment, and replicate, and a drop of 5% fast-green lactophenol was added to each slide to stain the pollen. The fast-green lactophenol stain was prepared by first dissolving 5 g phenol in 50 ml each of lactic acid and glycerol. To this solution was added 5 mg of fast-green stain.

After the addition of fast-green lactophenol stain to each slide, the pollen was vigorously mixed with the stain using a wooden toothpick. A new toothpick was used for each preparation. A coverslip was added and the pollen was examined with a Zeiss photomicroscope at 125 X magnification. Stained and unstained pollen grains were scored until a total of 1,000 pollen grains had been scored for each preparation. The number of stained pollen was recorded for each replicate of each treatment within each generation. A total of 309,000 pollen grains was scored from 103 treatments over nine generations.

Data for each replicate of each treatment, as per cent unstained pollen, were first transformed by taking the arcsin of the square-root of the percentage values (Steel and Torrie, 1960) and the transformed data were then statistically analyzed.

E. Evaluation of a known genetic marker

Brown floral keel tip color, a tetrasomically inherited dominant character, was found in the M_1, or treated parental generation. The mode of inheritance of this character has been previously established by Buzzell and Wilsie (1963) and Bubar and Miri (1965). This character was also found in subsequent progeny of the M_1, specifically, the M_2S, M_3S, M_2X and the M_3X generations. Plants in all of these generations were screened individually for the brown keel tip character. The number of plants having this character was recorded, according to replicate, treatment, and generation. The total number of plants per replicate was recorded as well. The number of plants displaying brown keel tips per replicate was divided by the total number of plants present in each replicate of each treatment, for all generations tested. In this fashion, the decimal frequency of plants having brown floral keel tips was obtained for each replicate of every treatment found in the five generations tested. The data were then statistically analyzed, after being transformed as in Section D.

F. Evaluation of mutant types

Plants in all generations derived from the M_1 generation, but excluding the M_1 generation, were screened at the four-leaf stage for the number of plants displaying chlorophyll deficiencies, along with abnormal leaves and stems. Those plants displaying these characteristics were isolated in the greenhouse within nylon mesh enclosures

with a two mm mesh, to keep out pollinating insects. Plants were maintained under these enclosures for one year of flowering. As well, any plants in the field with abnormal florets or a high level of sterility or dwarfism, were transferred and also isolated in the greenhouse within nylon mesh enclosures. M_1 and M_2S parents of plants (in the M_2S and M_3S generations, respectively) that were anomalous were also removed from the field, transplanted, and isolated in the greenhouse. Reciprocal crosses were made between plants in the same generation with the same type of anomaly and between plants in the same generation with different anomalies. Also, back-crosses were made between anomalous and parental types, reciprocally, in all combinations between plants in all generations.

All crosses were made utilizing the same procedure which consisted in emasculation of the female plant and placing pollen from the male plant onto the stigma of the female plant. Emasculation was carried out on immature florets about eight mm in length with standards still unopened. With a scalpel, a slit was made lengthwise along the base of the keel of the floret with the incision penetrating only the petal portion. A syringe with a 1 mm bore was fitted by rubber tubing to a suction pump, and the anthers to be emasculated were aspirated by means of the syringe-pump mechanism via the opening at the base of the keel. Emasculated florets were labelled with 1 cm^2 paper tags and misted twice daily with water to prevent drying. When standards from the emasculated florets had opened, fresh mature pollen was removed from the donor plant, placed at one end of a 0.5 x 4 cm strip of fine

sandpaper, and the sandpaper containing the pollen was gently rubbed on the stigma of the floret of the female plant. Except for continued misting of the pollinated florets at twice daily intervals, the florets were left undisturbed until mature pods had developed. The mature pods were harvested by hand and placed in envelopes. Seeds were surface broadcast in flats which were placed in a greenhouse.

When seedlings had reached the four-leaf stage, each flat was screened for the presence of plants with chlorophyll-deficiencies, or stem or leaf anomalies. The total number of seedlings in each flat, along with the total number of anomalous seedlings, were recorded according to parental types, crosses, and the phenotypes of each parent. A ratio of the number of anomalous plants to phenotypically normal ones was calculated from the data recorded. Individual seedlings were then transplanted into pots and allowed to grow to maturity and flower. When the seedlings were mature, they were examined for the presence of abnormal florets, complete sterility, and dwarf types. Each plant was recorded either as being phenotypically normal or as having one of the above abnormalities. A ratio was calculated between plants with floral anomalies, sterility, and dwarf types and phenotypically normal ones. Calculated ratios for anomalous progeny were compared with known ratios for reciprocal and back-crosses of disomically and tetrasomically inherited characters according to Allard (1960). A Chi-square test was performed to determine if ratios experimentally obtained differed significantly from known calculated or expected values.

G. Hydrocyanic acid glycoside content

All parental plants and progeny for all generations were screened for the quantitative presence of glycosides of hydrocyanic acid in the leaves as follows. From each replicate of each treatment for the nine generations, ten plants were chosen at random. Leaves located at the third to fifth nodes from the shoot apex were harvested and placed in 5 x 10 cm paper envelopes, which were labelled according to generation, treatment and replicate. Each envelope constituted one sample, and a total of 3,090 samples was obtained from 309 plots, comprising 103 treatments. From each sample 0.05 ± 0.005 g fresh weight of leaves was weighed and individual samples were placed in 5 ml capacity shell vials labelled with the corresponding generation, treatment and replicate.

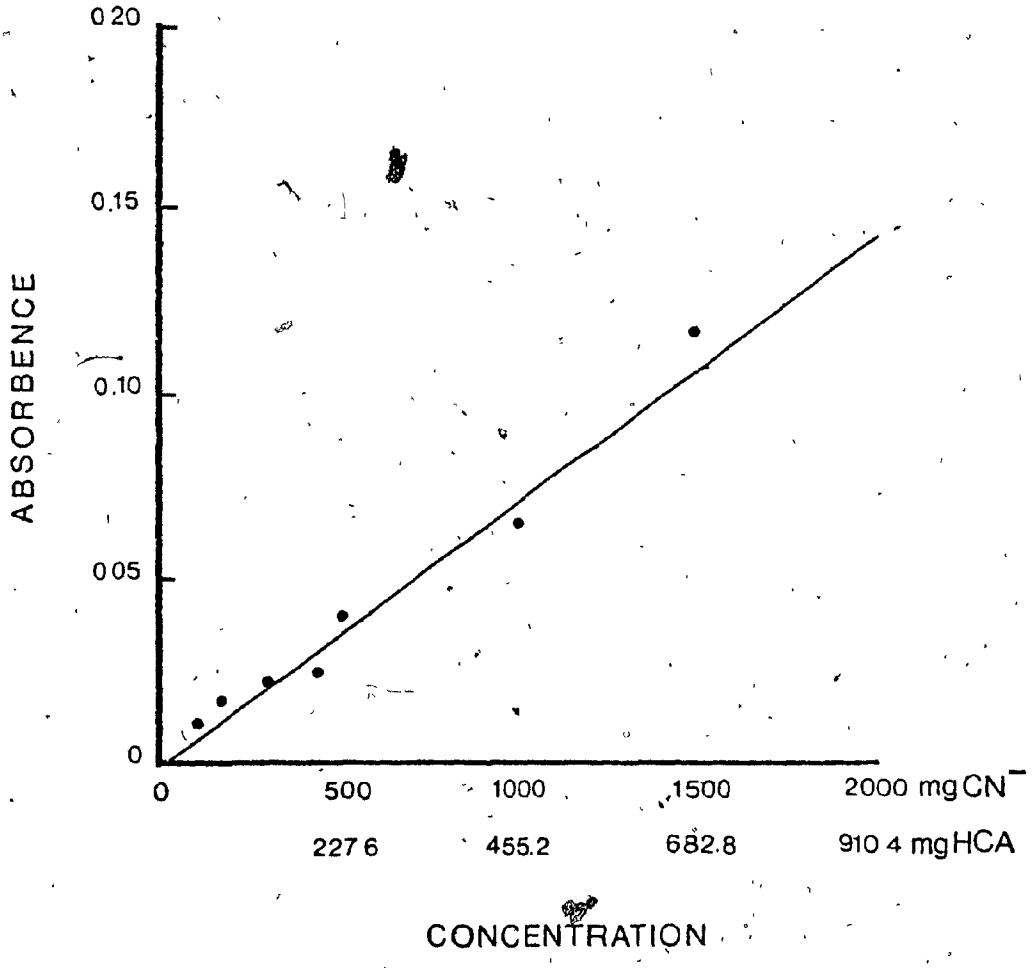
For the hydrocyanic acid glycoside (HCA) analyses, filter paper strips and sodium picrate solutions were prepared in the following manner. No.2 Whatman filter paper was cut into 1 x 4 cm strips. The picrate solution was prepared by the addition of 50.0 g sodium carbonate and 5.0 g of picric acid (Trinitrophenol) to 100 ml of distilled water, and filtered through No.3 Whatman filter paper. Strips of filter papers were left in solution for 5 minutes and air-dried for one-half hour.

To each vial, three drops of reagent-grade toluene were added, followed by the addition of a 1 x 4 cm strip of filter paper saturated with sodium picrate solution as described above. The vials were

tightly stoppered with new corks and incubated for 24 hours in the dark at 25°C. After incubation, the filter paper strips were removed from each vial, and air-dried for one-half hour. After drying, the individual strips were placed in paper envelopes, according to generation, treatment, and replicate. Strips from each replicate were placed in 5 ml shell vials containing 4.0 ml of 1:1 distilled water:acetone. Vials were tightly stoppered with new corks, and left standing for one-half hour at 25°C to allow for the dissolution of the chemicals from the filter-paper strips, after which they were removed. The solutions which remained were placed individually in 7 ml capacity colorimeter tubes which were read individually in a Spectronic 20 Colorimeter (Bausch and Lomb Optical Co.) at the spectral absorbance of 600 nanometers.

A reference standard was obtained by dissolving 0.0001, 0.005, 0.001, 0.025, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.0 g potassium cyanide, respectively, in each of 10 volumetric flasks with a 1.0 liter capacity with glass-distilled water to volume. This produced 5, 10, 25, 50, 100, 250, 500, 750, and 1,000 mg/liter concentrations of potassium cyanide, respectively. Four ml of each solution were placed in 5 ml capacity shell vials, to which were added single 1 x 4 cm strips of filter paper saturated with sodium picrate solution. Vials were tightly stoppered with new corks, and incubated in the dark at 25°C for 24 hours. After incubation, the filter-paper strips were removed and individual solutions were transferred to 7 ml capacity colorimeter tubes and a spectral reading with an absorbance at 600

Figure 1. Plot of mean values (•) and regression curve (-) obtained for absorbance vs. mg CN^- and mg hydrocyanoglycoside (HCA) per Kg fresh leaf weight.


0 20
0.15
0.10
0 05
0
ABSORBENCE
0
500
1000
1500
2000 mg CN⁻
227 6
455.2
682.8
910 4 mg HCA
CONCENTRATION

nanometers was made and recorded for each concentration. From these readings, an absorbance vs. concentration graph was constructed with absorbance in decimal units and concentration in milligrams per liter (mg/l) of potassium cyanide. Values obtained from experimental material in absorbance units were compared against the values obtained for the standard concentrations. The values extracted in this manner were used to estimate the amount of milligrams of free cyanide, as hydrocyanic acid (HCA), present in each sample per kilogram of fresh leaf weight. Regression analyses were performed on the data for the standard values, utilizing the regression analysis technique of Steel and Torrie (1960). Analysis yielded the linear model $X = 6825.94\ Y$, where X is the concentration of HCA in mg per kg of fresh leaves, and Y is the decimal absorbance value (Figure 1). This formula was utilized in obtaining estimated concentrations of free HCA in the leaves. The values were converted to mg HCA per kg of fresh weight of leaves, tabulated for each replicate, and both individual values and means were statistically analyzed.

H. Forage yield

The M_1 parental generation, as well as all selfed and crossed progeny, was cut for forage when fully mature in the second year. The exceptions were the final four groups, that is, the M_3I, M_4I, M_3O, and M_4O generations. Forage for these groups was cut when flowering had terminated at the end of the first growing season. For the M_1, M_2S, M_3S generations, all plants were cut to the crown, and each plant was placed

in an individual 50 x 75 x 3 cm screened drying tray, and labelled according to generation, treatment and replicate, as well as plant number. Trays were placed in forced-air drying ovens at 80°C for 12 hours. The dried forage samples were weighed to the nearest gram and the individual weights were recorded according to generation, replicate, and plant number.

Forage yields for the M_2X and M_3X generations were obtained by cutting ten plants, at random, to the crown, from each replicate of each treatment for both generations. Each plant was individually placed in a 50 x 75 x 3 cm screened-type drying tray, and each tray was labelled according to generation, treatment, and replicate. Trays were placed in forced-air drying ovens at 80°C for 12 hours. After removal from the ovens and cooled, the contents of each tray were weighed individually to the nearest gram. Individual weights were recorded according to generation, treatment, and replicate.

M_3O, M_4O, M_3I, M_4I forage yields were obtained by cutting all plants in each replicate of each treatment to the crown. Plants from each replicate were dried and weighed as for previous generations. The total weight for each plot of dry forage was determined and divided by the total number of plants in each plot to give the average weight per plant per plot. Data from all generations were then statistically analyzed.

I. Seed yield

1. Lotus corniculatus

In the M_1, M_2S, M_3S, M_2X and M_3X generations, 10 plants from each replicate of each treatment for each generation were harvested at random when the plants were fully mature in their second growing season. Individual plants were placed in 50 x 75 x 3 cm screened drying trays, labelled according to generation, treatment, and replicate and placed in forced-air drying ovens at 80°C for 12 hours. After the trays were removed, the contents were hand-sifted and placed in 10 x 15 cm paper envelopes which were labelled according to generation, treatment, and replicate. Contents of each envelope were placed on a 50 cm^2 gravity table and seeds of approximately 1.0 ± 0.05 mg were separated. Seeds were then returned to their envelopes, placed in a desiccator for one-half hour at 30°C, and weighed to the nearest 0.001 g. The approximate total number of seeds for each envelope was determined by obtaining ten 1,000-seed samples at random from the entire collection and weighing each sample to the nearest 0.001 g. An average value obtained for the 10 samples was determined as 0.997 ± 0.006 grams per 1000 seeds. This value was used as the 1000-seed weight reference from which the approximate number of seeds was determined for each envelope.

In the M_3I, M_4I, M_3O, and M_4O generations, all plants from every replicate were harvested in their first year and treated in the same manner as described above.

Data as number of seeds per plant for the M_1, M_2S, M_3S, M_2X, and M_3X generations, and as average number of seeds per plant for the M_3I, M_4I, M_3O, and M_4O generations were then statistically analyzed.

2. Lotus tenuis

In August of 1979, mature pods were hand-collected from all M_1 Lotus tenuis plants in the field and placed in 10 x 15 cm envelopes, according to treatment and replicate. All envelopes were placed in a desiccator at 30°C for 48 hours after which they were removed and the contents of each envelope placed individually on 30 cm diameter pans. The number of pods per envelope was counted and recorded, and seeds were hand-separated from individual pods. The seeds from each envelope were threshed on a 50 cm^2 gravity table, and seeds of uniform weight and size (1.0 ± 0.05 mg, 3 mm diameter) were separated from extraneous material. Each lot of seed was returned to its respective envelope, and the extraneous material discarded.

Ten 1000 lot seed samples were obtained, at random, from the M_1 seed collection, and each sample was weighed to the nearest milligram. The average weight of the ten samples was obtained, and served as the average weight per 1000 seeds. The contents of each envelope were then weighed to the nearest 1.0 mg, and the number of seeds per envelope calculated, using the average 1000 seed weight. Data were recorded according to treatment and replicate, for the number of seeds per plot, number of pods per plot, and the number of seeds per pod per plot. Data were then statistically analyzed.

J. Flowering frequency

The flowering frequencies in each generation were determined by daily recording the number of plants in flower in each replicate for all treatments. Recording of the data started when the first plant commenced flowering. Flowering was recorded in the first season for the M_3I, M_4I, M_3O and M_4O generations, and in the second season for the M_1, M_2S, M_3S, M_2X and M_3X generations. Recording ceased when the last plant had flowered for all treatments. The mean number of days to maximum flowering and the mean number of days to 50% flowering were calculated for all treatments, within each generation, based on the number of plants in flower on a daily basis over a fixed number of days for each generation. The fixed number of days was set by the interval between the first and last day of flowering, inclusively. These values were then statistically analyzed.

K. Pod dehiscence

On October 27, 1979, under conditions of frost and low humidity (-1.0°C, relative humidity of 35%), the total number of pods per plant in the M_1, M_2S, M_3S, M_2X and M_3X generations was recorded, as well as the number of dehisced pods per plant for each replicate of each treatment within each generation. An average per cent dehiscence value was determined for each replicate by dividing the number of dehisced pods per replicate into the total number of pods per replicate, and dividing this value by the number of plants in each replicate. The average

per cent dehiscence per plant was recorded according to generation, treatment, and replicate, transformed numerically, using the arcsin square root per cent transformation (Steel and Torrie, 1960), and statistically analyzed.

L. Studies on winterhardiness

1. Lotus corniculatus

All plants from the M_1, M_2S, M_3S, M_2X, and M_3X generations were counted in the field at the end of their first and second growing seasons, September, 1979 and September, 1980, respectively. Counts were made on the basis of the number of plants in each plot over both seasons. Values obtained in the second season were subtracted from values obtained in the first season, giving the numerical value of plants that did not survive the winter of 1979-80 in each plot. This value was divided by the number of plants present in each plot during the first season. The resulting decimal value obtained was the fractional portion of plants that did not survive the winter of 1979-80 in each plot. This value was subtracted from 1.00, giving that fraction of those plants that had overwintered in each plot. Data obtained on the fractional portion of overwintering plants were tabulated on the basis of treatment and replicate for each generation. The data were then transformed numerically, using the arcsin of the square root of each value according to procedures outlined in Steel and Torrie (1960) for statistical analysis of decimal values.

.2. Lotus tenuis

M_1 plants were allowed to overwinter during the winter of 1979-80. The number of plants per plot was recorded in August of 1979, and again in August of 1980. Values obtained for each plot in 1980 were divided by the 1979 values obtained for each respective plot. The resulting values were the decimal fraction of plants surviving the 1979-80 winter. These data were recorded according to treatment and replicate. The individual values were transformed using the arcsin of the square-root of each value to allow for statistical analysis of decimal values as specified in Steel and Torrie (1960). Data were then statistically analyzed.

M. Parent-progeny correlations

Parent progeny correlations were calculated for comparisons of significantly high and low yielding lines, parental lines, and progeny of significantly high and low yielding lines. Tested were lines showing significant values obtained in lines with high or low HCA content (Section G, Table XI), forage yield (Section H, Table XII), and seed yield (Section I, Table XIII).

Correlation coefficients (r) were obtained, and a probability of a greater r calculated, using the method explained in Steel and Torrie (1960), and utilizing the applications of correlation coefficients (r) on parent progeny comparisons as explained by Allard (1960). The procedure «corr» was utilized on McGill University's

IBM 360 interactive computer to analyze the data. The «corr» procedure is part of the McGill University computer library's Statistical Analysis Systems (SAS), developed by Goodnight et al. (1976) of the SAS Institute, Raleigh, North Carolina. The procedure was implemented as stated in the SAS '76 manual (Goodnight et al., 1976).

A total of 19 correlations was performed, with two correlations having r values greater than 0.9000 or less than -0.9000 and having a calculated probability of a greater r of less than 0.2000. These r values are given in Table XX, along with the generations being correlated and the mutagen and dosage responsible for significant increases or decreases in seed and forage yields and HCA content. Each correlation contained 18 observations.

N. Mutagenic efficiency ratios

The mutagenic efficiency coefficients of the five selected mutagens as well as control values, were determined over eight generations using the method of Konzak et al. (1965). This method expresses the mutagenic efficiency of any given physical or chemical mutagen as the ratio of the number of mutants obtained (in the progeny of treated organisms) to the number of parental individuals not surviving mutagenic treatment. This ratio, expressed as a decimal, is given in Table XXI for the five selected mutagens and controls for each of the eight generations along with their average ratios.

O. Statistical analysis procedures

Statistical analysis for all data was performed by means of a McGill University IBM 360 computer, using the Statistical Analysis Systems (SAS) computer library tape package developed by the SAS Institute, Raleigh, North Carolina. All data from the randomized complete block designs were analyzed using the «General Linear Models» (GLM) procedure from the SAS package. The GLM procedure performed an analysis of variance on treatment and replicate values, and computed an F-statistic to determine statistical significance between treatments and between replicates. Added to this was a «Duncan» procedure, which performed a Duncan's New Multiple Range Test on the means of the treatments and replicates on each set of data testing for statistical significance between the means generated for each set of data. The «GLM» and «Duncan» procedures were used from the SAS package, as outlined in the SAS '76 and SAS '79 manuals (Goodnight et al., 1976, 1979), and in accordance with standard statistical procedures. (Steel and Torrie, 1960; Duncan, 1975).

Certain data were not analyzed in the manner previously stated. These include the data obtained in Section F, which were analyzed using the «Freq» procedure in the SAS package. The «Freq» procedure contains a «chsq» option, which was used to perform a Chi-square test (Steel and Torrie, 1960) on the data. Chi-square and Probability of a greater Chi-square statistics generated were used to determine if ratios obtained were statistically significant from expected values. Also, no statistical methods were applied to the data generated in

Section N. Data on mutants obtained in Section F, part 1, were statistically analyzed using Friedman's rated Chi-square analysis (Friedman, 1937) as given in Steel and Torrie (1960). All data generating decimal or percentage values were transformed using the arcsin square-root transformation as given by Steel and Torrie (1960), and all data are expressed as values transformed back to the original decimal or per cent equivalent after analysis, in the tables, where applicable. All statistically significant values are significant with respect to control values for each set of data.

V. RESULTS

A. Seed germination rates

1. Lotus corniculatus

The toxic effects of the mutagenic treatments on seed germination were confined to two mutagens in the initially treated or M_1 generation. As may be seen from Table I, there were significant decreases in seed germination rates for both EMS and EC (see Appendix I, Tables 1 and 2) treated M_1 seed at doses of 0.01, 0.1, and 1.0%. Figures 2 and 3 show a sharp decrease between the dose range of 0.01 to 1.0% for EMS and EC treatments, respectively. Conversely, significant increases in seed germination rates were obtained for three mutagens in the M_2X generation. Table I refers to a 12 kR line from X-ray treatment, a 0.001% and 1.0% EMS line, and a 0.01% HU line; all show a significant enhancement of seed germination with an increase of at least 50% over the average value obtained for the M_2X control (see Appendix I, Tables 3, 4 and 5). No significant effects were obtained between control and treatment values for all other generations tested.

2. Lotus tenuis

Seeds treated with nine dosages of EMS in the M_2X showed a significantly lower germination rate in five of the nine dosages (Table II). Significant values were confined to dosages higher than

TABLE I. Seed germination rates expressed as per cent of total germinated seed for values significant from controls

Generation	Treatment	Dosage (%)[a]	Experimental value (%)
M_1	Control	0	54.9
	EMS	0.01	38.7*
		0.1	25.7*
		1.0	4.3*
	EC	0.01	32.0*
		0.1	18.7*
		1.0	7.0*
M_2S	Control	0	12.0 NS
M_3S	Control	0	68.5 NS
M_2X	Control	0	22.2
	X-rays	12	52.9*
	EMS	0.001	46.7*
		1.0	46.2*
	HU	0.001	46.7*
M_3X	Control	0	22.2 NS
M_4X	Control	0	26.0 NS
M_3O	Control	0	34.0 NS
M_3I	Control	0	47.8 NS
M_4O	Control	0	35.0 NS
M_4I	Control	0	42.0 NS

[a]Except X-rays, which are expressed in Kilorads (kR).

NS: No significance from controls.

*Significant at the 5% level.

Figure 2. Histogram of per cent seed germination vs. dosage for EMS-treated M_1 seeds, along with standard deviations (bars) for each value.

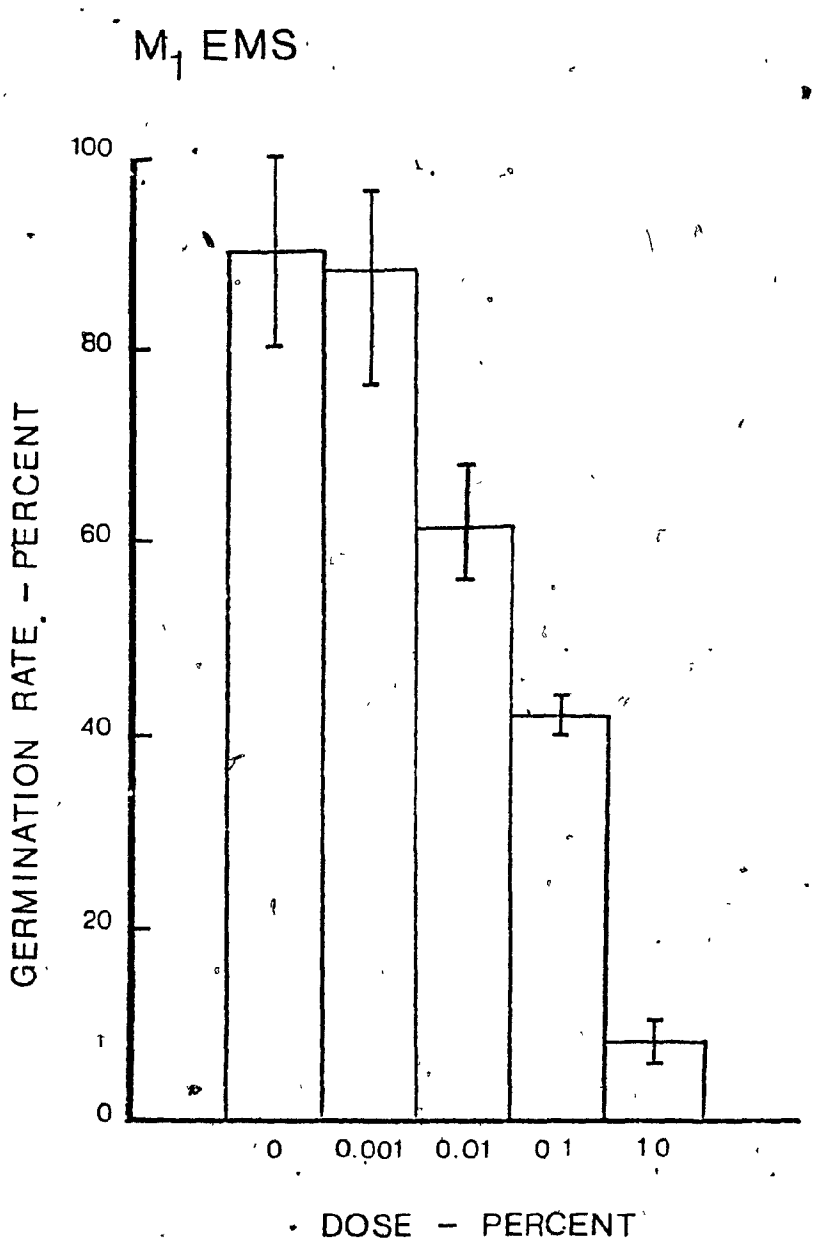

M1 EMS
100
80
60
40
20
0
GERMINATION RATE - PERCENT
0
0.001
0.01
0 1
1 0
DOSE - PERCENT

Figure 3. Histogram of percent seed germination vs. dosage for EC-treated M_1 seeds, along with standard deviations (bars) for each treatment.

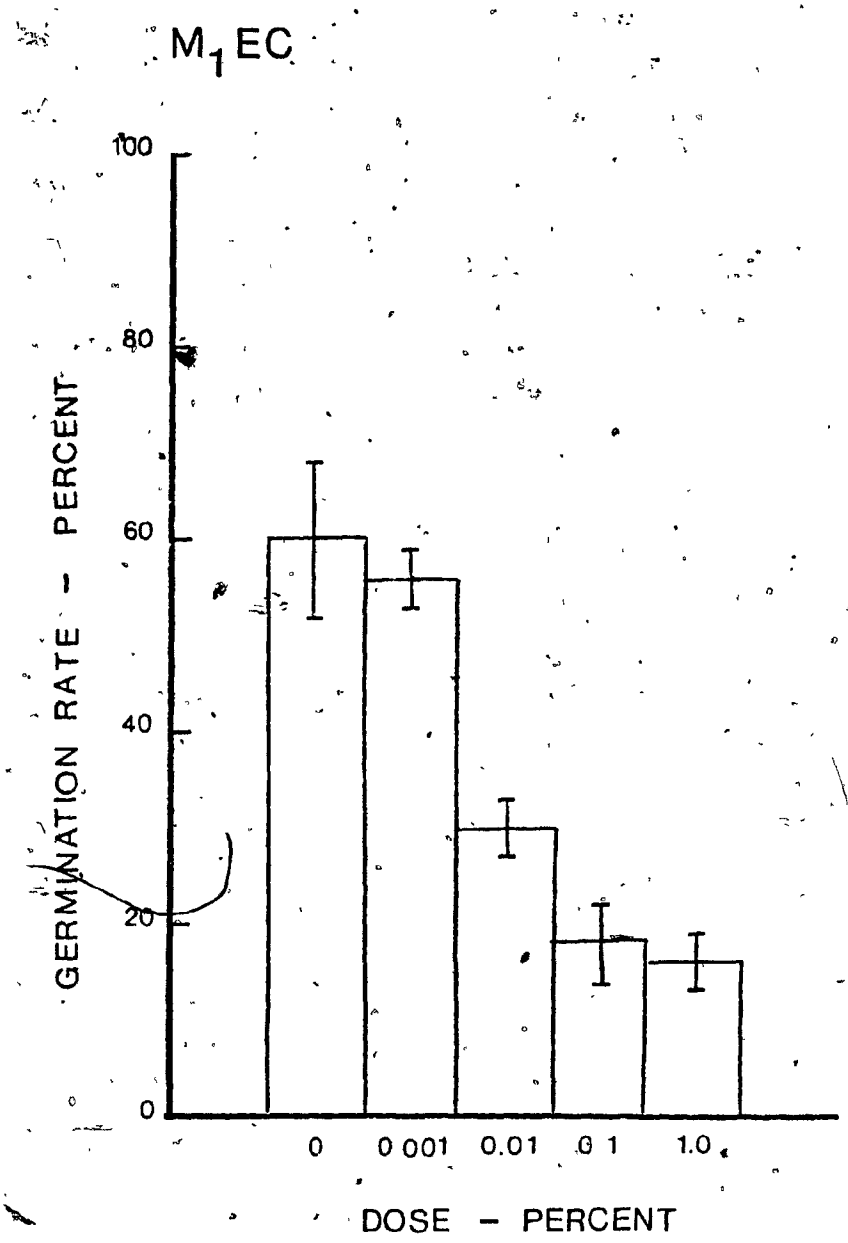

M1 EC
GERMINATION RATE - PERCENT
100
80
60
40
20
0
0
0 001
0.01
0 1
1.0
DOSE - PERCENT

TABLE II. Germination rate in Lotus tenuis after various treatments with EMS for the M_1 generation expressed as the percentage of total seeds germinated

Treatment (%)	Percent germination
Control	70.0
0.001	66.3
0.005	69.7
0.01	65.3
0.05	63.0*
0.1	60.0*
0.5	63.4*
1.0	63.2*
1.5	65.0
2.0	50.6*

*Significant at the 5% level.

0.01% EMS, with the exception of the 1.5% EMS treatment which was not significantly different from the control (see Appendix I, Table 6). The germination rate of initially treated M_1 seed did not differ significantly from the control germination rate whose mean value was 67.5%.

B. Seedling survival rates

1. Lotus corniculatus

Effects on the survival of seedlings to the four leaf stage after EC and X-ray treatment were confined to two of the nine generations (Table III). In the M_2S generation, two lines derived from X-ray treatment (6 and 12 kR), and two lines derived from EC treatment (0.001 and 1.0%) had a complete killing effect on the seedlings with 0% survival. These values were significant from the mean control value of 8.7% (see Appendix I, Table 7).

Significant decreases in seedling survival occurred for one treatment in the M_3O generation, namely 0.1% EC, which showed a drop in survival rate to 9.0% (see Appendix I, Table 8). No significant differences were observed in any of the other generations between control and treatment values for seedling survival rate. The control values for each generation were found to vary considerably with values ranging from 97.8% in the M_1 generation to a low of 8.7% in the M_2S generation.

TABLE III. Survival rates of seedlings to the four-leaf stage, expressed as the percentage of total surviving, for values significant from controls

Generation	Treatment	Dosage (%)[a]	Experimental value (%)
M_1	Control	0	97.8 NS
	Control	0	8.7 NS
	X-rays	6	0.0*
		12	0.0*
M_2S	EC	0.001	0.0*
		1.0	0.0*
M_3S	Control	0	12.6 NS
M_2X	Control	0	56.8 NS
M_3X	Control	0	59.0 NS
M_3O	Control	0	34.0
	EC	0.1	9.0*
M_4O	Control	0	35.0 NS
M_3I	Control	0	47.8 NS
M_4I	Control	0	42.0 NS

[a]Except X-rays, which are expressed in kilorads (kR).

NS: No significance between controls and all treatments.

*Significant at the 5% level.

2. Lotus tenuis

Mean values obtained for survival rates to the four leaf stage varied between 59.6 and 87.8% of the initially germinated seedlings (Table IV). The total range of values varied from 52 to 94%, with two values which were significantly different, namely the 0.1 and 2.0% EMS treatments (see Appendix I, Table 9).

C. Cytology

1. Lotus corniculatus

An analysis of the data for the Pollen Mother Cells (PMCs) of the control and treated plants for aberrant cells demonstrated that there were significant increases in the mean percent aberration rate in the M_1, M_3I and M_4I generations (Table V; see Appendix I, Tables 10-15). In the M_1 generation a significant increase in aberration rate occurred for all four doses in the EMS and the AP treated plants. From Figure 4, it may be seen that there were no dose effects due to the EMS treatments, whereas AP treatments (Figure 7) show a steady increase in the 0.001-0.1% dose range, followed by a decrease at the 1.0% dose level. EC treatment (Figure 5) shows a sharp increase in meiotic aberration rate at the 0.01% dose level. HU treatment (Figure 6) demonstrates a bimodal response to the dosage applied with sharp increases at the 0.001 and 1.0% dose levels. Significant increases in aberration rates were also found in the M_3I and M_4I generations for plants derived from treatment with 0.01% EMS. Significant effects were confined to the single doses in each case.

TABLE IV. Survival rates after EMS treatment of M_1 *Lotus tenuis* plants, expressed as the number of plants surviving to the four-leaf stage as percent of total.

Treatment (%)	Survival (%)	Range (%)
Control	87.4	75-94
0.001	83.4	73-91
0.005	87.8	83-91
0.01	82.2	77-91
0.05	79.2	72-86
0.1	74.8*	67-83
0.5	79.6	67-87
1.0	79.4	73-87
1.5	82.0	78-87
2.0	59.6*	52-66

*Significant at the 5% level.

TABLE V. Meiotic chromosome aberrations in Pollen Mother Cells expressed as percent aberrant cells for values significant from controls

Generation	Treatment	Dosage (%)	Mean percentage aberrancy
M_1	Control	0	3.33
	EMS	0.001	13.00*
		0.01	14.67*
		0.1	16.67*
		1.0	18.00*
	EC	0.01	15.33*
		0.1	22.00*
		1.0	22.67*
	HU	0.001	9.00*
		1.0	9.67*
	AP	0.001	6.00*
		0.01	7.33*
		0.1	9.00*
		1.0	13.33*
M_2S	Control	0	0.33 NS
M_3S	Control	0	1.00 NS
M_2X	Control	0	5.45 NS
M_3X	Control	0	1.33 NS
M_3O	Control	0	2.33 NS
M_3I	Control	0	3.00
	EMS	0.01	15.33*
M_4O	Control	0	2.33 NS
M_4I	Control	0	2.00
	EMS	0.01	8.00*

NS: No significance between controls and all treatments.

*Significant at the 5% level.

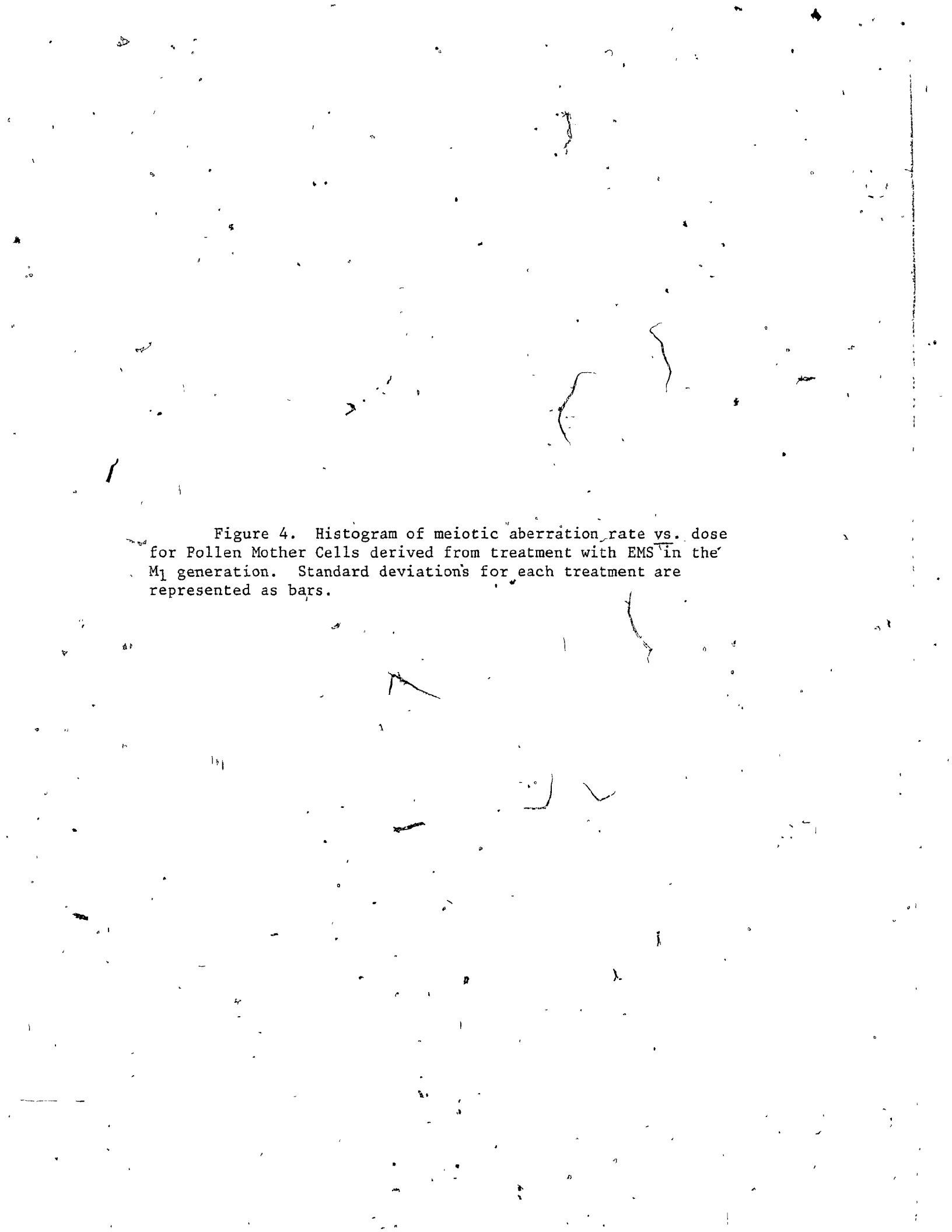


Figure 4. Histogram of meiotic aberration rate vs. dose for Pollen Mother Cells derived from treatment with EMS in the M_1 generation. Standard deviations for each treatment are represented as bars.

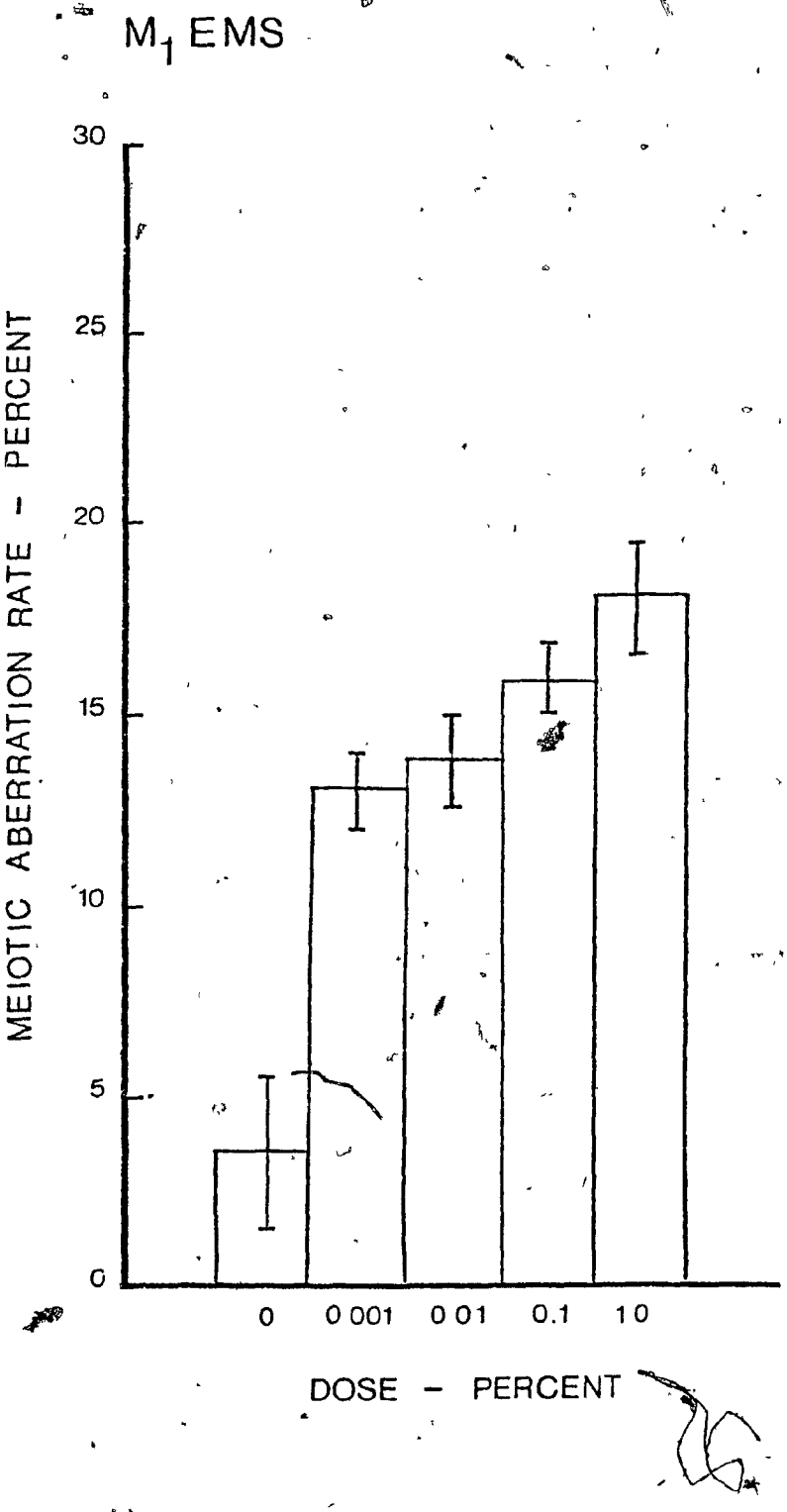

M1 EMS
MEIOTIC ABERRATION RATE - PERCENT
30
25
20
15
10
5
0
0
0 001
0 01
0.1
1 0
DOSE - PERCENT

Figure 5. Histogram of meiotic aberration rate vs. dose for Pollen Mother Cells derived from treatment with EC in the M_1 generation. Standard deviations for each treatment are represented as bars.

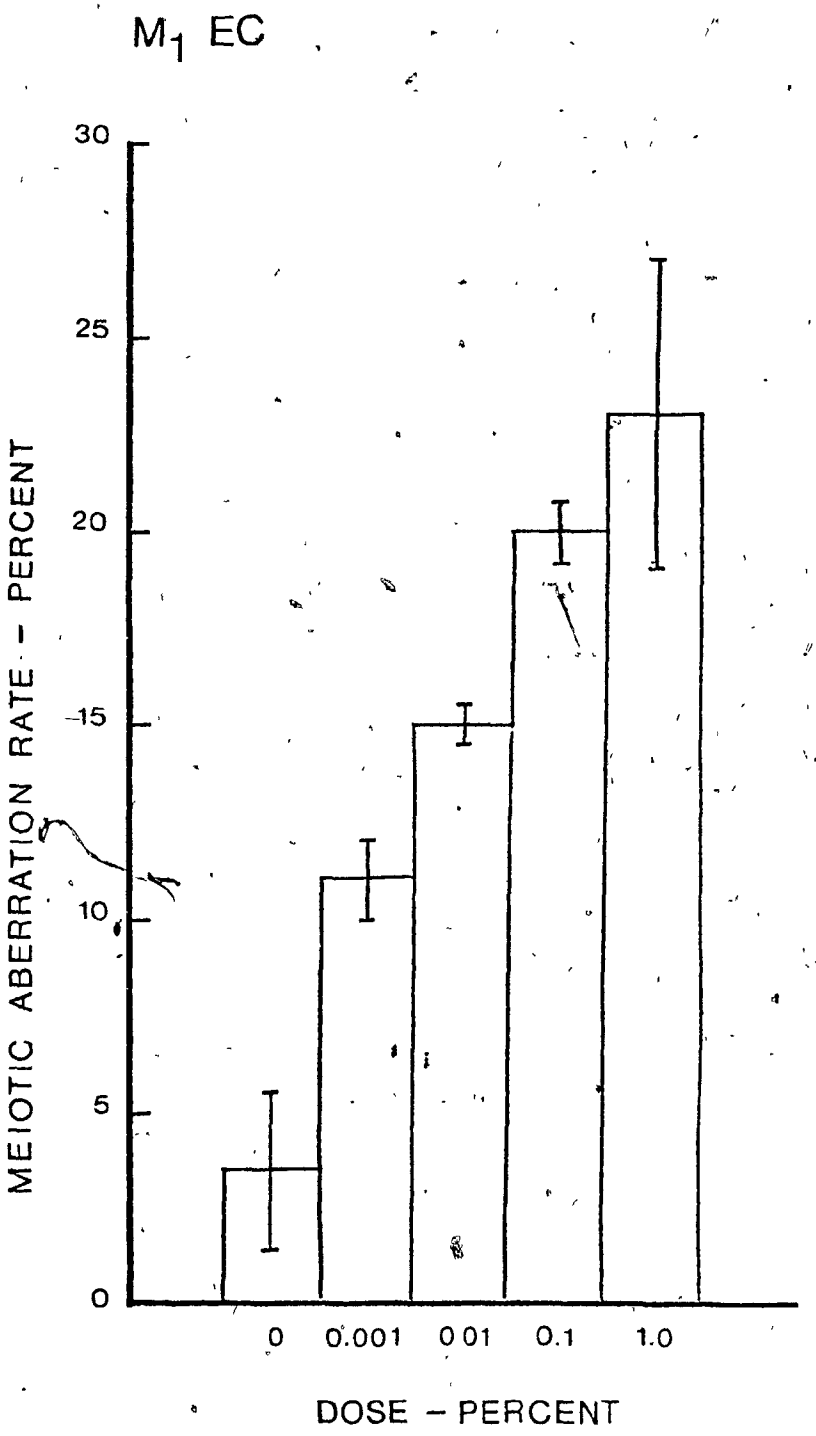
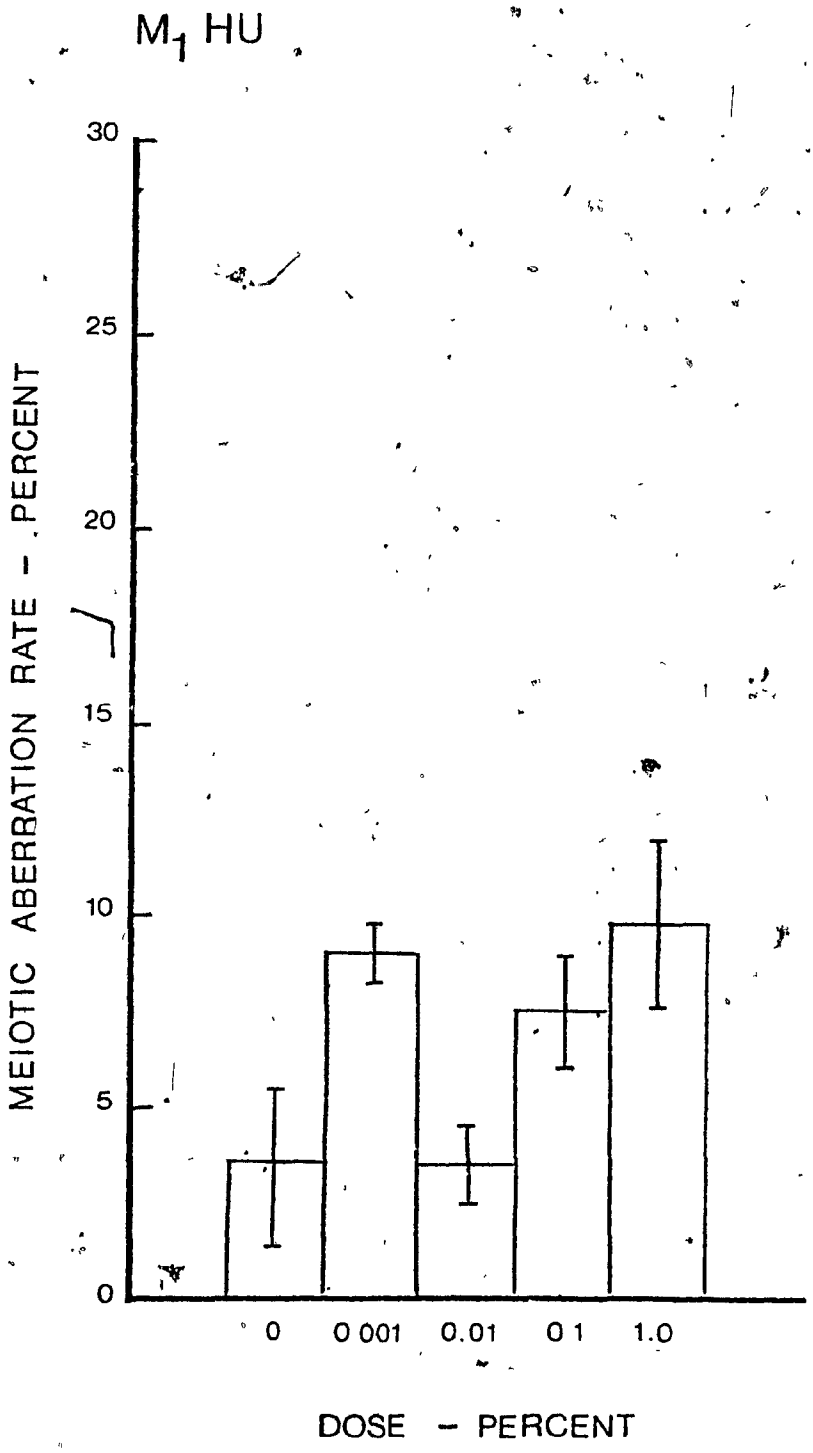

M1 EC
30
25
20
15
10
5
0
MEIOTIC ABERRATION RATE - PERCENT
0
0.001
0 01
0.1
1.0
DOSE - PERCENT

Figure 6. Histogram of meiotic aberration rate vs. dose for Pollen Mother Cells derived from treatment with HU in the M_1 generation. Standard deviations for each treatment are represented as bars.


M1 HU
MEIOTIC ABERRATION RATE - PERCENT
30
25
20
15
10
5
0
0
0 001
0.01
0 1
1.0
DOSE - PERCENT

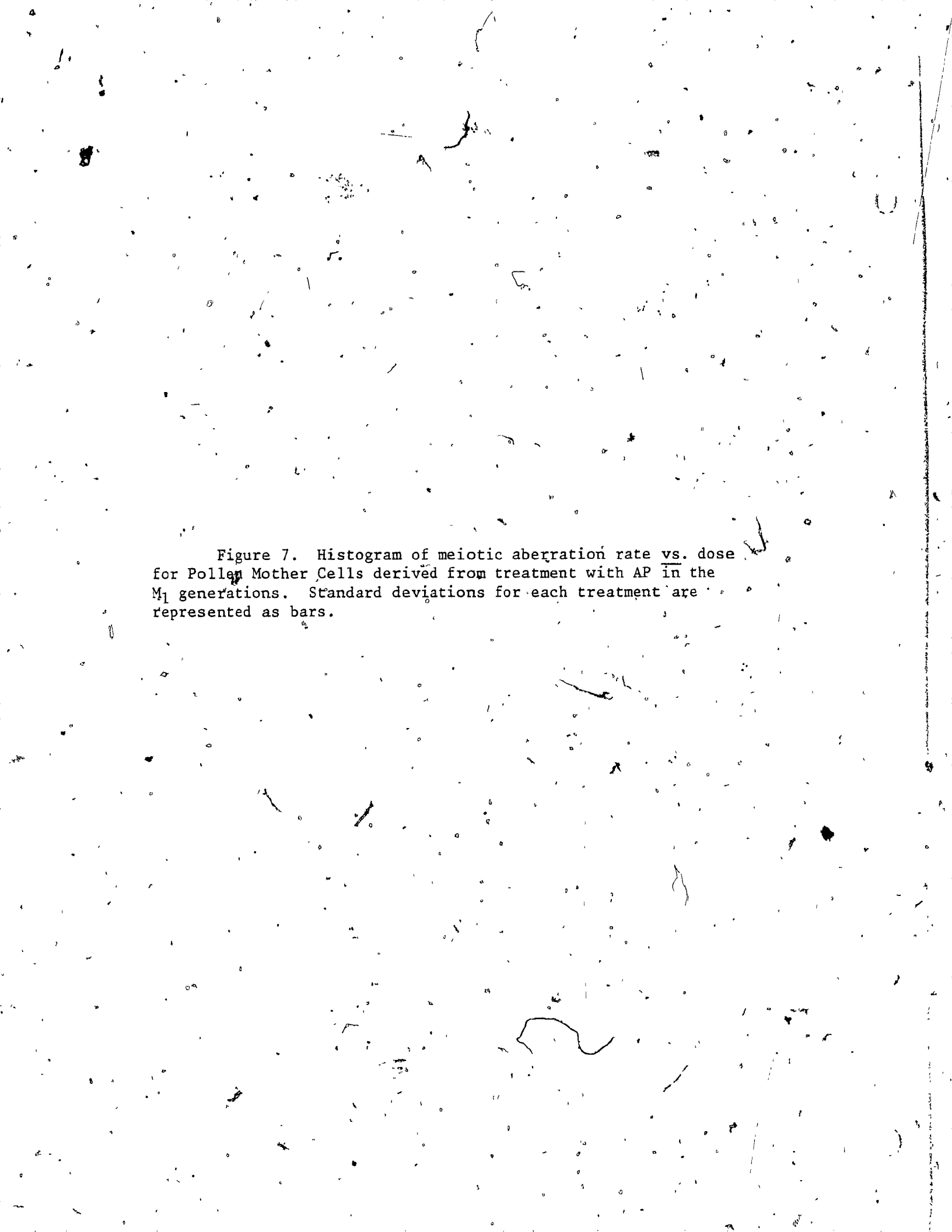


Figure 7. Histogram of meiotic aberration rate vs. dose for Pollen Mother Cells derived from treatment with AP in the M_1 generations. Standard deviations for each treatment are represented as bars.

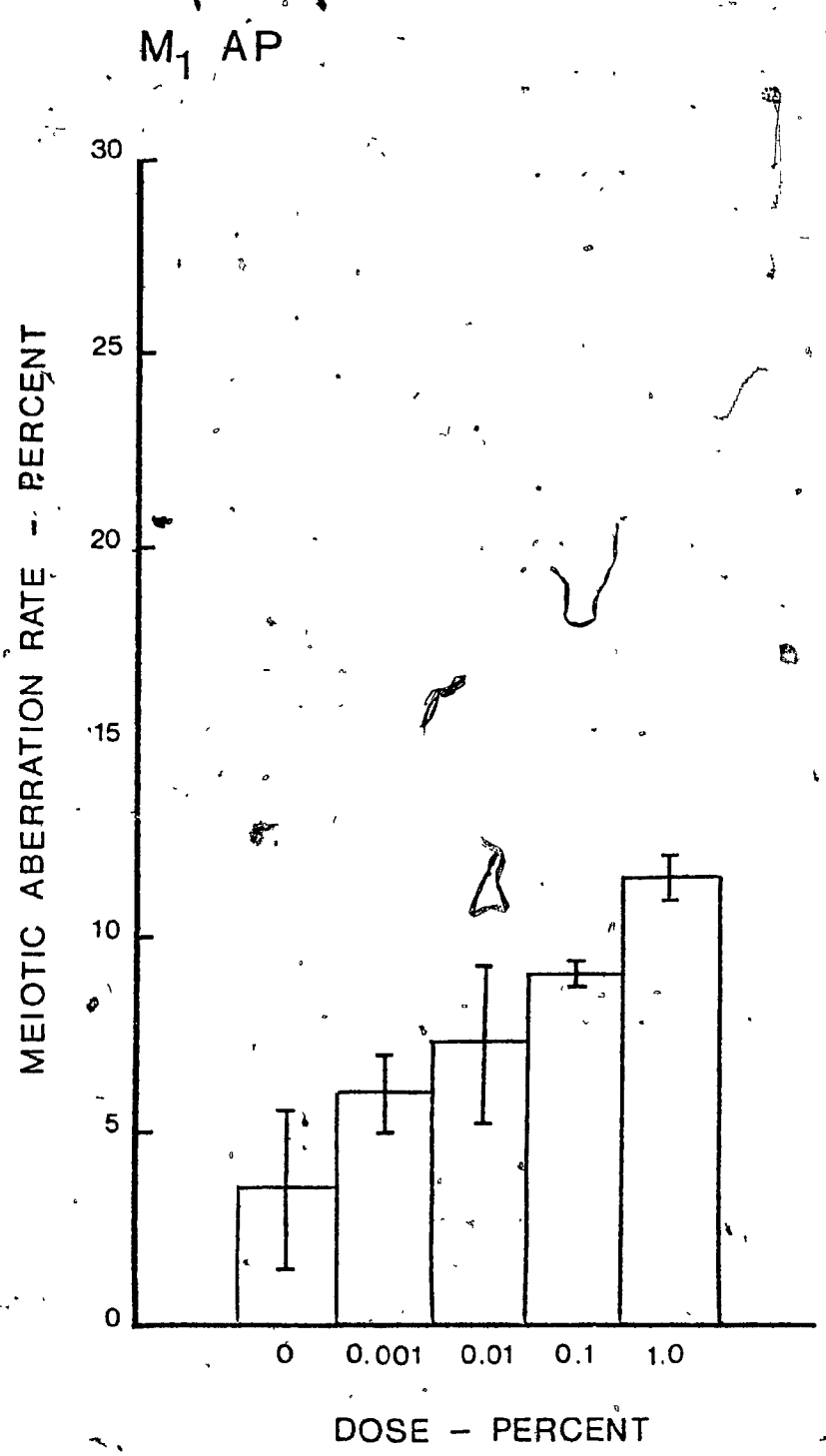
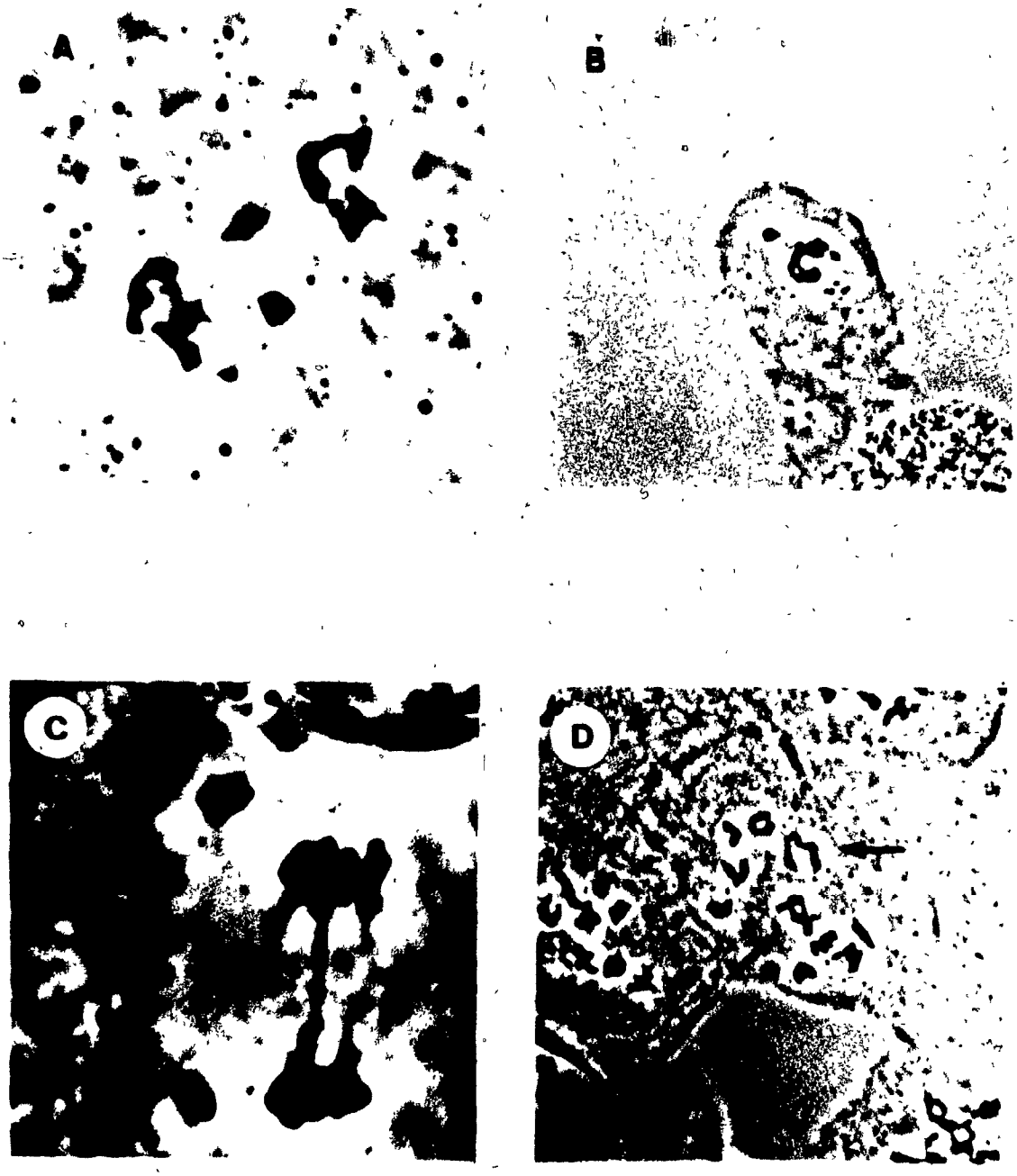

M_1 AP
MEIOTIC ABERRATION RATE – PERCENT
30
25
20
15
10
5
0
0
0.001
0.01
0.1
1.0
DOSE – PERCENT

Figure 8. Types of meiotic aberrations scored in PMCs: (a) Two laggards at Anaphase I, ca. 960 X; (b) Micronucleus at Interphase, ca. 420 X; (c) Bridge at Anaphase I, ca. 960 X; (d) Early Metaphase I showing ten figures, including two quadrivalents (arrows), ca. 420 X.


A
B
C
C
D

There were no significant differences between control and treatment values for the average percent aberrant cells in any of the other generations. Examples of the types of meiotic aberrations obtained are given in Figure 8.

2. Lotus tenuis

There were a significantly high number of meiotic aberrations induced in plants of L. tenuis derived from EMS treated seed in all but two treatments (Table VI). No relationship could be demonstrated between level of dosage and meiotic aberration rate. The types of anomalies induced did differ with respect to dosage with different proportions of the three types of meiotic aberrations and anomalies resulting from different dosages. The types of meiotic aberration encountered were quartet micronuclei (range between 0 and 18 per 300 cells scored), followed by quadrivalents in metaphase I (MI) (range 0 to 14 per 300 cells scored) and lastly, a few anaphase I (AI) bridges and laggards were noted for all treatments, with a maximum of nine observed in the 1.5% EMS line. Individual treatments did not differ significantly from one another, with the exception of the 0.005% EMS and the 0.5% EMS lines (see Appendix I, Table 16).

TABLE VI. Meiotic aberration rates for EMS derived Pollen Mother Cells of Lotus tenuis, for anomalies induced at metaphase I (MI), anaphase I (AI), quartet (Q), and for total anomalies induced, expressed as number of anomalous cells per 300 cells scored

Treatment (%)	Stage of meiosis				Mean of all values
	MI	AI	Q	Total	
Control	0	0	0	0	0.0
0.001	14	0	0	14	4.67*
0.005	6	0	0	6	2.00
0.01	1	1	13	15	5.00*
0.05	5	3	8	16	5.33*
0.1	6	0	14	20	6.67*
0.5	3	0	7	10	3.33
1.0	0	0	18	18	6.00*
1.5	4	9	0	13	4.33*
2.0	9	0	4	13	4.33*

*Significant at the 5% level.

D. Pollen stainability studies

From Table VII it may be seen that there were significant increases in the aborted pollen rate (as percent unstained pollen) for treatments in the M_1, M_3O, M_4O, M_3I and M_4I generations (see Appendix I, Tables 17-25). The most extensive effects were found in the M_1 generation where all five mutagens, namely, X-rays, EMS, EC, HU and AP were effective in significantly increasing the aborted pollen rate. Plants derived from X-ray treatments demonstrated a sharp and significant increase in aborted pollen rates at doses of 3 and 6 kR followed by a return to levels at or below control for the higher doses of 9 and 12 kR (Figure 9). The EMS and EC treated M_1 plants demonstrated a response for pollen abortion which was significant for all dosages. In EMS treated plants, there was a sharp increase in the rate of pollen abortion which occurred with the 0.01% treatment. This was followed by a levelling off in pollen abortion at around 30% for the 0.1 and 1.0% treatments (Figure 10). In the EC treated M_1 plants, there was an average increase in pollen abortion from 5 to 35% which occurred between controls and the 0.01% treatment, with a slight (not significant) decrease between the 0.001 and 1.0% treatments (Figure 11). The HU and AP treated M_1 plants did not show a significant response at any dosage. Both HU and AP treatments showed an overall rate of pollen abortion which was lower than X-ray, EMS or EC treatments. Within the HU treatments there was a significant increase in pollen abortion between the 0.001 and 0.1% levels, with a return to nonsignificant levels at 1.0% (Figure 12). The AP treatments produced significant

TABLE VII. Aborted pollen rate expressed as percent aborted pollen for all treatments showing significance from controls

Generation	Treatment	Dosage (%)[a]	Aborted pollen rate (%)
M_1	Control	0	14.6
	X-rays	3	19.3*
		6	24.7*
	EMS	0.001	28.3*
		0.01	44.3*
		0.1	32.3*
		1.0	36.4*
	EC	0.001	36.9*
		0.01	36.1*
		0.1	27.3*
		1.0	33.1*
	HU	0.001	19.6*
		0.01	20.4*
		0.1	21.4*
	AP	0.1	26.5*
		1.0	29.2*
M_2S	Control	0	6.3 NS
M_3S	Control	0	6.7 NS
M_2X	Control	0	19.6 NS
M_3X	Control	0	15.9 NS
M_3O	Control	0	1.6
	EMS	0.01	4.1*
M_4O	Control	0	2.0
	EMS	0.001	4.7*
		0.1	5.2*
M_3I	Control	0	1.9
	EMS	0.001	5.8*
		0.01	4.2*
		0.1	4.5*
M_4I	Control	0	1.7
	EMS	0.001	4.3*

[a]Except X-rays, which are expressed as kilorads (kR).

NS: No significance between controls and all treatment values.

*Significant at 5% level.

Figure 9. Histogram of percent aborted pollen vs. dose rate for X-ray treatment in the M_1 generation, along with standard deviation (bars).

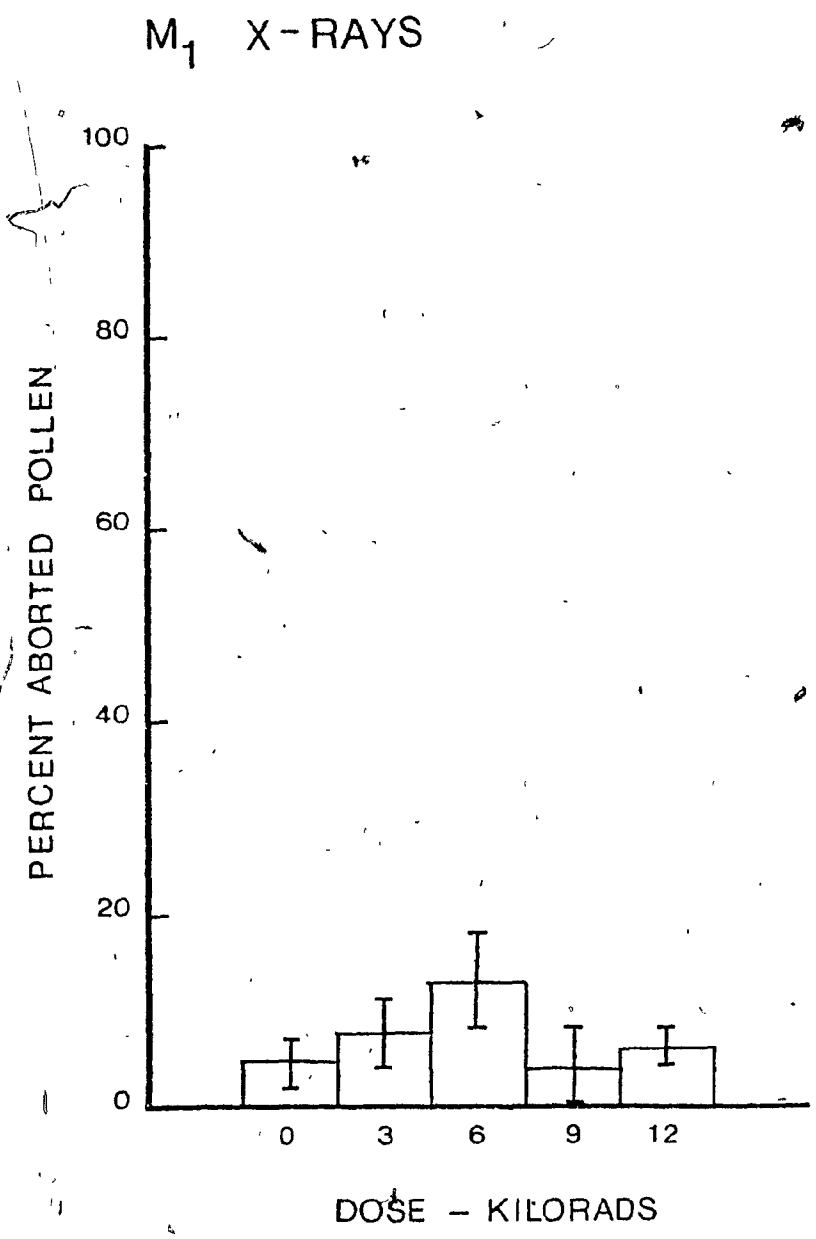

M1 X-RAYS
PERCENT ABORTED POLLEN
100
80
60
40
20
0
0
3
6
9
12
DOSE – KILORADS

Figure 10. Histogram of percent aborted pollen vs. dose rate for EMS treatments in the M_1 generation, along with standard deviations for each treatment (bars).

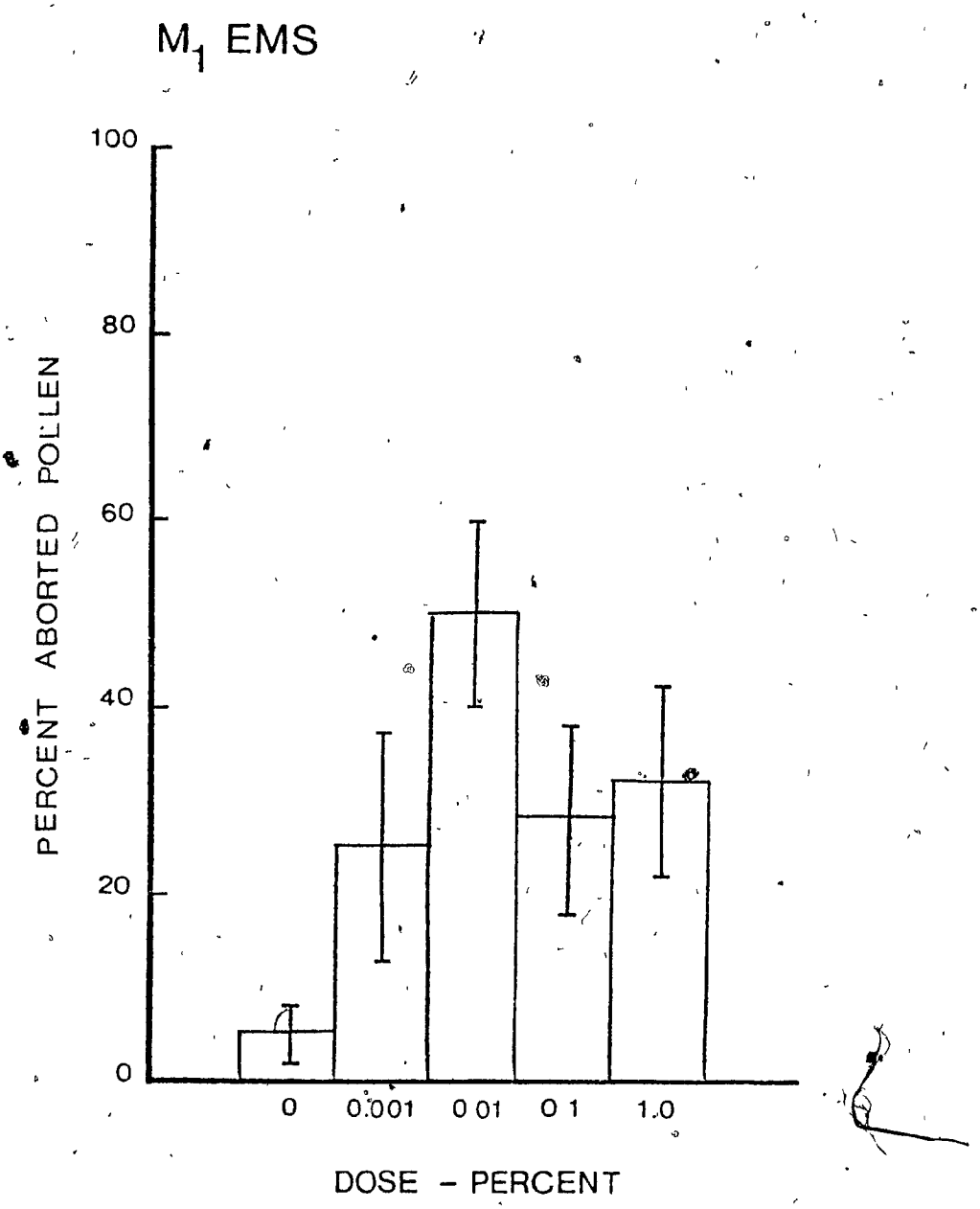

M1 EMS
PERCENT ABORTED POLLEN
100
80
60
40
20
0
0
0.001
0 01
0 1
1.0
DOSE – PERCENT

Figure 11. Histogram of percent aborted pollen vs. dose rate for EC treatments in the M_1 generation, along with the standard deviations for each treatment (bars).

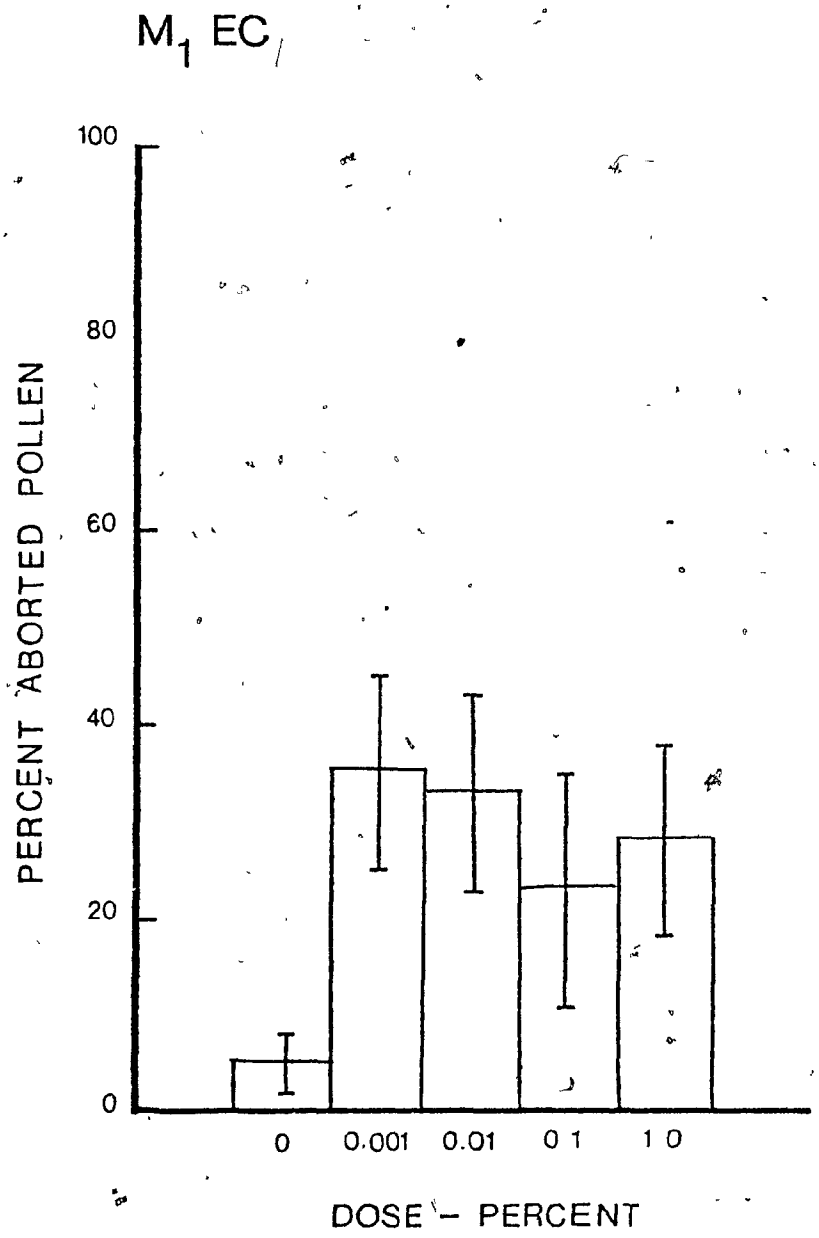

M1 EC
PERCENT ABORTED POLLEN
100
80
60
40
20
0
0
0.001
0.01
0 1
1 0
DOSE – PERCENT

Figure 12. Histogram of percent aborted pollen vs. dose rate for HU treatments in the M_1 generation, along with standard deviations for each treatment (bars).

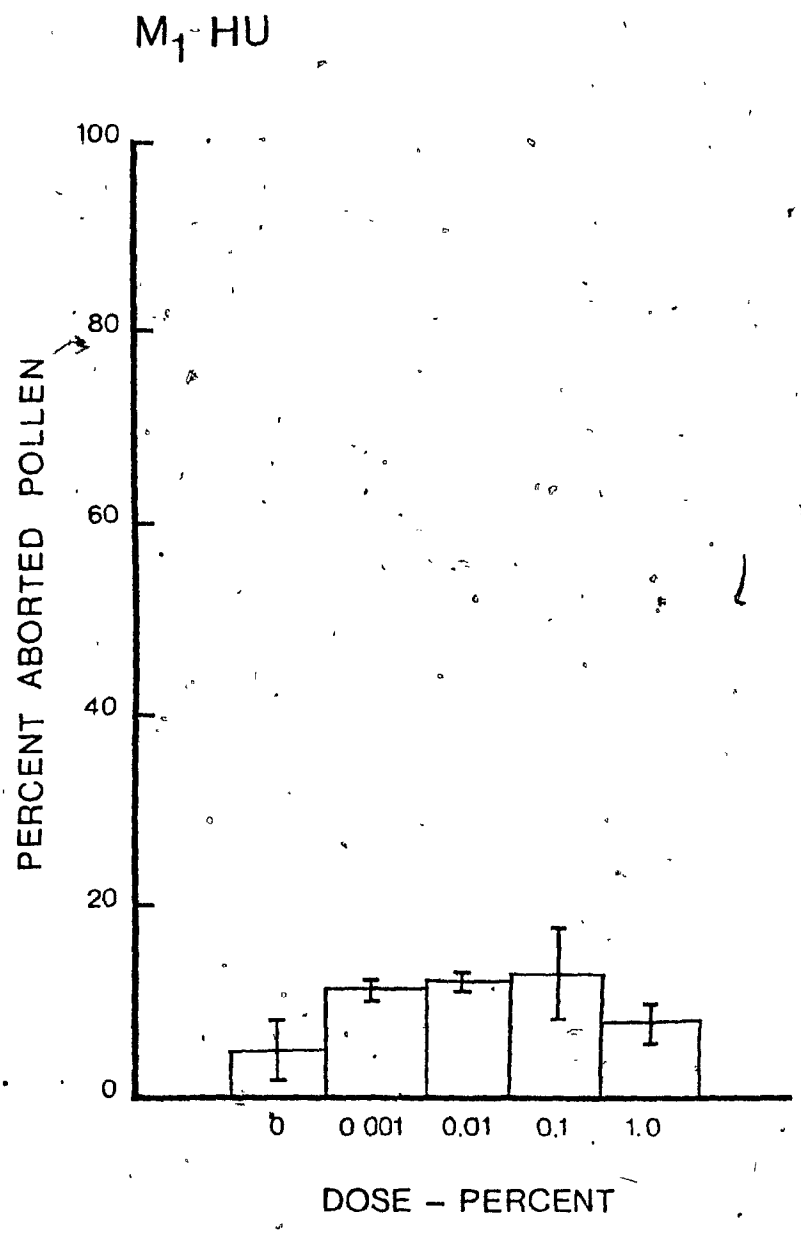
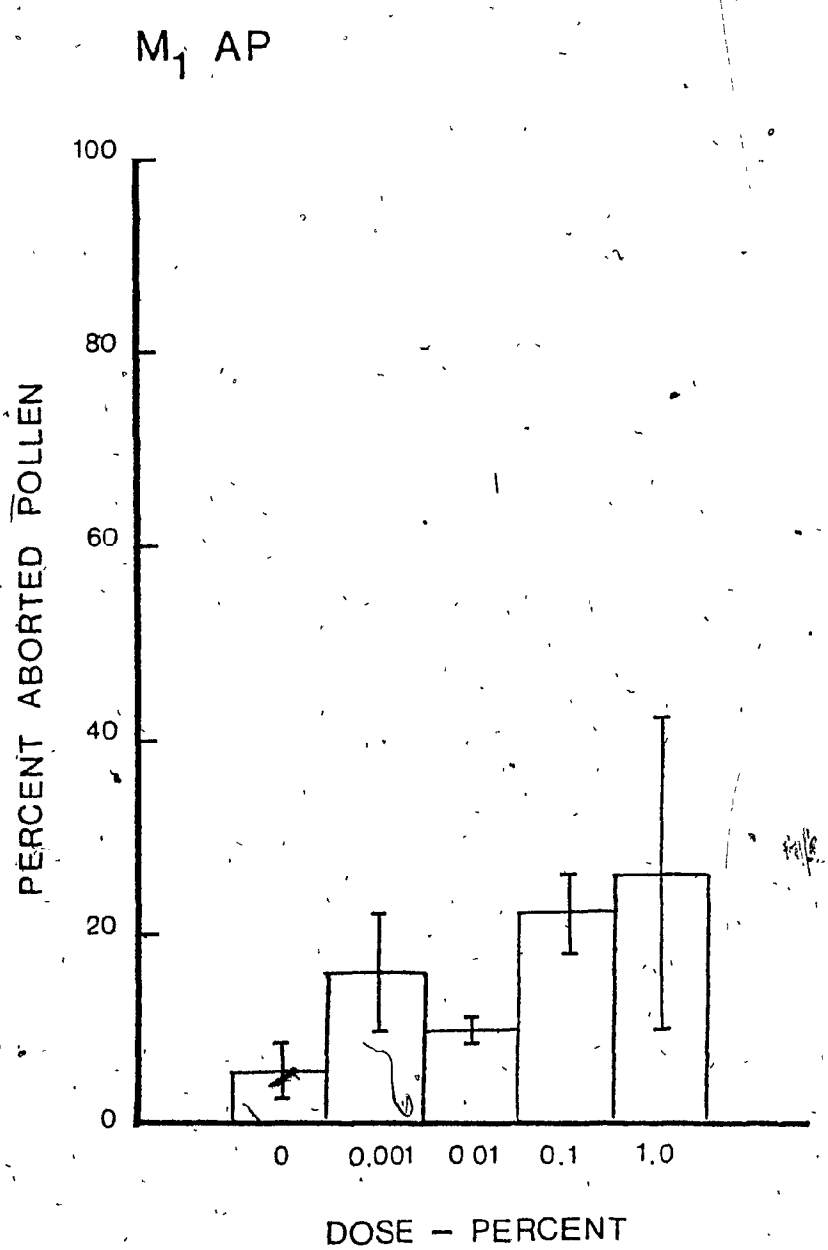

M1 HU
100
80
60
40
20
0
PERCENT ABORTED POLLEN
0
0 001
0.01
0.1
1.0
DOSE – PERCENT

Figure 13. Histogram of percent aborted pollen vs. dose rate for AP treatments in the M_1 generation, along with the standard deviations for each treatment (bars).


M1 AP
100
80
60
40
20
0
PERCENT ABORTED POLLEN
0
0.001
0 01
0.1
1.0
DOSE – PERCENT

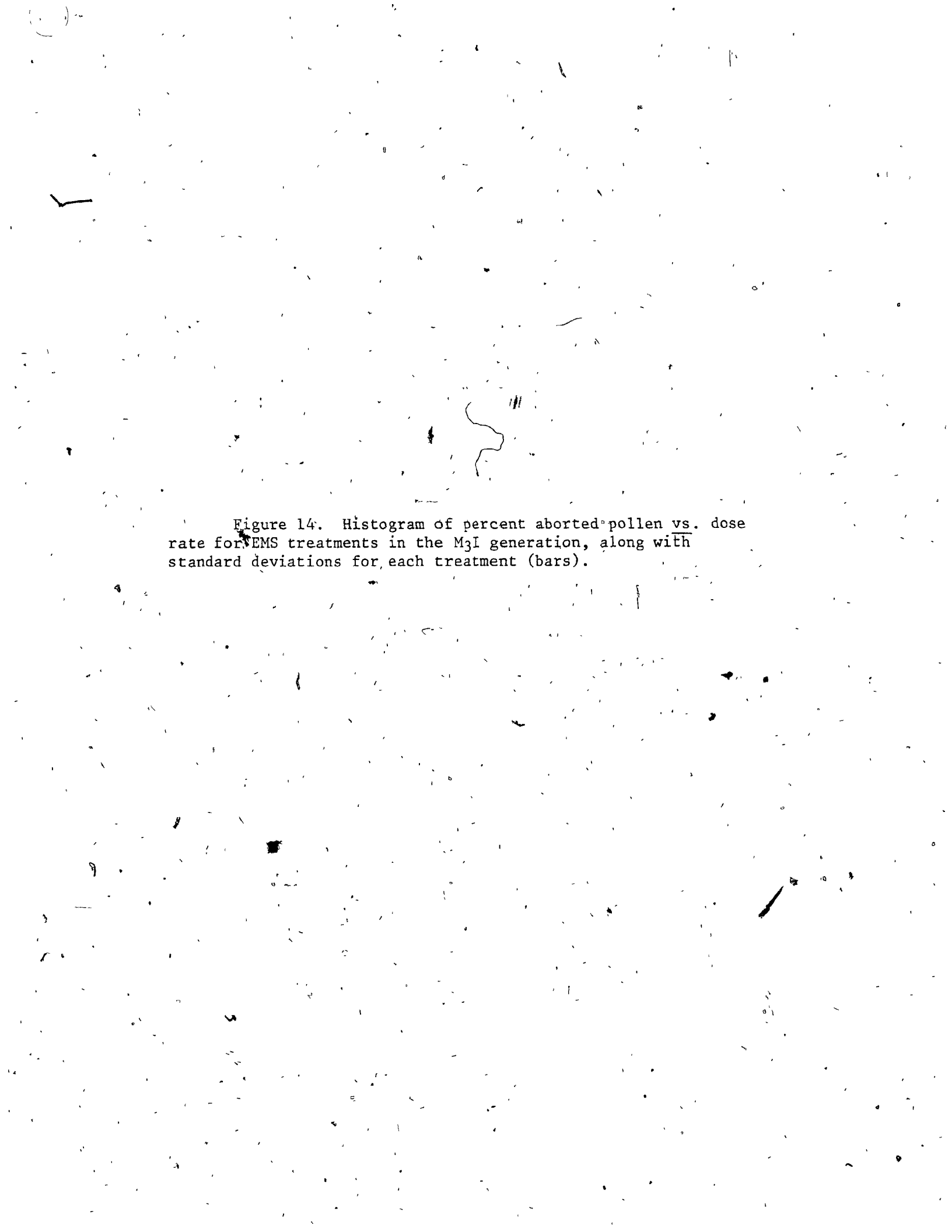
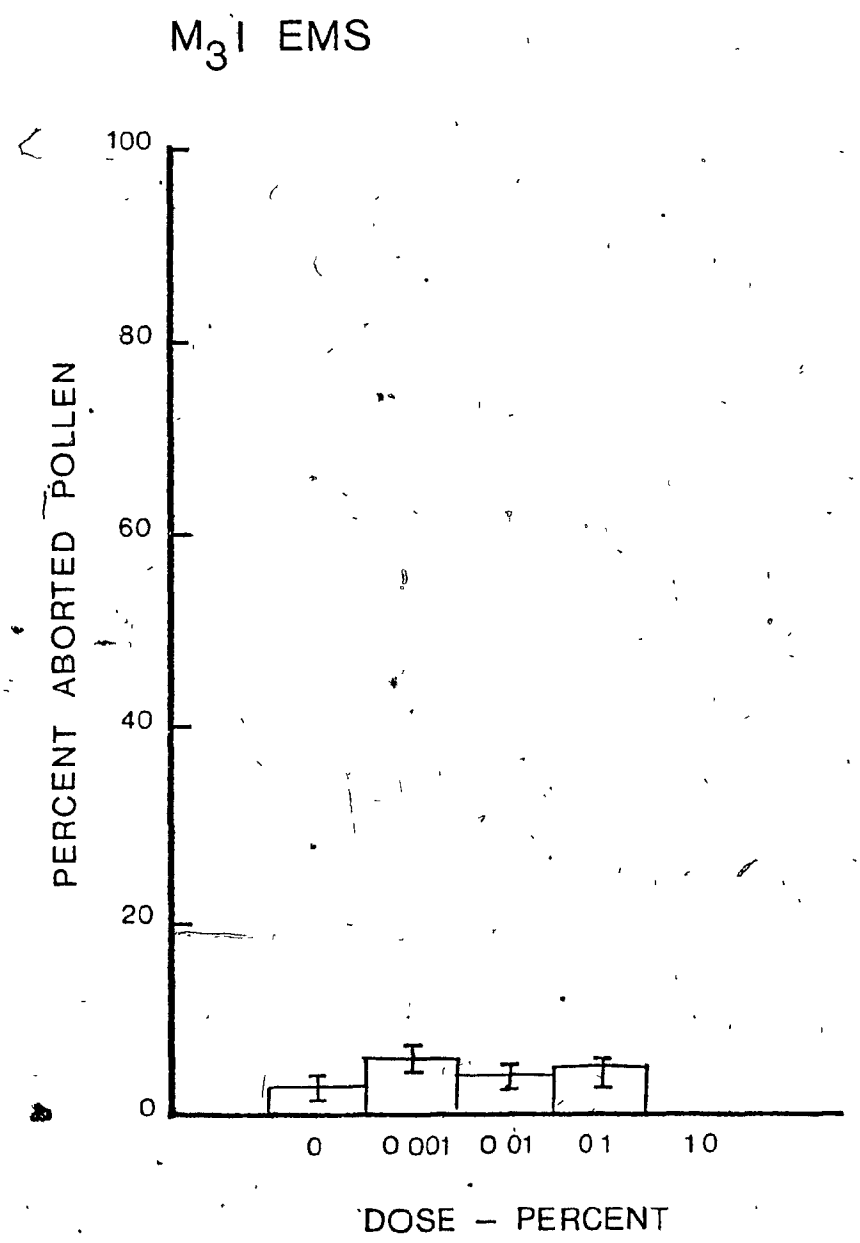


Figure 14. Histogram of percent aborted pollen vs. dose rate for EMS treatments in the M_3I generation, along with standard deviations for each treatment (bars).


M3I EMS
PERCENT ABORTED POLLEN
100
80
60
40
20
0
0
0 001
0 01
0 1
1 0
DOSE – PERCENT

effects at the two highest dosages, namely at 0.1 and 1.0%. There was a high level of variability with the latter dose which gave a standard deviation of approximately 36 (Figure 13).

Plants derived from treatments with EMS in the M_3O, M_4O, M_3I and M_4I generations also showed significant increases in aborted pollen rate for several dosages (Table VII). Those plants in the M_4O and M_3I generations demonstrated significant increases in pollen abortion rate for more than a single dose. M_3I EMS treated plants demonstrated an approximate two-fold increase between control and 0.001% treatment values, with this level of increase being maintained for the other doses tested (Figure 14). M_4O EMS treated plants demonstrated an approximate two-fold increase in pollen abortion rate, as well, for the two dose levels available for testing, that is the 0.001% and 0.1%. There were no significant differences between control and treatment values for all other generations.

E. Evaluation of a genetic marker; brown tip color

Gene frequencies were obtained for the brown floral keel tip color character as shown in Table VIII. The gene frequencies in each generation were calculated for all plants in all five generations.

The M_1, M_2S and M_3S generations had few assayable plants in each generation, due to the few plants which were also weak flowering. From the statistical analysis it was concluded that there was a high level of variability in gene frequency for the brown keel tip color for these

TABLE VIII. Gene frequencies of brown keel tip for five populations of Lotus corniculatus with five treatments per population

Generation	Control	Treatment					Average	Number of plants
		X-rays	EMS	EC	HU	AP		
M_1	0.185	0.400	0.381	0.330	0.136	0.200	0.270	125
M_2S	0.286	0.286	0.636	–	–	–	0.400	25
M_3S	0.286	–	0.353	–	–	–	0.320	24
M_2X	0.074	0.089	0.058	0.088	0.081	0.063	0.076	3098
M_3X	0.160	–	0.151	–	–	–	0.156	448

three generations; coefficients of variability ranged from approximately 45 to 145.5. An analysis of variance indicated no statistically significant differences between control and treated lines.

In contrast, the M_2X and M_3X generations had considerably larger sample sizes and showed lower gene frequencies for the brown keel tip character in contrast to those obtained in the M_1, M_2S and M_3S generations. Coefficients of variability ranged from 34 to 55% in the M_2X generation to a value of approximately 70% in the M_3X generation. No statistically significant differences were obtained between controls and treatments in either the M_2X or M_3X generations.

F. Evaluation of mutant types

1. Mutation rate

a. Lotus corniculatus

Friedman's (1937) ranked Chi-square test of the average number of qualitative mutants per 100 plants scored, indicated that significant increases in mutation rate vs. control occurred only in the M_3O generation (Table IX). Two types of mutants were scored, namely, the chlorotica (ct) and vestigial floret (vf) types (Figure 15). The highest mutation rates were found in X-ray and EMS treated lines of the M_3O generation, followed by EC and AP treatments, and lastly from HU treated progeny in the M_3O generation. All treatments were significant; a probability of a greater Chi-square value of 0.005 was calculated for X-ray and EMS treated lines, and a value of 0.05 for EC, HU and AP treated lines. No dose effects were observed in any of the lines of the M_3O generation.

TABLE IX. Mutation rate, expressed as the mean number of mutants per 100 plants for controls and five mutagens over eight generations

Generation and treatment	Number of mutants per 100 plants	Friedman's ranked Chi-square value	Probability of greater Chi-square value (3 degrees of freedom)
M_2X			
Control	0.78	-	-
X-rays	0.86	0.001	0.95
EMS	1.38	0.001	0.95
EC	0.47	0.001	0.95
HU	0.45	0.001	0.95
AP	0.97	0.001	0.95
M_3X			
Control	0.00	-	-
EMS	0.67	0.001	0.95
M_2S			
Control	0.00	-	-
EMS	0.33	0.001	0.95
M_3S			
Control	0.00	-	-
EMS	0.00	< 0.001	> 0.99
M_3O			
Control	0.33	-	-
X-rays	1.08	34.1	0.005
EMS	1.08	25.5	0.005
EC	0.75	10.8	0.05
HU	0.67	10.4	0.05
AP	0.75	10.7	0.05
M_3I			
Control	0.00	-	-
X-rays	0.33	0.001	0.95
EMS	1.00	0.001	0.95
HU	0.00	0.001	0.95
M_4O			
Control	0.33	0.001	0.95
EMS	0.50	0.001	0.95
M_4I			
Control	0.33	0.001	0.95
EMS	0.67	0.001	0.95

Figure 15. The two most frequently occurring mutants induced by mutagenesis in Lotus corniculatus: (a) Chlorotica (right), shown with a phenotypically normal plant (left). (b) Vestigial floret (top), with a phenotypically normal floret (bottom).

A

B

Coloured Paper
Papier de couleur

The qualitative mutation rates differed numerically from their respective controls in all generations by as wide a margin as 0 to 1.0%. None of these values was significant from each other owing to a preponderance of zeroes in the data and to a relatively small data base. The exceptions were the M_3O generation which was significant and the M_2X generation which, although containing a large data base, there was no statistical significance between treatments and control rates. The highest overall mutation rates were obtained in the M_2X ($\bar{x}$=0.82%) and M_3O ($\bar{x}$=0.78%) generations, whereas the lowest values were obtained in the M_2S and M_3S generations with respective values of 0.17% and 0%. The sample size of the treated and control lines varied from 600 to 900 plants in the M_2S, M_3S, M_3X, M_3I, M_4O, and M_4I generations, to 1800 plants in the M_2X and M_3O generations. Background (control) mutation rates, for the two mutant characters (ct and vf), ranged from 0% to 0.78%, being highest in the open-pollinated progeny and lowest in the selfed and outcrossed progeny derived from the selfed M_2S and M_3S generations.

b. Lotus tenuis

In the M_2X generation of L. tenuis only 137 plants were produced, the majority of which were derived from the 0.05% and 0.1% EMS treatments. A total of 17 anomalous plants (chlorophyll deficient and morphological abnormalities) were obtained from two lines derived from EMS treatments. Eleven of these «mutants» were derived from treatment with 0.05% EMS and six from the 0.1% treatment. None of the other seven treatments resulted in the production of any mutants. From the

statistical analysis, no significant increase in mutation rate could be determined for the two lines containing anomalous plants when compared with controls or other treatments.

2. Segregation ratios of mutant types

Segregation ratios from parent-progeny crosses for putative qualitative mutants were obtained from M_3 progeny derived from various treated lines (Table X). Although parent-progeny crosses and selfings in every combination were attempted, only three types of crosses and selfings were successful in the production of progeny. These were (1) parent X mutant (M_2S X M_3S), (2) parent-selfed (M_2S), and (3) mutant selfed (M_3S). Three putative mutant types were studied, namely, chlorotica (ct), vestigial floret (vf), and dwarf (d). The ct and vf types were derived from X-ray and EMS treatments, while the d type arose in progeny from all mutagen treatments, except from the EC treatments.

The EMS derived ct type has a 20:1 segregation ratio for normal:ct from a parent X mutant backcross. This ratio approximates the expected 27:1 ratio for a triplex X nulliplex cross, assuming random chromatid assortment (Chi-square probability = 0.1966). Selfing the parent, however, did not result in the expected normal:ct ratio, and no progeny were produced upon selfing the ct type. The X-ray derived ct type produced a normal:ct ratio of 6.1:1, upon selfing the parent. This closely approached the expected ratio of 3:1 for a selfed simplex (Chi-square probability of 0.0782). Crossing the parent with

TABLE X. Segregation ratios for parent-progeny crosses and selfed induced mutant types

Mutant type	Cross or selfing	Mutagen	Ratio observed normal: mutant	Ratio expected normal: mutant	Expected genotype	Chi-square	Probability of greater Chi-square value
Chlorotica (ct)	M_2S (selfed)	EMS	700:3 (233.3:1)	1:0	Triplex[a]	161936.3	< 0.005
	M_2S X M_3S (mutant)		60:3 (20:1)	27:1	Triplex X nulliplex[b]	1.815	0.1966
	M_3S (selfed mutant)		No progeny	0:1	Nulliplex	-	-
Chlorotica (ct)	M_2S (selfed)	X-rays	412:68 (6.1:1)	3:1	Simplex[a]	3.203	0.0782
	M_2S X M_3S (mutant)		660:92 (7.1:1)	1:1	Simplex X nulliplex[a]	37.21	< 0.005
				5:1	Duplex X nulliplex[a]	0.882	0.4006
	M_3S (selfed)		No progeny	0:1	Nulliplex	-	-
Vestigial floret (vf)	M_2S (selfed)	X-rays	40:2 (20:1)	20.8:1	Duplex[b]	0.0308	0.8739
	M_2S X M_3S (mutant)		No progeny	3.7:1	Duplex X nulliplex[b]	-	-
	M_3S (selfed)		No progeny	0:1	Nulliplex	-	-

table continued

TABLE X (continued)

Mutant type	Cross or selfing	Mutagen	Ratio observed normal: mutant	Ratio expected normal: mutant	Expected genotype	Chi-square	Probability of greater Chi-square value
Vestigial floret (vf)	M_2S (selfed)	EMS	73:4 (18.3:1)	20.8:1	Duplex[b]	0.3005	0.6261
	M_2S X M_3S (mutant)		No progeny	3.7:1	Duplex X nulliplex[b]	-	-
	M_3S (selfed)		No progeny	0:1	Nulliplex	-	-
Dwarf (d)	M_2S (selfed)	X-rays	155:2 (77.5:1)	35:1	Duplex[a]	51.6071	< 0.005
	M_3S (mutant-selfed)		No progeny	0:1	Nulliplex	-	-
	M_2S X M_3S (mutant)		81:2 (40.5:1)	5:1	Duplex X nulliplex[a]	252.05	< 0.005
Dwarf (d)	M_2S (selfed)	EMS	198:4 (49.5:1)	35:1	Duplex[a]	6.007	0.0158
	M_3S (mutant)		161:0	0:1	Nulliplex	25921.0	< 0.005
	M_2S X M_3S (mutant)		106:0	5:1	Duplex X nulliplex	2040.2	< 0.005

table continued

TABLE X (continued)

Mutant type	Cross or selfing	Mutagen	Ratio observed normal: mutant	Ratio expected normal: mutant	Expected genotype	Chi-square	Probability of greater Chi-square value
Dwarf (d)	M_2S (selfed)	HU	111:4 (27.8:1)	35:1	Duplex[a]	1.4811	0.2326
				20.8:1	Duplex[b]	2.3558	0.1382
	M_3S (mutant selfed)		84:0	0:1	Nulliplex	7056.0	< 0.005
	M_2S X M_3S (mutant)		No progeny	5:1	Duplex X nulliplex[a]	-	-
Dwarf (d)	M_2S (selfed)	AP	95:5 (19:1)	20.8:1	Duplex[b]	1.1571	0.2676
	M_3S (mutant selfed)		No progeny	0:1	Nulliplex	-	-
	M_2S X M_3S (mutant)		No progeny	3.7:1	Duplex X nulliplex[b]	-	-

[a]Random chromosome assortment.

[b]Random chromatid assortment.

the ct type produced a 7.1:1 ratio of normal:ct progeny. This ratio does not fit the expected 1:1 ratio for a simplex X nulliplex cross assuming random chromosome assortment, but it does fit an expected 5:1 ratio for a duplex X nulliplex cross assuming random chromosome assortment, with a Chi-square probability of 0.4006. The ct type did not produce progeny upon selfing.

Upon selfing the parents of the vestigial floret (vf) putative mutant type for both X-ray and EMS derived lines, a normal:vf ratio of 20:1 and 18.3:1, respectively, were obtained. This closely approximates the expected 20.8:1 ratio for a selfed duplex assuming random chromatid assortment. Chi-square probability values of 0.8739 and 0.6261 were obtained for the ratios of 20:1 and 18.2:1, respectively. The vf type was found to be completely sterile and therefore could not be selfed or crossed.

The dwarf (d) putative mutant type was obtained in progeny from X-ray, EMS, HU and AP treatments. Selfing of HU and AP derived parents produced progeny with ratios of normal:d of 27.8:1 and 19:1, respectively. This approximates the 35:1 and 20.8:1 segregation ratios for a selfed duplex for random chromosome and chromatid assortment, respectively. However, upon selfing the HU d type, 84 phenotypically normal progeny were obtained, which is contrary to expected values for a selfed nulliplex. Furthermore, X-ray and EMS derived d types did not conform to expected ratios for progeny obtained from parent X d, selfed parent, or selfed d types. The ct and vf mutants are shown in Figure 15.

G. Hydrocyanoglycoside content

The deposition of hydrocyanoglycosides (HCA) in the leaves of Lotus corniculatus cultivar «Mirabel» was found to be inherited in a quantitative fashion (Figure 16). Significant changes in HCA content were obtained in three generations, namely the M_2X, M_3O and M_3I (see Appendix I, Tables 26-30) with no significant changes in HCA content between control and treatment values for any other generation (Table XI). Significant increases in HCA content were obtained from X-ray and AP treatments in the M_2X generation as well as for plants derived from HU treatments in the M_3I generation. One EMS treatment in the M_2X generation, as well as two EC treatments in the M_3O generation demonstrated a significant decrease in HCA content.

Three groups of treatments, in three generations, demonstrated significant increases or decreases over two or more dosages. These were the AP treated lines in the M_2X generation, EC treated lines in the M_3O generation, and HU treated lines in the M_3I generation. An increase in HCA content was induced in the AP-treated M_2X and the HU-treated M_3I generations. All dosages were effective in inducing a quantum increase in HCA content for AP-treated M_2X plants, with all dosages demonstrating a significant increase with respect to control levels, but not with respect to each other (Figure 17). Plants derived from treatment with HU in the M_3I generation demonstrated induced increases in HCA content for the 0.001 and 0.01% treated lines, and returning to control levels for the 0.1% treated line (Figure 19). No progeny were obtained for 1.0% treatment with HU in the M_3I

Figure 16. Frequency polygon for hydrocyanoglycoside (HCA) content in leaves of Lotus corniculatus, superimposed on a histogram, for each of three consecutive, open-pollinated generations, sampled at random. Each generation represented includes (a) HCA content in the M_1 (parental) generation, (b) HCA content in the M_2X generation, and (c) HCA content in the M_3O generation. Plotted are frequency vs. mg HCA per kg of fresh leaf. N = the number of plants tested.

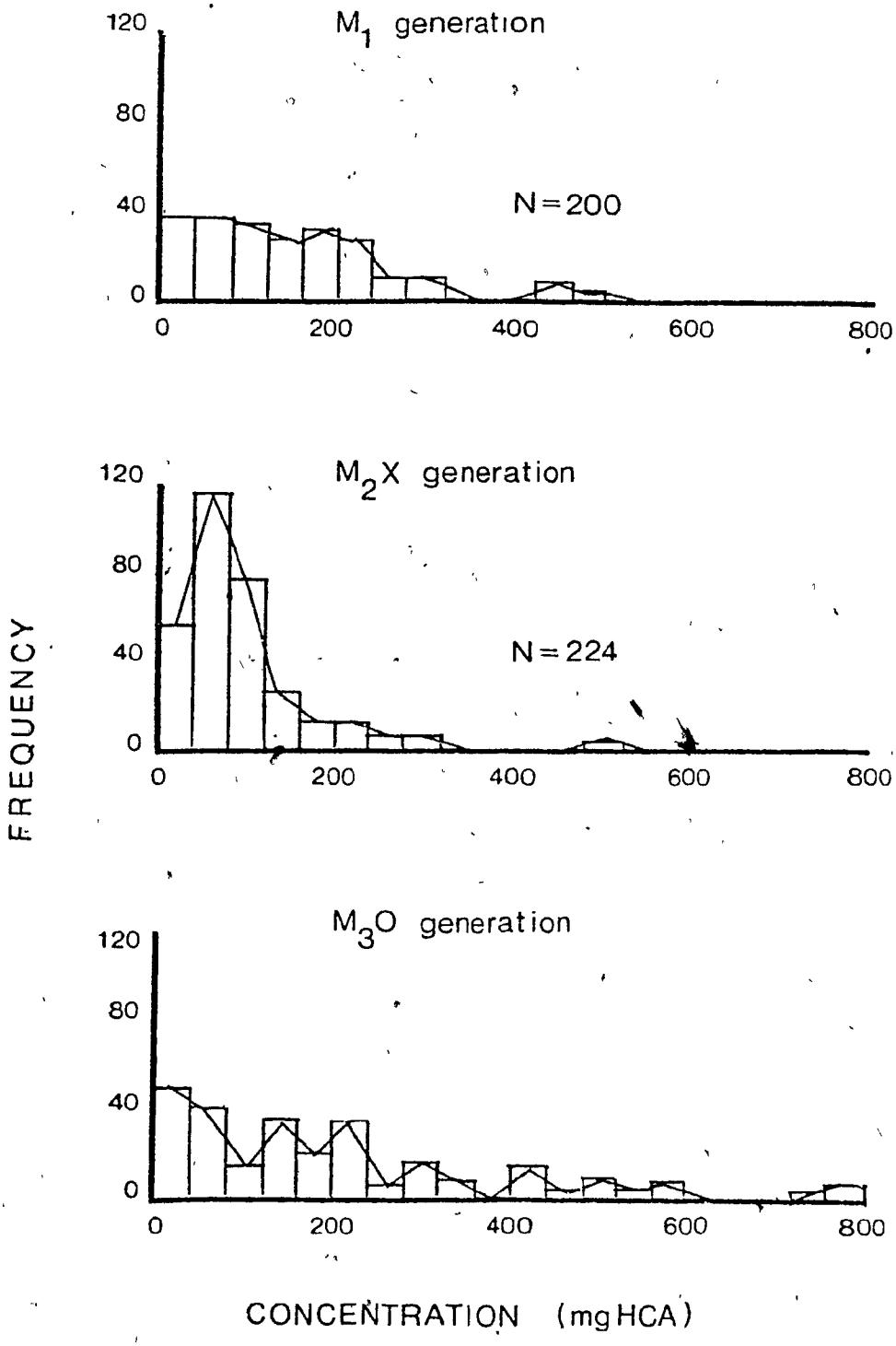

M1 generation
120
80
40
0
N=200
0
200
400
600
800
M2X generation
120
80
40
0
N=224
0
200
400
600
800
FREQUENCY
M3O generation
120
80
40
0
0
200
400
600
800
CONCENTRATION (mg HCA)

TABLE XI. Hydrocyanic glycoside (HCA) content, expressed as the mean value in mg hydrocyanic acid per kg fresh leaf matter, for all treatments showing significance with respect to controls

Generation	Treatment	Dosage (%, except X-rays, kR)	HCA content (mg/kg)
M_1	Control	0	86.2 NS
M_2S	Control	0	182.2 NS
M_3S	Control	0	134.2 NS
M_2X	Control	0	74.0
	X-rays	3	131.3*
	EMS	0.1	29.5*
	AP	0.001	99.3*
		0.01	104.4*
		0.1	110.6*
		1.0	96.8*
M_3X	Control	0	177.1 NS
M_3O	Control	0	208.2
	EC	0.1	36.4*
		1.0	14.7*
M_4O	Control	0	168.3 NS
M_3I	Control	0	114.8
	HU	0.001	326.0*
		0.01	301.5*
M_4I	Control	0	230.9 NS

NS: No significance between controls and all other treatments.

*Significant at the 5% level.

Figure 17. Histogram of hydrocyanoglycoside (HCA) content (as mg HCA per Kg fresh leaf) vs. dose levels for plants derived from treatment with AP in the M_2X generation, along with plots of the standard deviation for values obtained at each dose, indicated as bars.

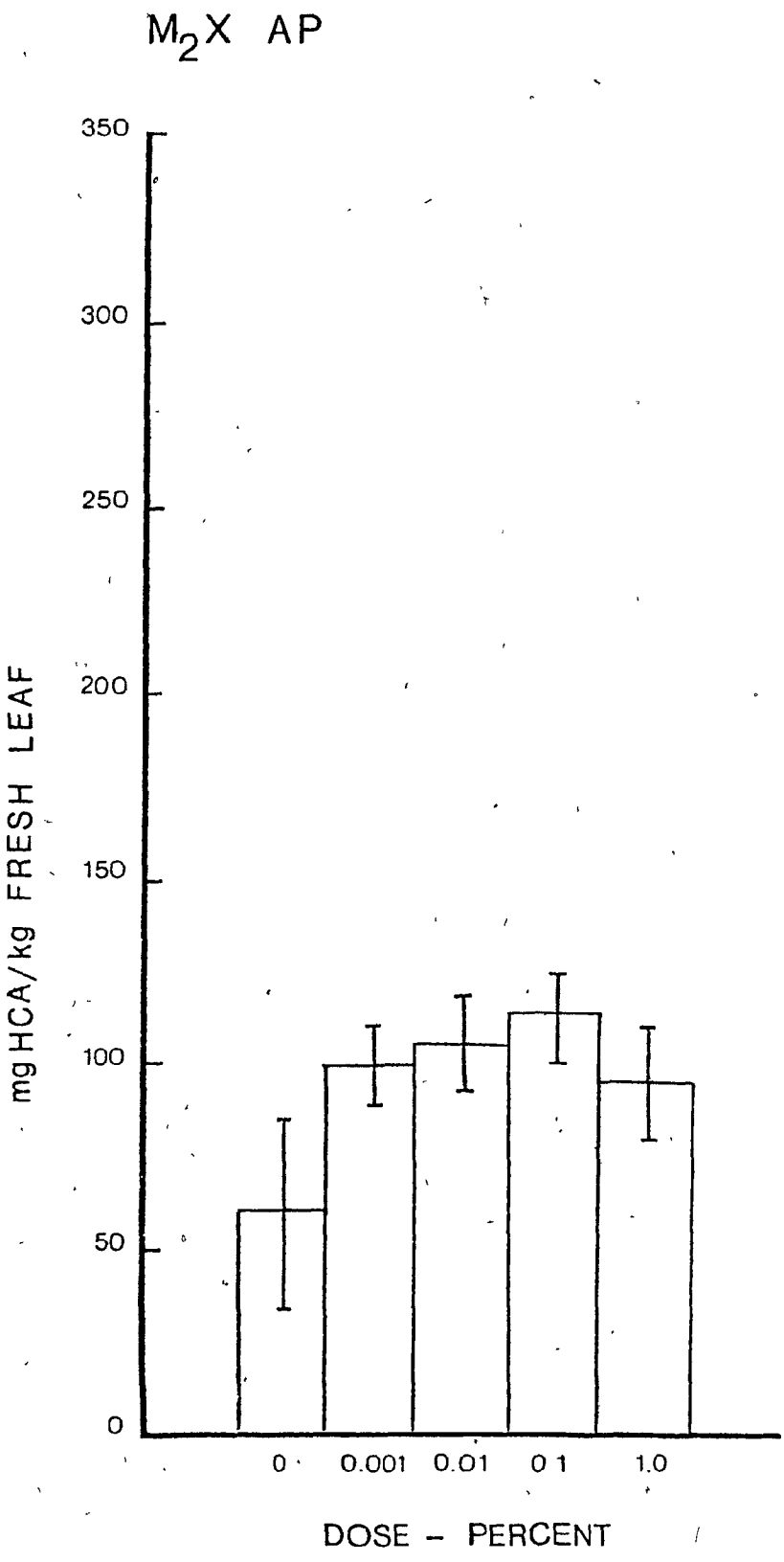

M2X AP
350
300
250
200
150
100
50
0
mg HCA/kg FRESH LEAF
0
0.001
0.01
0 1
1.0
DOSE – PERCENT

Figure 18. Histogram of hydrocyanoglycoside (HCA) content (as mg HCA per Kg fresh leaf) vs. dose levels for plants derived from treatment with EC in the M_3O generation, along with plots of the standard deviation for values obtained at each dose, indicated as bars.

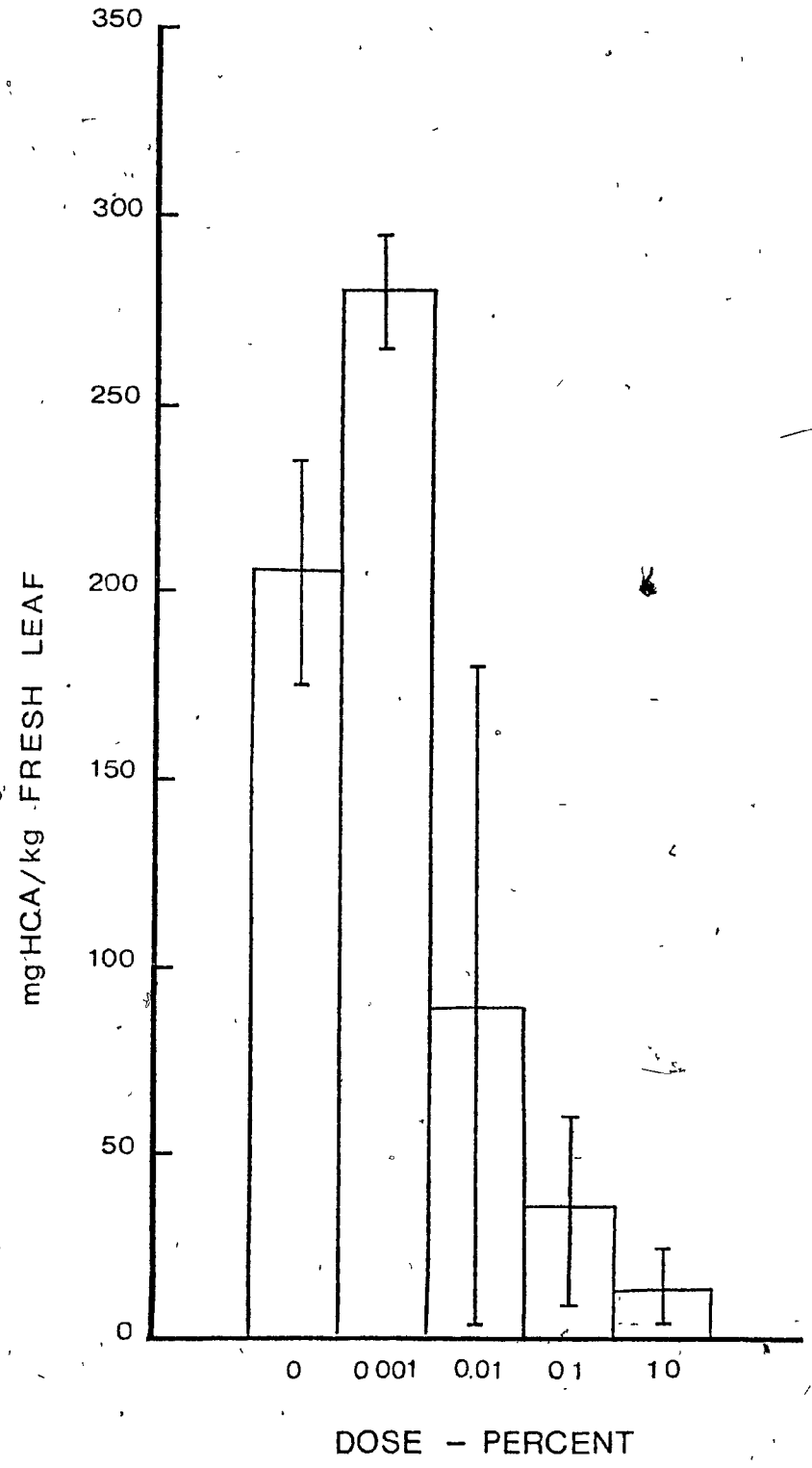
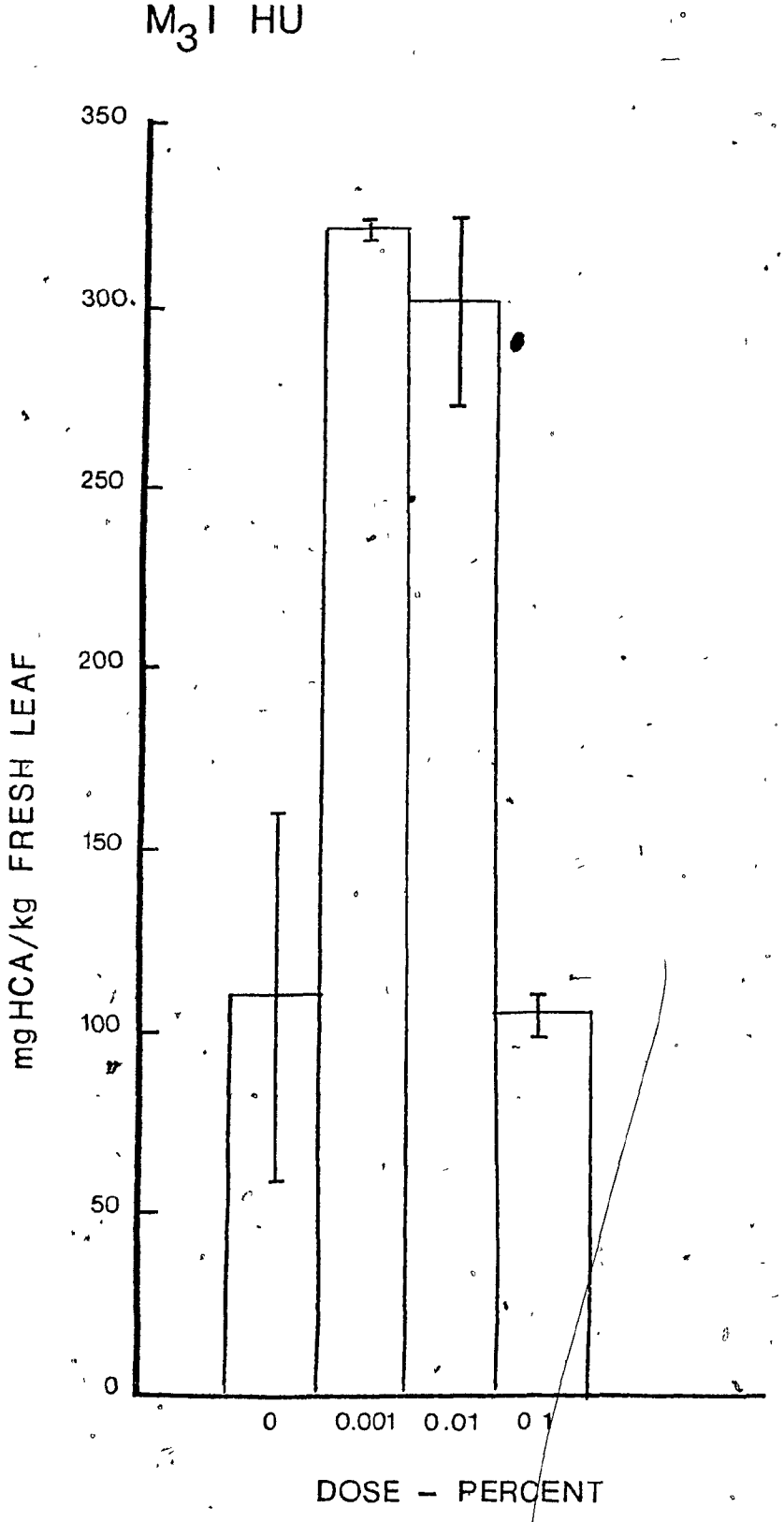

M_3O EC
350
300
250
200
150
100
50
0
mg HCA/kg FRESH LEAF
0
0 001
0.01
0 1
1 0
DOSE – PERCENT

Figure 19. Histogram of hydrocyanoglycoside (HCA) content (as mg HCA per Kg fresh leaf) vs. dose levels for plants derived from treatment with HU in the M_3I generation, along with plots of the standard deviation for values obtained at each dose, indicated as bars.


M3 I HU
350
300
250
200
150
100
50
0
mg HCA/kg FRESH LEAF
0
0.001
0.01
0 1
DOSE – PERCENT

generation. EC treated plants in the M_3O generation demonstrated a sharp decrease in HCA content for the two highest doses, namely, 0.1 and 1.0% (Figure 18). A third dose, the 0.01%, also showed a sharp decrease in mean value, but a large standard deviation of greater than 100 indicated a high degree of variation at this dose. From a statistical analysis it was found that this value was not significant from control levels.

Significant effects in the M_2X generation obtained in X-ray, EMS and AP treated progeny were not transmitted to the M_3O progeny, and no significant differences between control and treatment values were obtained for any of the other six generations tested.

H. Forage yield

There was a significant increase in the mean forage yield over control values in one open-pollinated generation, namely the M_3O generation (Table XII). A mean dry forage yield of 41.3 grams per plant was obtained for the 0.1% EC-treated line, a significant increase of more than twice the mean control value of 16.0 grams (see Appendix I, Table 31). The 0.1% EC-treated parental line in the M_2X generation did not show any significant increase. Likewise, there were no significant differences in mean forage yields between control plants and treatments for any of the other eight generations. An overall increase in mean forage yield was noted for the M_2S and M_3S generations vs. the M_1 (initially treated generation) and all other generations. An average 3.5 and 7.0 fold increase in yields occurred in the M_2S and M_3S generations, respectively,

TABLE XII. Forage yield, expressed as the mean dry weight (in grams) per plant, for all values that are significant from controls

Generation	Treatment	Dosage (%)	Dry weight (g/plant)
M_1	Control	0	35.5 NS
M_2S	Control	0	124.7 NS
M_3S	Control	0	253.7 NS
M_2X	Control	0	21.6 NS
M_3X	Control	0	17.1 NS
M_3O	Control	0	16.0
	EC	0.1	41.3*
M_4O	Control	0	14.3 NS
M_3I	Control	0	20.3 NS
M_4I	Control	0	13.2 NS

NS: No significance between controls and all treatments.

*Significant at the 5% level.

when measured against yields in the M_1 and later generations. The increased yields were an overall effect and not due to any mutagenic treatments, since no significant differences were found between control and treatment values for M_2S and M_3S plants; these increases in yield were not transmitted to the progeny of the M_2S or M_3S generations.

I. Seed yield

1. Lotus corniculatus

Statistical analysis of the mean number of seeds per pod per plant indicated that the values were significantly different from those of the control for three treated lines (Table XIII). The first line, derived from treatment with 0.1% EMS in the M_3S generation, had a two-fold increase in seed yield over the M_2S control values. A similar effect was found for plants derived from treatment with 0.001% EMS in the M_3X generation which showed a two-fold increase in seed yield when compared with M_3X control values. A third line, derived from treatment with 0.01% EC in the M_2X generation, showed a significant and drastic drop of 92.6% in seed yield when compared with the values obtained for the M_2X control (see Appendix I, Tables 32-34). No significant changes in seed yield were found for the parents or the progeny of these three lines or in any of the other lines.

TABLE XIII. Seed yield, expressed as the average number of seeds per pod per plant, for all values that are significant from controls

Generation	Treatment	Dosage (%)	Mean number of seeds/pod/plant
M_1	Control	0	11.9 NS
M_2S	Control	0	15.9 NS
M_3S	Control	0	4.0
	EMS	0.1	9.6*
M_2X	Control	0	6.8
	EC	0.01	0.5*
M_3X	Control	0	5.4
	EMS	0.001	12.9*
M_3O	Control	0	26.0 NS
M_4O	Control	0	28.3 NS
M_3I	Control	0	27.4 NS
M_4I	Control	0	16.6 NS

NS: No significance between controls and all treatments.

*Significant at the 5% level.

2. Lotus tenuis

Seed yield varied greatly for the treated and untreated lines of L. tenuis, as may be seen in Table XIV. The total number of seed obtained from cross-pollination varied from a low of 44 seeds from 7 pods after a treatment with 0.001% EMS, to a high of 1975 seeds from 286 pods from treatment with 0.005% EMS. No significant differences were obtained between controls and treatments for the total number of seeds or the total number of pods. Average seed yield, expressed as the mean number of seeds produced per pod per plant, was found to vary from 4.6 to 9.0, and no significant differences were obtained between treatment and control values.

3. Selfed seed production

Values obtained for selfed seed production are given in Table XV for 20 mutagenic treatments and controls in the M_1, and for four mutagenic treatments and controls in the M_2S generation. All M_1 seed treated with AP, as well as those treated with 1.0% EMS, did not produce any selfed progeny after one flowering cycle. All other treatments in the M_1 showed reduced selfed seed set over control M_1 values with the exception of the 9 kR treated line which produced a 10-fold increase in the number of selfed seed set per plant. However, the total number of selfed seed produced was lower in the 9 kR treated line in comparison with the M_1 control. Viable (germinating) seeds were recovered in all selfed seed producing M_1 lines, with the greatest production in the control M_1 line, followed by HU, EC, X-ray and EMS lines, in decreasing order. After one cycle of selfing, M_2S derived

TABLE XIV. Open-pollinated seed yield in the M_2X generation of *Lotus tenuis* from EMS treatment (30 plants per treatment sampled)

Treatment (% EMS)	Number of seeds	Number of pods	Seeds/pod/plant
Control	543	118	4.6
0.001	44	7	6.3
0.005	1975	286	6.9
0.01	361	44	8.2
0.05	237	37	6.4
0.1	1884	309	6.1
0.5	2302	465	5.0
1.0	470	78	6.0
1.5	1144	164	7.0
2.0	819	91	9.0

TABLE XV. Selfed seed production for M_1 and M_2S treated and untreated lines of Lotus corniculatus

Treatment	Number of seeds	Number of plants (per 100)	Number of seeds per plant	Percent germination
M_1				
Control	330	68	4.9	14.5
kR				
3	40	11	3.6	2.7
6	9	3	3.0	0.9
9	132	3	44.0	11.5
12	7	3	2.3	0.0
EMS				
0.001	13	4	3.3	3.9
0.01	1	1	1.0	0.0
0.1	28	12	2.3	8.2
1.0	0	–	–	–
EC				
0.001	19	9	2.1	0.0
0.01	1	1	1.0	0.0
0.1	0	–	–	–
1.0	4	1	4.0	25.0
HU				
0.001	10	10	1.0	30.0
0.01	11	11	1.0	0.0
0.1	12	12	1.0	75.0
1.0	0	–	–	–
AP				
0.001	0	–	–	–
0.01	0	–	–	–
0.1	0	–	–	–
1.0	0	–	–	–
M_2S				
Control	85	27	3.1	4.7
EMS				
0.001	181	15	12.1	14.4
0.01	47	19	2.5	57.4
0.1	347	52	6.7	24.1
1.0	0	–	–	–

control plants showed a reduced selfing capacity in comparison with the parental M_1 controls, but the number of seeds per pod in the M_2S generation (3.1 seed/pod) was not significantly different from the M_1 control group (4.9 seeds/pod). In contrast, EMS derived M_2S plants did show a numerical increase in total number of selfed seed, number of plants selfed per 100 plants, number of selfed seeds per plant, and the percentage viable (germinating) seeds in comparison with the parental M_1 EMS treated plants. There were no statistically significant differences between any values in the M_1 and M_2S lines derived from treatment with EMS.

J. Flowering frequency

The two methods utilized in assessing the flowering frequencies of the 103 treated and control lines of Lotus corniculatus cv. «Mirabel,» over the nine generations were the mean number of days to peak, or maximum flowering for control and significant values, and secondly, the mean number of days to 50% flowering (Table XVI). Only the 0.0001% and 0.01% EMS treated lines in the M_4O generations in which the peak flowering cycles occurred about 2 to 3 days earlier than the controls were significant (Appendix I, Table 36). Lines in ascending parental order in the M_3X, M_2S and M_1 generations, did not show significant changes in their flowering cycle.

Significant changes in flowering cycle were also found in the M_3O generations (see Appendix I, Table 35). There was a late flowering line derived from treatment with 0.1% EC. These late flowering 0.1%

TABLE XVI. Flowering frequency, expressed as the mean number of days to maximum or peak flowering and the mean number of days to 50% flowering, for all significant values, along with the ranges for non-significant values

Generation	Treatment	Dosage (%)	Mean days to maximum flowering (Range)	Mean days to 50% flowering (Range)
M_1	Control	0	9.2(8.3- 9.7)NS	6.4(3.0- 9.0)NS
M_2S	Control	0	9.5(9.5- 9.6)NS	9.0(4.0-15.0)NS
M_3S	Control	0	9.0(9.0-11.0)NS	8.0(8.0-14.0)NS
M_2X	Control	0	9.0(8.5- 9.5)NS	10.8(5.0-11.0)NS
M_3X	Control	0	9.7(9.5-10.2)NS	8.8(9.0-10.0)NS
M_3O	Control	0	5.2(3.5- 7.7)NS	11.9
	EC	0.1	--	18.8*
M_4O	Control	0	8.3	9.2(7.0-10.0)NS
	EMS	0.001	5.5*	--
		0.01	6.1*	--
M_3I	Control	0	4.8(3.1- 7.6)NS	9.6(9.0-13.0)NS
M_4I	Control	0	6.0(4.4- 6.4)NS	11.7(11.0-13.0)NS

NS: No significance between controls and all treatments.

*Significant at the 5% level.

EC derived plants in the M_3O did not contain late flowering types in the parental M_2X EC lines. No significant differences were noted for all other generations.

K. Pod dehiscence

Pod dehiscence was highly variable, with the percentage of dehisced pods per plant ranging between 0 and 75.8% with a mean of 34.5% for five generations. An analysis of variance further confirmed the variability of this character; a standard deviation of 21.28 and a coefficient of variability of 68.26 were obtained for the combined data over five generations. From Table XVII it may be seen that there were no significant differences between treatments and control values in each generation. Likewise, no significant differences were found even after the data had been transformed using the arcsin of the square root of the percent dehisced values.

L. Winter hardiness

1. Lotus corniculatus

Winter hardiness, expressed as the percentage of plants over-wintering the 1979-80 season, varied from 72.8 to 100% in the different treatments over five generations (Table XVIII). From the statistical analyses there were no significant differences between control and treated lines in any generation from the minimum of 300 plants tested in the M_3X and M_3S generations to the 1800 plants tested in the M_1 and M_2X generations. Mean values of treated and untreated lines per

TABLE XVII. Pod dehiscence expressed as the mean dehisced pods per plant for values tested, along with the range of each value

Generation	Treatment	Mean percent dehisced pods	Range[a]
M_1	Control	39.2	6.1-63.4
M_2S	Control	17.9	9.7-38.7
M_3S	Control	24.2	12.9-38.1
M_2X	Control	31.3	0.0-64.7
M_3X	Control	30.7	14.4-75.8

[a]No significance found between controls and all treatments.

TABLE XVIII. Winter hardiness expressed as the percentage of plants overwintering two successive seasons for five mutagens over five generations

Generation	Control	Mutagen					Mean
		X-rays	EMS	EC	HU	AP	
M_1	93.3	97.7	95.6	97.6	96.1	96.0	96.3
M_2S	97.1	98.0	97.8	99.9	99.3	100.0	98.7
M_3S	95.0	--	96.5	--	--	--	96.3
M_2X	93.1	88.6	84.4	91.0	89.0	92.4	89.7
M_3X	76.4	--	72.8	--	--	--	74.6

generation were used for statistical analysis; the coefficients of variability for the winter hardiness character ranged from approximately 26.6 to 51.4. Only the M_3X generation showed any substantial reduction in winter hardiness with an average 15.1% decrease over the next lowest level in the M_2X generation. The average level in the M_3X generation was not statistically different from any other generation.

2. Lotus tenuis

Winter hardiness of Lotus tenuis was found to be very low in the M_1 generation. The total number of plants which survived through one winter ranged between 2.5 and 15.4% (Table XIX). There were no significant differences between control and treatment values, as well as between individual treatments. No dose effect relationship could be determined between dosage and winter hardiness.

M. Parent-progeny correlations

The correlation coefficients (r), obtained in Table XX for a comparison of lines containing significantly high or low levels of HCA content, forage and seed yields, and their respective parental and filial lines, ranged greatly in values obtained, with respect to probability values for a greater r. A total of four parent-progeny correlations demonstrated strong or significant correlations (probably of a greater r less than or equal to 0.0500), while two correlations were found to be moderate (probability of a greater r between 0.0501 and 0.3000). The remaining 13 correlations (of the 19 performed) did not exhibit any moderate or strong levels.

TABLE XIX. Winter hardiness in Lotus tenuis EMS-treated and untreated lines, expressed as the percentage of the total number overwintering two consecutive seasons

Treatment EMS (%)	Survival (%)	Range (%)
Control	15.4	0.0-23.1
0.001	15.4	15.4
0.005	10.2	7.7-15.4
0.01	10.2	7.7-15.4
0.05	10.2	0.0-15.4
0.1	7.7	0.0-23.1
0.5	10.2	7.7-15.4
1.0	7.7	0.0-15.4
1.5	2.5	0.0- 7.7
2.0	10.2	7.7-15.4

TABLE XX. Correlation coefficients (r) for parent-progeny correlations of lines showing significant increases or decreases in values for quantitative characters indicated

Mutagen and dosage (%)	Generations compared	r value	Probability of a greater r
	HCA content		
EMS 0.1	M_1 X M_2X*	-0.99538	0.0612[a]
	M_2X* X M_3O	0.68332	0.5211
EC 0.1	M_2X X M_3O*	0.61428	0.5789
1.0	M_2X X M_3O*	0.86603	0.3333
HU 0.001	M_2S X M_3I*	0.00000	1.0000
0.01	M_2S X M_3I*	0.91603	0.2628[b]
AP 0.001	M_1 X M_2X*	-0.40088	0.7374
	M_2X* X M_3O	-0.43967	0.7102
0.01	M_1 X M_2X*	0.25103	0.8391
	M_2X* X M_3O	0.30073	0.8055
0.1	M_1 X M_2X*	0.26442	0.8296
	M_2X* X M_3O	0.99982	0.0122[a]
1.0	M_1 X M_2X*	-0.67821	0.5255
	M_2X* X M_3O	0.99734	0.0465[a]
	Forage yield		
EC 0.1	M_2X X M_3O*	0.29299	0.4442
	Seed yield		
EMS 0.001	M_2S X M_3X*	-0.47292	0.4211
0.1	M_2S X M_3S*	0.07050	0.9103
	M_3S* X M_4I	-1.00000	0.0000[a]
EC 0.01	M_2X* X M_3O	-1.00000	0.0000[a]

*Indicates generation with treated line containing significant values.

[a]Indicates strong correlation.

[b]Indicates moderate correlation.

Two parent-progeny comparisons were found to be highly negatively correlated. These include (1) a high seed yielding line derived from 0.1% EMS treatment in the M_3S generation and its M_4I filial line, and (2) a low seed yielding line derived from 0.01% EC treatment in the M_2X generation and its M_3O filial line.

Only two parent-progeny comparisons were found to be strongly positively correlated. These were two high HCA-containing lines in the M_2X generation, derived from treatment with 0.1 and 1.0% AP and their respective M_3O filial lines. Both lines and their progeny demonstrated a probability of a greater coefficient of less than 0.05. A third parent-progeny comparison was found to be moderately correlated, that being a 0.01% HU-treated line in the M_3I generation and its M_2S parental line.

N. Mutagenic efficiency ratios

Mutagenic efficiency ratios were lowest in the control groups of each generation (Table XXI). The exceptions were in the M_2X and M_3O generations with control values of 0.111 and 0.013, respectively. The M_2X generation control ratio was actually higher than that of the X-ray, EMS or the EC progeny. In the case of the M_3O controls, however, all mutagenic treatments had higher mutagenic efficiency ratios than the control value of 0.013.

TABLE XXI. Mutagenic efficiency ratios for five treatments over eight generations

Generation	Control	Treatments					Average
		X-rays	EMS	EC	HU	AP	
M_2S	0.000	0.000	0.044	0.000	0.000	-	0.007
M_3S	0.000	-	0.000	-	-	-	0.000
M_2X	0.111	0.024	0.074	0.014	0.237	0.222	0.114
M_3X	0.000	0.000	0.031	0.000	0.000	0.000	0.005
M_3O	0.013	0.185	0.181	0.107	0.028	0.045	0.096
M_4O	0.000	-	0.041	-	-	-	0.002
M_3I	0.000	0.031	0.163	-	0.000	-	0.032
M_4I	0.000	-	0.086	-	-	-	0.014
Average	0.016	0.030	0.078	0.015	0.033	0.033	

When averaged over the eight generations, the most efficient mutagen was EMS, followed by HU, AP, X-rays, and EC, in decreasing order. The generations demonstrating the highest mutagenic efficiency ratios (averaged over five mutagens, plus one control group) were the M_2X and M_3O. These were followed by the M_3I, M_4I, M_2S, M_3X, M_4O and M_3S generations in order of decreasing values.

VI. DISCUSSION

A. Seed germination rates

1. Lotus corniculatus

The positive relationship between plant injury and lethality in the M_1 generation following mutagenic treatment has been known for some time (Gaul, 1959). One method for estimating plant lethality has been determining the germination rate after seed treatment. Gaul (1963), using barley (Hordeum vulgare L.) as the test organism, determined the effects of X-rays and EMS on seed germination rate and its relationship to mutation production. He determined that, for both X-rays (0-40 kR dose range) and EMS (0-1.0% dose range) there was a direct correlation between increase in dosage and decrease in germination rate. This decrease in germination rate simultaneously resulted in an increase in mutation rate. This relationship between dosage, germination rate, and mutation rate could be graphically represented in the form of a sigmoid curve. Gaul found also that EMS had a more drastic effect on germination rate than did X-rays and that X-rays had a higher degree of lethality when the treated plants were at the seedling stage. Blixt (1976) found a less drastic but similar effect after gamma-irradiation (0-60 kR range) of hexaploid Phleum pratense L. ($2n = 6x = 42$), and a much reduced effect after treatment with EMS (0 to 1.0% dose range). Blixt found the mutation frequency to be highest at the lowest dose

(5 kR) for gamma-rays with a ten-fold increase over control levels, whereas EMS failed to produce a significant increase in mutation frequency. Thus, prediction of mutation frequency via dose-response effects from the germination rates was possible for diploid barley (Gaul, 1963), but not possible for hexaploid Phleum (Blixt, 1976).

Treated plants of Lotus corniculatus show a decrease in germination rate per increase in dosage for the M_1 generation after treatment with EMS and EC. Both mutagens gave a response similar to that obtained by Gaul (1963) for EMS-treated barley, and by Blixt (1976) for gamma-ray treated Phleum. In the present study, there was a sharp drop in germination rates at the highest dosage (1.0%). EMS-treated Phleum (Blixt, 1976) did not give the same response as was found for L. corniculatus for either EMS or EC treatments. A comparison of seed germination rates in Lotus to mutation rates indicated that the inverse relationship suggested by Gaul (1963) for germination rates and mutation rates does not apply to this species, nor do the dose-effect relationships found by Blixt (1976) in gamma-irradiated Phleum. The mutation rates obtained in the present study for all treatments were dose-independent and could not be predicted from effects on germination rates obtained for the M_1 generation. Therefore, in this species, effects on seed germination are not necessarily related to any lethal effects which may reflect the level or predictability of mutational events. Such lethal effects may be the result of toxicity.

Unexpected results were obtained for X-ray, EMS, and HU-treated seed in the M_2X generation, where different dosages of these mutagens significantly enhanced the germination rates. The progeny of these affected lines, in the M_3O generation, did not have the increases in seed germination rates found in the parental lines. This increase in seed germination rate was not transmitted and consequently the effects do not appear to be due to any mutational events.

2. Lotus tenuis

The decrease in seed germination rate in L. tenuis with increasing dosage of EMS was similar to the observations of Gaul (1963), for EMS-treated barley seed, and Blixt and Gottschalk (1975), for EMS-treated peas (Pisum species). These authors found a corresponding increase in mutation rate with an increase in dosage and decrease in germination rate. In L. tenuis, no selfed progeny were produced, as had been the case for barley and peas. The progeny that were produced from open-pollination were so few in number that an accurate estimate of the number and types of segregating variants was not possible. Any variants that were obtained were either completely sterile or died before reaching maturity. As a result, these variants could not be assessed as to whether or not they were actually mutants. Therefore, no conclusions can be drawn on the relationship between seed germination and mutation rates in this species.

B. Seedling survival rate

1. Lotus corniculatus

Gustafsson (1954) found for a number of crop species treated with various physical and chemical mutagens, that a decrease in viability of the treated plants was dose-dependent. This dose-dependent effect was also related to mutation rate in the progeny, with an increase in dosage usually producing an increase in mutation rate. Gregory (1955) found that X-irradiation of groundnuts (Arachis hypogaea L.) produced effects similar to that observed by Gustafsson. This was amongst the first reports on the relationship between viability of mutagenically treated plants and mutation rate in the offspring for a polyploid (tetraploid) legume. Van der Walt (1962) found that a decrease in viability, resulting from irradiation of Arachis, was not confined to the treated generation, but extended to the progeny as well. Blixt (1976), using gamma-irradiated hexaploid Phleum pratense, reported that there was a marked decrease in the numbers of M_3 selfed progeny which were dose-dependent, and that the decrease in viability was associated with a corresponding increase in mutation rate which was also dose-dependent. In L. corniculatus, X-irradiation resulted in a decrease in seedling survival rate which was a fairly accurate measure of viability since all plants which grew beyond the seedling stage survived to maturity. The observed decreases in seedling survival rate did not, however, correspond to increases in mutation rate at the same dosages, and both seedling survival rate and mutation rate were independent of dosage. Therefore, the decrease in viability observed

from X-irradiated L. corniculatus could not be used in predicting mutational events in the progeny of lines displaying reduced viability.

No previous study has been carried out on the effects of EC on seedling survival rate as has been carried out in this study with L. corniculatus. With EC treatment the effects were similar to that obtained for X-irradiated L. corniculatus with decreases in seedling survival rate not corresponding to any increase in mutation rate, and neither rates being dependent on dosage. The one exception was found in two treated lines in the M_3O generation. A significant decrease in seedling survival rate in these two lines corresponded to a significant increase in mutation rate in the same generation. These observed changes were not dose-dependent. Therefore, as with X-rays, EC-induced decreases in seedling survival rate are independent of dosage and do not effectively predict the mutation rate nor the most effective dosage found in the progeny.

2. Lotus tenuis

Seedling survival was significantly reduced for two lines of L. tenuis derived from EMS treatment in the M_2 generation from open pollination. No significant changes in seedling survival rate were found in the M_1 generation. These findings do not agree with those reported by Gaul (1970a) and Konzak et al. (1971), studying barley, Blixt and Gottschalk (1975), Pisum, Fowler and Stefansson (1975), Brassica, and Blixt (1976), Phleum. These authors found that decreases in seedling survival rate or viability in these diploid self-fertilizing species were confined to the M_1 generation, or to the M_2 generation

upon selfing. Further, seedling survival or viability was dosage dependent which was not the case in L. tenuis. The actual presence of mutants in the progeny of treated L. tenuis plants could not be demonstrated due to insufficient numbers in the progeny and the death of putative mutants prior to maturity before any breeding could be attempted. Therefore, no relationship between dosage, seedling survival and mutation rates could be determined for this species.

C. Cytology

1. Lotus corniculatus

Meiotic aberrations have been reported in a number of species after exposure to physical and chemical mutagens (Delaunay, 1930; Sapehin, 1930; Catcheside, 1945; Tsuchiya, 1960; Sparrow, 1961; Evans, 1962; Goud, 1967). An increase in meiotic aberration rate has been found to correspond to an increase in mutation rate with increasing dosage (Gustafsson and von Wettstein, 1958; Nagaraja and Natarajan, 1965). No previous work has been done on the meiotic effects of physical and chemical mutagens with L. corniculatus. However, some results obtained in this species were found to have similarities to those which have been reported in other species. In a number of polyploid species it has been observed that a non-linear relationship between dosage and mitotic aberration rate results from EMS treatment and that the relationship between mitotic aberration and mutation rates is non-linear and complex (Moutschen et al., 1964; Gaul, 1970b; Auerbach and Kilby, 1971; Wanjari and Kutarekar, 1977). In L. corniculatus,

treatment with EMS gave a non-linear response to increasing meiotic aberration rate with increasing dosage. Mutation rate was independent of increasing meiotic aberration rate or dosage.

Treatment of L. corniculatus with X-rays likewise did not respond in a manner similar to that reported in other species. Delaunay (1930) and Fröier et al. (1941) found that X-ray dosages between 5 and 25 kR produced as much as 96% aberrant cells in hexaploid wheat (Triticum aestivum L.). Significant increases in chromosome aberrations from X-irradiation have been reported in many other species as well (Gaul, 1970a). In contrast, L. corniculatus did not respond with a significant increase in meiotic aberration rate, after X-irradiation with 3 to 12 kR dosages. The amount of radiation required to elicit a response is known to vary for a number of agricultural species, with some species, such as Brassica campestris, tolerating dosages in excess of 100 kR without any noticeable detrimental effects (Gustafsson, 1944). A similar situation may exist in L. corniculatus, with dosages of X-rays exceeding the upper dosage range of 12 kR being required before any meiotic effects can be detected. Despite the lack of meiotic effects, a significant number of mutants was recovered in the progeny of X-irradiated plants of L. corniculatus. Therefore, mutations were induced without any chromosome rearrangements or any other chromosomal changes being evident. The lack of meiotic disturbances after X-irradiation does not, therefore, mean that mutational events have not taken place. It is recommended that meiotic aberration rate not be used as the sole

criterion for determining the presence of induced mutations in L. corniculatus from X-irradiation.

No previous study has been carried out on the meiotic effects of EC in a higher plant. Cytologically-related effects of EC have been shown in mitotic cells of barley and Vicia faba by a number of investigators, where EC has been reported to be clastogenic (Natarajan and Ramanna, 1966; Sturelid and Kihlman, 1975; Hartley-Asp, 1976). The clastogenic properties of EC are evident in L. corniculatus, and the response to meiotic aberration rate with increasing dosage is similar to that obtained in L. corniculatus for EMS treatments. As with EMS treatment, there is a non-linear relationship between dosage and meiotic aberration rate, and an increase in meiotic aberration rate or increase in dosage does not result in a subsequent increase in mutation rate. Therefore, mutation rate cannot be predicted from meiotic aberration rates after EC treatment in L. corniculatus, as is the case after EMS treatment.

Of the two remaining mutagens studied, HU and AP, HU is known to cause chromosome aberrations in a number of species (Kihlman et al., 1968; Timson, 1975). In this study, HU caused meiotic anomalies in L. corniculatus at a relatively low but significant level. The relationship between meiotic aberration rate, dosage, and mutation rate has not been studied previously, and, in L. corniculatus, meiotic aberration rate has been found to be independent of dose or mutation rate. Therefore, meiotic aberration rate cannot be used to predict the mutation rate in the progeny of HU-treated L. corniculatus plants.

No previous work has been done on the meiotic effects of AP in L. corniculatus. This mutagen displays some clastogenic properties, but largely affects meiotic pairing. AP appears to increase the level of multivalent associations without causing desynapsis. This is in contrast to EMS, EC, or HU, which all induce a higher level of clastogenesis and other chromosomal anomalies. There is a non-linear dose effect for meiotic aberration rate resulting from AP treatment, but this does not correspond to a similar increase in mutation rate. There is a significant increase in mutation rate as a result of AP treatment, since this increase is dose-independent, thus the dose cannot be used to predict mutation rate on the basis of the meiotic aberration rate as has been found for X-ray, EMS, EC, and HU treatments.

2. Lotus tenuis

The meiotic effects of EMS-treated M_1 plants of L. tenuis were demonstrated for the first time in this species. EMS treatments in other diploid species have shown that an increase in dosage results in a generally linear increase in meiotic or mitotic aberration rates with dosages around 1.0% EMS having fairly drastic effects. This increase in aberration rate with increasing dosage was usually associated with a proportional increase in mutation rate in the progeny (Moutschen et al., 1964; Shevchenko, 1968; Nagy-Porpaczy, 1974; Rehmatulla and Gostimskii, 1976). Lotus tenuis displayed a quantum increase in meiotic aberration rate which was not dependent on dosage. No mutation rate data were available for comparison with meiotic aberration rate or dosage.

A comparison of L. tenuis with L. corniculatus showed that the diploid L. tenuis has a lower rate of EMS-induced meiotic aberrations than L. corniculatus. Similarly, Fröier et al. (1941) found that a 10 kR dosage of X-rays applied to diploid Triticum monococcum L. and tetraploid T. dicoccum L. induced a lower chromosome aberration rate in the diploid T. monococcum. In both studies, the chromosomes of the tetraploids were more sensitive to mutagenic effects than their respective related diploid species. However, this increase in sensitivity did not correspond to a proportional increase in mutation rate.

D. Pollen stainability studies

Sterility caused by exposure to mutagens can be the result of a number of factors. The most important of these is disruption of the normal cell cycle and the production of chromosome aberrations in the meiocytes of treated plants. These aberrations have, in turn, been found to be the cause of reduced fertility or complete sterility in a number of plant species (Auerbach, 1976). Changes in the genes governing the meiotic cell cycle have also been shown to be of major importance as a cause of induced sterility in plants exposed to various mutagens. Eckberg (1969) reported that X-rays caused sterility in barley (Hordeum vulgare) via the induction of translocations and inversions in the meiocytes which could lead to cell death. He further noted that EMS worked primarily in the zygotes of treated reproductive cells, causing zygotic lethality. However, both mutagens were shown to

produce recessive mutations which induced male sterility or complete sterility in the progeny. Gaul et al. (1966) and Sato and Gaul (1967) showed that sterility caused by induced chromosomal aberrations and gene mutations was present in the same lines of EMS-treated barley. Sterility as a result of these causes also has been noted for hexaploid common wheat, Triticum aestivum, by Sasakuma et al. (1978). Mutagen-induced sterility is not confined to the M_1 generation, but has been shown to persist to various degrees for several generations (Gaul and Mittelstenscheid, 1960).

In L. corniculatus sterility was estimated by examining stained and unstained pollen. This method has been found to give a reliable estimate of the degree of pollen sterility caused by chromosomal aberrations (Swanson, 1957). All five mutagens used in this study induced significant levels of unstained pollen in the M_1 generation indicating a potential reduction in fertility had been brought about. The tetrasomic mutant, vf, induced in this study from both EMS and X-ray treatment, was completely sterile. Thus both partial sterility and complete sterility have been induced from EMS and X-ray treatments in L. corniculatus, as has been reported for EMS- and X-ray-treated barley and wheat in previous studies.

The effects of EC, HU, and AP on inducing aborted pollen has not been previously studied. The effects of EC are similar to those of EMS in inducing significant increases in aborted pollen in L. corniculatus. The mutagens HU and AP have a significant, but less drastic effect on the induction of aborted pollen than either EMS or EC and these mutagens differ also in their response to dosage.

Earlier studies on the relationship between induced sterility in the M_1 and mutation rate in the progeny of treated plants have shown that an increase in the level of sterility with increasing dosage corresponded to an increase in mutation rate. This relationship was found to be more or less linear for diploids but more complex in polyploids (Gaul, 1975, 1963; Burnham et al., 1954; Tsuchiya, 1960). In L. corniculatus, significant increases in the levels of aborted pollen were not necessarily correlated to increasing dosage, and mutation rate was found to be independent of the aborted pollen rate. This mutual independence of induced sterility and mutation rate has been found in the tomato, Lycopersicon esculentum L. (Hildering and van der Veen,1966) and in a number of other species (Rao and Jawa, 1976). Thus, sterility, as estimated by the level of aborted pollen, is a poor means of estimating mutation rate in L. corniculatus.

E. Evaluation of the floral genetic marker: brown keel tip

Since Stadler and Roman (1948) first used the aleurone gene A in Zea mays as a genetic marker to study the effects of mutagens on a single gene system, the utilization of such genetic systems to study the effects of mutagens has been successfully implemented in other species (Hagberg et al., 1958; Lundqvist et al., 1968; Tuleen et al., 1968). Single gene systems have been studied in L. corniculatus by carrying out specific crosses, but the effects have not been assayed on a population level. In L. corniculatus, a floral genetic marker brown keel tip has been shown to be a tetrasomic dominant character

(Buzzell and Wilsie, 1963; Bubar and Miri, 1965). In this study, the brown keel tip character did not vary significantly in frequency in the population after treatment with all five mutagens. Selfing and outcrossing of treated and untreated lines had a greater effect than any of the mutagens in changing the frequency of brown keel tip in the population. Therefore, conventional methods of selection through selfing and outcrossing appear more efficient in altering the keel tip color gene frequencies than mutagenic treatments in L. corniculatus.

The failure of the mutagens in altering the gene frequencies of brown floral keel tip color may be due, in part, to the following: (1) the lack of adequate progeny in selfed lines to accurately test the effects of each mutagen; (2) an insufficient number of generations to allow for full segregation of the keel tip color character; and (3) the lack of controlled crosses in the experiment which could not be practically implemented in the field.

F. Evaluation of mutant types

1. Mutation rate

a. Lotus corniculatus

A Friedman's ranked Chi-square Test (Friedman, 1937) was necessary in assessing the significance of the levels of mutation frequencies for the various treatments over the eight generations due to the low mutation rates encountered in the treated and untreated lines (5 mutants, or less, per replicate per treatment). The small

numerical values obtained, including the preponderance of zeroes, meant that the data obtained were not normally distributed, and could not be transformed to conform to normal distribution. Therefore, the Friedman's ranked Chi-square Test was selected, based on the recommended use of this test for these types of data (Steel and Torrie, 1960).

The mutants induced were largely chlorophyll-deficient types which formed about 95% of all the mutants that were recovered. The largest proportion of these were induced by X-rays and EMS, which is consistent with previous findings showing that X-rays and EMS produce mainly chlorophyll-deficient mutants in crop plants (Gustafsson, 1947; Sigurbjörnsson and Micke, 1969, 1974; Micke, 1970; Blixt and Gottschalk, 1975; Blixt, 1976). Mutation rates induced by EC, HU, and AP were consistently lower than those produced by either X-rays or EMS. This would concur with previous findings that EC, HU, and AP have weaker mutagenic properties than X-rays or EMS (Auerbach, 1976).

The mutants were exclusively tetrasomic recessives. No disomically inherited characters controlling any changes in morphology or growth habit were found in either the treated or untreated lines in any generation. This would indicate that disomic genes governing visible mutations in L. corniculatus are rare. This contrasts to earlier findings by Dawson (1941) and Donovan (1957) who reported evidence for both disomic and tetrasomic inheritance in this species. Since the characters tested by Dawson and Donovan were not investigated in this study, it can only be assumed that disomic inheritance occurs

at a very low frequency in L. corniculatus. This pattern of inheritance has also been found for alfalfa (Medicago sativa) which like L. corniculatus is also a tetraploid (Bingham, 1980).

The average mutation rate obtained from X-ray and EMS treatment in L. corniculatus varied between an average of 0.67 and 1.08%. This was within the range of mutation rates obtained for two other tetraploid legumes, including alfalfa (Childers and McLennan, 1961) and the peanut Arachis hypogaea (Patil, 1966; Ashri and Levy, 1978).

b. Lotus tenuis

The lack of sufficient numbers of progeny, along with the lack of any statistical significance for an increase in the number of mutants, indicated that the data obtained did not provide sufficient information to base any definite conclusions as to whether or not EMS effectively induces mutation in L. tenuis.

2. Segregation ratios of morphological mutants

A total of seven types of morphological «mutants» segregated in the M_3 and M_4 generations. Four of these «mutants,» including a semi-chlorophyllous mosaic, an extended internode and variegated and pinnate-leaved types, did not survive to maturity and, hence, could not be used for determining their inheritance. However, similar types were induced by physical and chemical mutagens in other species and were found to be true mutants (Kobayashi, 1958; Blixt and Gottschalk, 1975; Fowler and Stefansson, 1975; Blixt, 1976; Mouli and Patil, 1976).

Three other «mutant» types survived to maturity. They include a chlorophyll-deficient chlorotica (ct) type, first described by von Rosen (1942), a vestigial floret (vf), and some dwarf (d) types. These were found to be tetrasomic recessives, although in the case of chlorotica and dwarf mutants, segregation ratios suggested the influence of one or more additional genes. From the limited success of the crosses and selfings carried out the precise nature of additional genetic factors could not be determined. Chamberlain (1960), in screening large populations of L. corniculatus, reported a chlorophyll-deficient mutant very similar to the ct mutant found in the present study, and he determined that it was a recessive. The chlorophyll-deficient mutant described by Chamberlain occurred in a natural population of L. corniculatus, as was a chlorophyll-deficient mutant found by Bubar and Miri (1965) which they determined to be a tetrasomic recessive. The chlorophyll-deficient mutant of Bubar and Miri was also similar to the one obtained in the present study. Since such a mutant exists in natural populations of this species, including the untreated lines in the present study, it is considered that mutagenic treatments applied to L. corniculatus induced a significant increase in the ct mutant. Mutants of a similar nature have been reported in Medicago. They have been shown to have a tetrasomic recessive mode of inheritance with one or more modifyer genes (Childers and McLennan, 1961; Buss, 1977). Chlorophyll-deficient mutants have been induced in other tetraploid legumes also. Gamma and X-irradiation of Arachis species has produced tetrasomic recessive mutants similar to the ct mutant in L. corniculatus (Van der Walt, 1962; Gustafsson and Gadd, 1965b; Patil,

1966; Tai et al., 1977). Mutagenic treatments applied to tetraploid Lupinus species resulted in genetically similar mutant types as had been produced in Arachis (Gustafsson and Gadd, 1965a; Porsche, 1967).

The vf mutant obtained in L. corniculatus has not been previously reported in this species. A naturally-occurring vestigial floret mutant has been reported in the tetraploid legume Medicago sativa and has been found to be a recessive character. Unlike the vf mutant obtained in L. corniculatus, the Medicago mutant was not completely sterile, and a more complete study was possible. It was determined that the character was controlled by two tetrasomic recessive and two disomic recessive genes (Dudley and Wilsie, 1957). There is no evidence to indicate that any disomic genes are involved in the vf mutant in Lotus, but results obtained in Medicago suggest the possibility exists for disomic genes being involved in the inheritance of the vf mutant in Lotus. Mutants displaying reduced floral parts have been induced by X-rays, in wheat (Triticum aestivum) (MacKey, 1954), and in oats (Avena sativa) with EMS (Cummings et al., 1978). These mutants were recessives, involving genes segregating at the disomic, tetrasomic, and hexasomic levels. Therefore, at present, it can only be stated that the vf mutant in Lotus is an induced mutant involving at least one major gene inherited as a tetrasomic recessive.

The d mutant in Lotus demonstrates a mode of inheritance that would suggest at least one major gene, inherited as a tetrasomic recessive with the possible addition of one or more modifyer genes. Dwarf mutants were also obtained from natural populations, arising

spontaneously in Medicago sativa. Different dwarfs displayed different modes of inheritance, demonstrating a mixture of disomic and tetrasomic inheritance. In addition, there was some evidence of quantitative inheritance (Busbice, 1965). There was no evidence of disomic inheritance for the d mutant in Lotus, but restrictions on the breeding of the d mutant did not allow its precise mode of inheritance to be established. It is considered that there is at least one major gene involved which is inherited as a tetrasomic recessive. Dwarf mutants have been produced in a number of other crop species, and have been shown to be inherited either as recessives or in a quantitative manner (Sigurbjörnsson and Micke, 1969, 1974; Worland et al., 1980).

G. Hydrocyanic glycoside content

The leaves of Lotus corniculatus contain a glycoside complex of hydrocyanic acid (HCA) which has been known to display various levels of toxicity to ruminants after the ingestion of the leaves of this fodder crop (Finnemore and Cooper, 1938; Seaney and Henson, 1970). Since this characteristic is of agronomic importance, genetic studies have been undertaken to determine its mode of inheritance with the aim of modifying or eliminating the toxic effects. Dawson (1941) found this character to be a tetrasomic dominant, which was confirmed by de Nettancourt and Grant (1964). In 1967, Grant and Sidhu showed the quantitative nature of this character in Lotus species by demonstrating the presence in the leaves of various levels of HCA. This quantitative aspect was further elucidated by Ogilvie (1970) who found that the

presence of HCA in the leaves of L. corniculatus was not completely determined by a single dominant gene, and that the mode of inheritance was influenced by environmental factors which were complex in nature. Bansal (1970), in an independent study, demonstrated that the enzyme responsible for the deposition of HCA in the leaves of L. corniculatus was quantitatively inherited. The actual presence of HCA in the leaves of this species also appears to be quantitatively inherited. A random sampling of three successive generations revealed that variation for this character is continuous and skewed towards the low levels of HCA content over all three generations. Skewness indicated dominance for this character for low HCA levels. This is in accordance with the model postulated by Allard (1960) for quantitative characters demonstrating complete dominance in the filial generations with approximately 75% heritability or a strong degree of genetic influence. The skewness for this character may be due to varietal influence, as varietal differences within Lotus species are known to occur (Grant and Sidhu, 1967; Urbanska-Worytkiewicz and Wildii, 1975). Other synthetic and natural varieties of this species may not display the same distribution pattern for the HCA character as has been found for the cultivar «Mirabel.»

Treatment of Lotus seed with chemical and physical mutagens altered the mean HCA levels in several generations. Such changes have been successfully induced for biochemical characters in other polyploid legumes (Gustafsson and Gadd, 1965a; Ved Brat and Collins, 1976; Ripa and Orbidane, 1978; Hussein and Abdalla, 1979). However, in the case of L. corniculatus, changes in HCA content occurred as early as the M_2

generation, whereas the segregation of induced quantitative variants in polyploid species normally occurs in the M_3 generation or beyond (Gregory, 1965; Gustafsson and Gadd, 1965a; Blixt, 1976). Also, the high and low HCA levels induced in the parental lines in the present study were not recovered *per se* in the progeny of these lines, although parent-progeny correlations point to a high degree of transmission of this character. Since experimentation was limited to the M_4 generations by practical limitations, complete segregation for induced quantitative variation may not have taken place to that point and may require further segregation for clearly segregating high or low HCA containing lines to appear. This has been shown in other polyploid legumes for the character displaying induced quantitative variation (Gregory, 1955, 1960; Gustafsson and Gadd, 1965a, 1965b). The evidence suggests that inheritance of induced quantitative changes in HCA content for *L. corniculatus* was at least partially successful, and the successful utilization of induced mutant HCA lines is possible in this species.

H. Forage yield

Forage yield in *L. corniculatus* is a quantitatively inherited, highly variable character (Seaney and Henson, 1970; Keoghan and Tossell, 1974) with a fairly high degree of heritability (broad sense) at about 68% (Sandha *et al.*, 1977), and good general combining ability (Miller, 1968; Keoghan and Tossell, 1974; Khayrallah, 1979). Therefore, forage yield has been improved in *L. corniculatus* by conventional breeding methods. Induction of increased forage yield *via* exposure to mutagens

has not been previously attempted in L. corniculatus. One line created with EC in the M_3 generation did produce a significant increase in forage yield that was more than 2.5 times greater than the control. An increase of this magnitude has not been previously reported in this species, but mutant lines demonstrating superior forage yields have been successfully induced in other forage crops with physical and chemical mutagens (Gustafsson and Gadd, 1965a, 1965b; Sigurbjörnsson and Micke, 1969; Scossiroli, 1970). The mutant lines obtained in other species did not fully segregate until the M_4 generation, or later, usually in inbred lines. Since the «mutant» line obtained in Lotus was from open-pollinated progeny, complete segregation of this line, as well as the evaluation of the heritability, could not be accomplished at the same rate as was found for inbred mutant lines in other studies. Therefore, the exact nature of the high forage yielding line in L. corniculatus could not be fully evaluated in the M_3 generation, and it is suggested that evaluation of this line be carried out to the M_4 or later generations.

I. Seed yield

1. Lotus corniculatus

The agronomic character seed yield has been found to be highly variable in L. corniculatus with yields varying by more than 50% in natural and synthetic varieties (Bresciani and Frakes, 1973). Seed yield has been shown to be a quantitative character demonstrating a broad sense heritability estimate of 64%, indicating moderate genetic

influence (Sandha et al., 1977). Conventional methods of breeding have increased seed yield in L. corniculatus cv. «Leo» by as much as 158% after 2 cycles of recurrent selection (Sandha and Twamley, 1973).

Increasing seed yield by the induction of quantitative variation has not been attempted previously in L. corniculatus. Lines with both induced increases and decreases in seed yield were produced. Reduced seed yield was reported in the M_2 and M_3 generations in oats (Avena sativa) after EMS treatments (Jalani et al., 1979) and, likewise, reduced seed yields occurred in the M_2 generation of L. corniculatus in this study. However, the reduced yields in Lotus were induced by EC and not EMS. This would indicate that EC has the same effect of reducing seed yields in Lotus as EMS does in oats. The exception is that the induced seed yield reduction in oats was transmitted to the progeny, whereas in the case of Lotus it was not. Therefore, the induced reduction in seed yield in L. corniculatus from EC treatment appears to be a transient effect and not necessarily the effect of the treatment.

EMS did not induce any reduction in seed yield, as was found in oats. Although increased seed yield in L. corniculatus does not agree with the findings of Jalani et al. (1979) for EMS-treated oats, high seed-yielding lines have been reported to be induced from EMS treatment for pearl millet (Pennisetum americanum, Singh et al., 1976) and wheat, Triticum aestivum (Raina et al., 1978; Siddiqui et al., 1980). These high seed-yielding mutant lines of millet and wheat resulted after inbreeding, and the high seed-yielding character was transmitted to the selfed progeny of mutant lines. The high seed-yielding lines of

L. corniculatus which were not transmitted were derived through open-pollination. It has been shown that induced increases in seed yields in open-pollinated crops may not be apparent for ten generations. This may be due to the increase in heterozygosity resulting from open-pollination and subsequent reassortment of mutant genes involved in the control of the seed yield character (Gregory, 1965; Gardner, 1969). An evaluation of the high-seed yielding lines in L. corniculatus for many successive generations may be required before mutant lines of any practical value can be obtained. This approach does not appear to have any advantage in producing improvements in seed yield over conventional methods in terms of the number of generations required. However, it may prove advantageous over conventional methods in terms of the magnitude of increase. For example, Sandha and Twamley (1973) obtained a 153% improvement in seed yield, after two generations, whereas in this study an increase in seed yield of approximately 260% was obtained in L. corniculatus after two generations.

2. Lotus tenuis

In diploid crop species the use of EMS has generally been successful in producing mutant lines that are superior in seed yield (Gustafsson, 1947; Gelin, 1954; Gaul, 1965; Scossiroli, 1970; Sigurbjörnsson and Micke, 1969). In L. tenuis, seed yields were very poor, and independent of any mutagenic treatment with EMS. The cause of the poor seed set could not be determined, but both treated and untreated plants of L. tenuis had reduced vigor. Therefore, the effects of EMS on seed yield in L. tenuis could not be ascertained.

3. Selfed-seed production in Lotus corniculatus

Lotus corniculatus is an almost exclusively outcrossing species as was first demonstrated by Silow (1931) who obtained only a few seeds per hundred florets in plants isolated from insects and other pollinating vectors. Giles (1949) confirmed that the self-sterility found in this species was the result of an S-allele self-incompatibility system. The S-allele incompatibility system allowed for a wide variety of selfed-seed set, ranging from 8.8 to 49.1% pod set, and a range of 0.9 to 4.8 seeds per pod, as was shown by Brandenburg (1961), for the cultivars «Manden» and «Granger.» The cultivar «Mirabel» used in the present study likewise showed a considerable variation with 0 to 44 seeds per plant. Reduced vigor associated with inbreeding in this species (Seaney, 1967) was also observed in selfed plants in the present study. An attempt to induce increased self fertility by producing S-locus mutations in L. corniculatus was unsuccessful, despite an apparently successful increase in selfed-seed set for a 9 kR X-ray treated line. This line did not produce any mature progeny, and it could not be established if an induced increase in self-fertility had occurred. Ramulu (1980) also was unable to increase self-fertility in Nicotiana alata by failing to induce S-locus mutations with EMS. Many other mutant types were produced in N. alata, but S-locus mutants were not obtained. The S-allele self-incompatibility system of L. corniculatus does not, therefore, appear to be effectively altered by mutagenic treatments.

J. Flowering frequency

Flowering frequency is a quantitatively-inherited character in L. corniculatus with a moderate genetic component (broad sense heritability estimate of 49%), and which displays heterosis (Buzzell and Wilsie, 1965). Conventional methods of breeding have produced early flowering lines of L. corniculatus, but these show a wide variation for flowering time (Miller, 1968). This wide variation for flowering time has also been found in this study in which two early and one late flowering lines were induced from mutagenic treatments. No previous work has been done on the induction of flowering frequency in Lotus. The early and late flowering lines obtained flowered between 2 and 7 days earlier or later than the untreated lines. The maximum range of days for early or late flowering obtained by this method is less than that obtained by Miller (1968), from conventional methods. These conventionally-bred progeny flowered two to three weeks earlier than control lines. Gamma-ray induced early flowering mutants of Medicago were also found to flower two to three weeks earlier than untreated lines, although, in this case, breeding and selection were continued to the M_7 generation to obtain complete segregation of the mutants (Brock et al., 1971). X-rays and EMS have also been shown to induce changes in flowering time by a factor of two to three weeks in a number of species (Wellensiek, 1961; Brock, 1976; Ramakanth et al., 1977; Ahmed and Ahmad; 1979). Early flowering lines in this study of only two to three days were induced with EMS. This would indicate that early flowering induced by EMS treatments in this species was not as

effective as conventional methods in producing early-flowering lines in other species.

There have been no previous reports of EC inducing late flowering in higher plants as has been carried out in this study with L. corniculatus. The EC-induced late flowering line flowered seven days later than the control lines, which is similar to that obtained in Medicago (Brock et al., 1971) after three generations of conventional breeding. Early flowering mutants of Medicago were evident in the M_3 generation, and continued to segregate until the M_7 generation. Likewise, late flowering mutants of L. corniculatus were also evident in the M_3 generation. However, it was not possible to continue breeding of the late-flowering L. corniculatus to the M_7 generation as was done in Medicago. Also, the conventional methods of Miller (1968) produced lines of L. corniculatus for early flowering within four generations that showed a greater level of improvement than that obtained from EC treatment for late flowering. Therefore, although early and late flowering lines can be produced from mutagenic treatments in L. corniculatus, these lines do not display the magnitude of change in flowering time obtained by other investigators nor reduce the number of generations required to obtain early or late flowering lines that would prove useful in agricultural applications.

K. Pod dehiscence

Under controlled environmental conditions, Metcalfe et al. (1957) reported pod dehiscence to vary between 8 and 100% for a single vegetative clone of L. corniculatus cv. «Empire.» They found pod dehiscence to be influenced to a great extent by relative humidity. Peacock and Wilsie (1957) showed pod dehiscence to be a quantitative character, and that there was a wide range of variation for pod dehiscence in a large number of clones of L. corniculatus. They selected an F_1 clone with 17% enhanced indehiscence over non-selected plants. In utilizing clonal and open-pollinated plants of L. corniculatus, Gershon (1961) also found wide variation for the pod dehiscence character, but he was unable to select for increased indehiscence in open-pollinated lines. Hood (1964) found that the lengthy flowering cycle in certain clones of L. corniculatus also contributed to the degree of pod dehiscence, along with the relative humidity. Pod dehiscence was also reported to vary greatly between different lines within a single cultivar (Badcock, 1973).

The induction of increased pod indehiscence with physical and chemical mutagens in L. corniculatus has not been studied previously. A high degree of variability was encountered for pod dehiscence in this study as has been found in previous studies. Neither mutagenic treatments nor breeding methods were found to significantly enhance pod indehiscence in treated or untreated lines of L. corniculatus. Therefore, mutagenic treatments do not appear to be an effective method of increasing pod indehiscence in this species. The method of

Peacock and Wilsie (1957), using selections from vegetative clones, appears to be the method of choice for breeding increased indehiscence in L. corniculatus due to the fact that it is the most successful method that has been attempted to date in this species.

L. Winter hardiness

1. Lotus corniculatus

The cold-resistance or winter hardiness character has been shown to be a quantitatively inherited character in L. corniculatus, and varied greatly for different strains and varieties. Mass and recurrent selection techniques were found to be effective breeding materials in L. corniculatus for increased winter hardiness as well (Rachie and Schmidt, 1955). In the present study, at least 73% of all plant lines tested survived temperatures in the field of -20°C. Most lines of cultivar «Mirabel» had over-wintering survival rates of 85% or better. Therefore, this cultivar of L. corniculatus was already well-suited to winter conditions of this region (Ste-Anne de Bellevue, Québec). Mutagenic treatments were ineffective in increasing winter hardiness, presumably due to a high level of winter hardiness already present in this cultivar. It is suggested that non-winter hardy lines be utilized in any future attempts at evaluating the effects of mutagenesis on the winter hardiness character in this species.

2. Lotus tenuis

Narrow-leaf trefoil (Lotus tenuis Waldst. et Kit.) is generally less winter hardy than birdsfoot trefoil (Lotus corniculatus) under field conditions (Seaney and Henson, 1970). In this study, L. tenuis was much less winter hardy than L. corniculatus under the same field conditions. Increased cold-tolerance has been induced successfully with various mutagens in Pisum species, indicating that this character can be altered by mutagenic treatments in a diploid leguminous species (Andeweg and Kooistra, 1962). Treatments with EMS were not successful in increasing the level of hardiness in L. tenuis. M_1 plants did not show any significant increase in winter hardiness and M_2X plants did not survive overwintering. This would indicate that winter conditions in the area of Ste-Anne de Bellevue, Québec, were too severe for this species, and lines from other sources of L. tenuis should be tested.

M. Parent-progeny correlations

The varying degrees of transmission of induced quantitative changes, as a result of genotype, environment, selection of mutagen and specific treatment conditions on quantitative characters have been observed in studies involving a number of crop species (Gregory, 1960; Sigurbjörnsson and Micke, 1969, 1974; Blixt and Gottschalk, 1975; Kaul and Matta, 1976). As a means of assessing the degree of transmission of induced quantitative changes for quantitative characters in this study, a parent-progeny correlation (Falconer, 1960; Steel and Torrie, 1960) was performed to estimate the degree of transmission of induced

changes. As was found in other species, induced quantitative changes in L. corniculatus were transmitted in varying degrees, ranging from nil to nearly complete transmission as estimated by correlation coefficients on parent-progeny comparisons. The variability in the transmission of induced quantitative changes displayed in L. corniculatus has also been found in a number of outcrossing and polyploid species (Gardner, 1969; Larik et al., 1980). Parent-progeny correlations in the present study were able to identify a number of mutant lines that demonstrated a high level of transmission of quantitative mutant characters. The application of parent-progeny correlations in the identification quantitative mutant characters that may be transmitted has, therefore, been shown to be of potential practical use in the mutation breeding of L. corniculatus.

N. Mutagenic efficiency ratios

As a means of assessing which mutagen produced the highest level of mutations with the least detrimental effects in studies involving a number of mutagens, Konzak et al. (1965) devised the Relative Mutagenic Efficiency Ratio (RME). This ratio is a measure of mutation rate per level of lethality induced by a given mutagen. Nilan et al. (1965) reported that RME values in barley were generally higher for physical mutagens than for chemical mutagens. The mutagens EMS and ethylene imine were found to have the highest RME values of a number of alkylating mutagens tested in barley (Ramanna and Natarajan, 1965). In Lycopersicon species, Majid (1975) found that various radiations, including gamma

and X-rays, were more efficient than EMS. Conger and Carabia (1977) showed that EMS had a relatively high RME value compared with other chemical mutagens in treated corn, Zea mays L. RME ratios were also determined for the effects of gamma-rays, EMS, and hydrazine on quantitative characters in Phaseolus aureus. All three mutagens attained highest RME values, depending on which quantitative character was being analysed. Haynes and Eckardt (1979), in summarizing previous studies involving the calculation of RME ratios, stated that physical mutagens were generally found to be more efficient than chemical mutagens, and that different test organisms gave different RME values for the same group of mutagens tested at the same dosages.

RME values have not been estimated previously in L. corniculatus, nor are there any reports on RME values for EC, HU, and AP for any test organism to the knowledge of the author. RME values in L. corniculatus varied considerably, depending on the generation being tested. In averaging the RME values for each mutagen over all eight generations tested, it was found that EMS was the most efficient mutagen in this species. However, only one generation, the M_3O, gave significantly high mutation rates, and, therefore, the most accurate RME values. In the M_3O generation, X-rays were slightly more efficient than EMS, placing it as the most efficient mutagen for the induction of qualitative mutants in L. corniculatus. These findings generally agree with previous ones which show X-rays to be more efficient than EMS. EC also had a relatively high RME value, in comparison with X-rays and EMS, and could, therefore, prove useful in

inducing qualitative mutants in L. corniculatus. HU and AP were not efficient mutagens and their use in inducing qualitative mutants in L. corniculatus is not recommended.

VII. SUMMARY AND CONCLUSIONS

The tetraploid outcrossing nature of Lotus corniculatus has made genetic studies and improvement of agronomic characters difficult in this species. As a result, a study of induced mutagenesis was undertaken in an effort to determine if qualitative mutants could be produced for use as genetic markers and to determine if quantitative characters of agronomic value could be improved by the recovery of superior mutant lines. Effects of the mutagens on the cytology, survival, and related parameters on the mutant plants or lines and their respective parents were also studied. This was done in order to determine if changes observed for these characteristics could be used to predict the rate of induced qualitative and quantitative mutants in this species.

Germination, seedling survival, meiotic aberration, and aborted pollen rates could not be used to predict the most effective dose that would produce a significantly high rate of qualitative and quantitative mutation. Significant changes in the rate of germination, seedling survival, meiotic aberrations, and aborted pollen were related to significant increases in qualitative mutation rate. This relationship was found to be independent of dosage, and was not always consistent nor completely reliable as a means of detecting potential qualitative or quantitative mutants.

Inheritance studies of three qualitative mutants indicated that all were tetrasomic recessives. Only one mutant, chlorotica (ct), was produced in a high frequency. No disomically-inherited characters were detected in some 22,000 progeny. This would support previous studies which have shown that Lotus corniculatus displays largely tetrasomic inheritance for qualitative characters. The mutation specturm, that is the variety of mutant types produced, was also found to be narrow for this species. An attempt to increase homozygosity in L. corniculatus by selfing did not result in an increase in the mutation frequency over that which resulted from open-pollination. This was due to detrimental effects, resulting from selfing, that is decreased size and fitness of the selfed populations. Therefore, in order to increase the mutation frequency in this species, it is necessary to utilize much larger population sizes than for an inbreeding species. Thus, a population size of at least an order greater than that used in this study is considered necessary, and this may prove impractical.

The induction of quantitative variation was more successful than for qualitative mutants. Of the six agronomic characters studied, namely cyanoglycoside content (HCA), seed yield, forage yield, flowering time, pod dehiscence, and winter hardiness, four of these responded to mutagenic treatments. The HCA character responded favorably, yielding most of the mutant lines obtained in this study, with some lines displaying a high level of inheritance for the induced changes in HCA content. Flowering frequency, seed yield, and forage

yield responded to a limited extent. The two remaining agronomic characters, pod dehiscence and winter hardiness, were not responsive to mutagenic treatments. Most induced quantitative changes occurred in the M_3 or M_4 generations, which did not allow for the testing of the progenies of these generations of plants, since this study was terminated at the M_4 generation. Other studies have shown that seven or more generations may be required after initial mutagen treatments before any independently-segregating mutant lines could be recovered. Some lines in L. corniculatus displayed a fair degree of transmission of induced quantitative characters to their progeny, indicating that continued segregation of these lines could prove worthwhile for obtaining stable mutant lines for use in plant breeding applications.

APPENDIX I

Analysis of Variance and Duncan's New Multiple Range Test Tables for Significant Treatments

TABLE 1. Analysis of variance for M_1 EMS germination rate

Source	DF	SS	MS	F	PR.GR.F
Model	6	7590.13	1265.02	8.44	0.0041
Error	8	1199.20	149.90		
Total	14	8789.33			
Rep.	2	180.13		0.60	0.5713
Treatment	4	7410.00		12.36	0.0017

R^2 = 0.8636 C. V. = 31.66 Mean = 38.67 SD = 12.24

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 8 MS = 149.9

Grouping	Mean	Dose (%)
A	63.33	Control
A		
A B	61.33	0.001
B		
B C	38.67	0.01
B C		
B C D	25.67	0.1
D		
D	4.33	1.0

TABLE 2. Analysis of variance for M_1 EC germination rate

Source	DF	SS	MS	F	PR.GR.F
Model	6	5777.00	962.83	7.03	0.0106
Error	7	958.50	136.93		
Total	13	6735.50			
Rep.	2	678.75		2.48	0.1535
Treatment	4	5098.25		9.31	0.0062

R^2 = 0.8577 C.V. = 32.06 Mean = 36.50 SD = 11.70

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 7 MS = 136.93

Grouping	Mean	Dose (%)
A	60.00	0.001
A		
A	55.00	Control
B	32.00	0.01
B		
B	18.67	0.1
B		
B	7.00	1.0

TABLE 3. Analysis of variance for M_2X X-ray germination rate

Source	DF	SS	MS	F	PR.GR.F
Model	8	3146.57	393.32	4.15	0.0075
Error	16	1517.52	94.85		
Total	24	4664.09			
Rep.	4	978.00		2.58	0.0773
Treatment	4	2168.57		5.72	0.0047

R^2 = 0.6746 C.V. = 27.64 Mean = 35.23 SD = 9.74

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 16 MS = 94.85

Grouping	Mean	Dose (kR)
A	46.66	12
A		
A B	41.56	9
A B		
A B	38.80	6
B		
B C	27.96	Control
C		
C	21.18	3

TABLE 4. Analysis of variance for M_2X EMS germination rate

Source	DF	SS	MS	F	PR.GR.F
Model	8	4166.77	520.85	3.52	0.0155
Error	16	2370.80	148.17		
Total	24	6537.57			
Rep.	4	1046.01		1.76	0.1853
Treatment	4	3120.75		5.27	0.0067

R^2 = 0.6374 C.V. = 36.03 Mean = 33.79 SD = 12.17

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 16 MS = 148.18

Grouping	Mean	Dose (%)
A	46.68	0.001
A		
A	46.22	1.0
A		
A B	30.34	0.01
B		
B	27.96	Control
B		
B	17.74	0.1

TABLE 5. Analysis of variance for M_2X HU germination rate

Source	DF	SS	MS	F	PR.GR.F
Model	8	2361.98	295.25	4.57	0.0066
Error	14	904.71	64.62		
Total	22	3266.69			
Rep.	4	438.60		1.70	0.2065
Treatment	4	1923.39		7.44	0.0020

R^2 = 0.7231 C.V. = 27.08 Mean = 29.68 SD = 8.04

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 16 MS = 64.62

Grouping	Mean	Dose (%)
A	35.48	0.001
A B	31.28	Control
A B	31.00	0.1
A B	29.65	0.01
B	22.16	1.0

TABLE 6. Analysis of variance for L. tenuis germination rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	1403.06	107.93	5.65	0.0001
Error	36	687.16	19.09		
Total	49	2090.22			
Rep.	4	34.48		0.45	0.7705
Treatment	9	1368.58		7.97	0.0001

R^2 = 0.6713 C.V. = 6.86 Mean = 63.65 SD = 4.37

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 19.09

Grouping	Mean	Dose (%)
A	70.01	Control
A	69.70	0.005
A	66.30	0.001
A B	65.29	0.01
A B	64.98	1.5
B	63.41	0.5
B	63.16	1.0
B	63.05	0.05
B	60.00	0.1
C	50.58	2.0

TABLE 7. Analysis of variance for M_2S seedling survival rate

Source	DF	SS	MS	F	PR.GR.F
Model	20	183.68	9.18	3.10	0.0015
Error	36	106.56	2.96		
Total	56	290.25			
Rep.	2	20.77		3.51	0.0405
Treatment	18	162.91		3.06	0.0021

R^2 = 0.6329 C.V. = 146.37 Mean = 1.18 SD = 1.72

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 56 MS = 2.96

Grouping			Mean	Dose
A			7.50	0.01% HU
A	B		6.25	Control
A	B		3.75	9 kR X-rays
	B	C	1.75	3 kR X-rays
	B	C	1.00	1.0% HU
	B	C	0.50	0.001% HU
		C	0.00	1.0% EC
		C	0.00	0.001% EC
		C	0.00	12 kR X-rays
		C	0.00	6 kR X-days

TABLE 8. Analysis of variance for M_3O survival rate

Source	DF	SS	MS	F	PR.GR.F
Model	6	2372.70	395.45	7.12	0.0037
Error	10	555.30	55.53		
Total	16	2928.00			
Rep.	3	893.20		5.36	0.0185
Treatment (T^2)	1	1365.70		24.59	0.0006

R^2 = 0.8103 C.V. = 26.61 Mean = 28.00 SD = 7.45

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the Means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 10 MS = 55.53

Grouping	Mean	Dose (%)
A	37.00	0.01
A		
A	32.00	1.0
A		
A	30.75	Control
A		
A	29.75	0.001
B	9.00	0.1

TABLE 9. Analysis of variance for M_1 L. tenuis survival rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	56.54	4.35	2.23	0.0290
Error	36	70.28	1.95		
Total	49	126.82			
Rep.	4	8.92		1.14	0.3523
Treatment	9	47.62		2.71	0.0161

R^2 = 0.4458 C.V. = 72.02 Mean = 1.94 SD = 1.39

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 70.28

Grouping	Mean	Dose (%)
A	87.80	0.05
A	87.40	Control
A B	83.40	0.001
A B	82.20	0.01
A B	82.00	1.5
A B	79.60	0.5
A B	79.40	1.0
A B	79.20	0.05
B	74.80	0.1
C	59.60	2.0

TABLE 10. Analysis of variance for M_1 EMS meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	4	403.73	100.93	18.69	0.0001
Error	10	54.00	5.40		
Total	14	457.73			
Rep.	2	0.00			
Treatment	4	403.73		18.69	0.0001

R^2 = 0.8820 C.V. = 17.69 Mean = 13.13 SD = 2.32

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 10 MS = 5.40

Grouping	Mean	Dose (%)
A	18.00	1.0
A		
A B	16.67	0.01
A B		
A B	14.67	0.1
B		
B	13.00	0.001
C	3.33	Control

TABLE 11. Analysis of variance for M_1 EC meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	4	671.07	167.77	15.07	0.0003
Error	10	111.33	11.13		
Total	14	782.40			
Rep.	2	0.00			
Treatment	4	671.07		15.07	0.0003

R^2 = 0.8577 C.V. = 21.95 Mean = 15.20 SD = 3.34

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 10 MS = 11.13

Grouping	Mean	Dose (%)
A	22.67	0.01
A		
A	22.00	0.1
B	15.33	1.0
B		
B	11.00	0.001
C	5.00	Control

TABLE 12. Analysis of variance for M_1 HU meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	4	127.60	31.9	12.59	0.0006
Error	10	25.33	2.53		
Total	14	152.93			
Rep.	2	0.00			
Treatment	4	127.60		12.59	0.0006

R^2 = 0.8343 C.V. = 25.40 Mean = 6.26 SD = 1.59

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 10 MS = 2.53

Grouping	Mean	Dose (%)
A	9.67	1.0
A		
A	9.00	0.001
A		
A B	6.67	0.1
B		
B C	4.00	0.01
C		
C	2.00	Control

TABLE 13. Analysis of variance for M_1 AP meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	4	217.73	54.43	14.32	0.0004
Error	10	38.00	3.80		
Total	14	255.73			
Rep.	2	0.00			
Treatment	4	217.73		14.32	0.0004

R^2 = 0.8514 C.V. = 26.11 Mean = 7.47 SD = 1.95

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 10 MS = 3.80

Grouping	Mean	Dose (%)
A	13.33	0.1
B	9.00	1.0
B		
B	7.33	0.01
B		
B	6.00	0.001
C	1.67	Control

TABLE 14. Analysis of variance for M_3I EMS meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	5	314.08	62.82	16.51	0.0019
Error	6	22.83	3.81		
Total	11	336.92			
Rep.	2	5.17		0.68	0.5423
Treatment (T^2)	1	290.71		76.39	0.0001

R^2 = 0.9322 C.V. = 29.63 Mean = 6.58 SD = 1.95

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 6 MS = 3.81

Grouping	Mean	Dose (%)
A	15.33	0.01
B	4.33	0.001
B		
B	3.67	0.1
B		
B	3.00	Control

TABLE 15. Analysis of variance for M_4I EMS meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	5	87.75	17.55	6.38	0.0215
Error	6	16.50	2.75		
Total	11	104.25			
Rep.	2	1.50		0.27	0.7703
Treatment (T^2)	1	60.22		21.90	0.0034

R^2 = 0.8417 C.V. = 44.22 Mean = 3.75 SD = 1.66

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 6 MS = 2.75

Grouping	Mean	Dose (%)
A	8.00	0.01
B	4.00	0.001
B		
B	2.00	Control
B		
B	1.00	0.1

TABLE 16. Analysis of variance for L. tenuis M_1 meiotic aberrations

Source	DF	SS	MS	F	PR.GR.F
Model	9	104.96	11.66	3.16	0.0265
Error	14	51.67	3.69		
Total	23	156.63			
Rep.	2	1.00		0.14	0.8744
Treatment	7	103.96		4.02	0.0128

R^2 = 0.6701 C.V. = 46.57 Mean = 4.13 SD = 1.92

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 14 MS = 3.69

Grouping	Mean	Dose (%)
A	6.67	0.1
A	6.00	1.0
A	5.33	0.05
A	5.00	0.01
A	4.67	0.001
A	4.33	1.5
A	4.33	2.0
A B	3.33	0.5
B	2.00	0.005
B	0.00	Control

TABLE 17. Analysis of variance for M_1 X-rays aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	1514.95	116.53	7.96	0.001
Error	36	527.00	14.64		
Total	49	2041.95			
Rep.	9	86.71		0.66	0.7402
Treatment	4	1428.24		24.39	0.0001

R^2 = 0.7419 C.V. = 23.77 Mean = 16.10 SD = 3.83

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 14.64

Grouping	Mean	Dose (kR)
A	24.68	6
B	19.33	3
C	14.61	Control
C D	12.20	12
C D	9.67	9

TABLE 18. Analysis of variance for M_1 EMS aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	6401.99	492.46	4.56	0.0001
Error	36	3885.57	107.93		
Total	49	10287.56			
Rep.	9	1583.36		1.63	0.1437
Treatment	4	4818.64		11.16	0.0001

R^2 = 0.6223 C.V. = 33.36 Mean = 31.14 SD = 10.40

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 107.93

Grouping	Mean	Dose (%)
A	44.25	0.01
A A B	36.35	1.0
B B B	32.26	0.1
B	28.25	0.001
C	14.61	Control

TABLE 19. Analysis of variance for M_1 EC aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	4621.56	335.50	1.82	0.0787
Error	36	7051.14	195.86		
Total	49	11672.69			
Rep.	9	1237.40		0.70	0.7028
Treatment	4	3384.16		4.32	0.0059

R^2 = 0.3959 C.V. = 47.25 Mean = 29.62 SD = 14.00

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 195.87

Grouping	Mean	Dose (%)
A	36.90	0.001
A		
A	36.07	0.01
A		
A	33.09	1.0
A		
A	27.33	0.1
B	14.61	Control

TABLE 20. Analysis of variance for M_1 HU aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	675.55	51.97	2.02	0.0475
Error	36	924.19	25.67		
Total	49	1599.74			
Rep.	9	201.44		0.87	0.5584
Treatment	4	474.11		4.62	0.0041

R^2 = 0.4228 C.V. = 28.18 Mean = 17.98 SD = 5.07

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 25.67

Grouping	Mean	Dose (%)
A	21.37	0.1
A		
A	20.36	0.01
A		
A	19.61	0.001
B	14.61	Control
B		
B	13.95	1.0

TABLE 21. Analysis of variance for M_1 AP aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	13	1752.60	134.82	1.23	0.2972
Error	36	3934.79	109.30		
Total	49	5687.39			
Rep.	9	534.89			
Treatment	4	1217.71		2.79	0.0410

R^2 = 0.3082 C.V. = 45.49 Mean = 22.98 SD = 10.45

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 36 MS = 109.30

Grouping	Mean	Dose (%)
A	29.16	1.0
A		
A	26.47	0.1
A		
A B	22.84	0.01
A B		
A B	21.83	0.001
B		
B	14.61	Control

TABLE 22. Analysis of variance for M_3O EMS aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	5	29.00	5.80	2.20	0.1439
Error	9	23.75	2.64		
Total	14	52.74			
Rep.	2	1.81		0.34	0.7183
Treatment (T^3)	1	19.45		7.37	0.0238

R^2 = 0.5497 C.V. = 17.14 Mean = 9.48 SD = 1.62

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 9 MS = 2.64

Grouping	Mean	Dose (%)
A	11.63	0.01
A		
A B	10.20	0.1
B		
B	10.17	0.001
B		
B	8.20	1.0
B		
B	7.20	Control

TABLE 23. Analysis of variance for M_4O EMS aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	4	25.86	6.47	13.92	0.0129
Error	4	1.86	0.46		
Total	8	27.72			
Rep.	2	8.23		8.86	0.0339
Treatment (T^2)	1	10.52		22.64	0.0089

R^2 = 0.9330 C.V. = 17.23 Mean = 3.96 SD = 0.68

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 4 MS = 0.46

Grouping	Mean	Dose (%)
A	5.20	0.1
A		
A	4.67	0.001
B	2.00	Control

TABLE 24. Analysis of variance for M_3I EMS aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	5	29.56	5.91	11.41	0.0051
Error	6	3.11	0.52		
Total	11	32.67			
Rep.	2	5.73		5.53	0.0435
Treatment (T^3)	1	22.04		42.54	0.0006

R^2 = 0.9049 C.V. = 17.59 Mean = 4.09 SD = 0.7198

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 6 MS = 0.52

Grouping	Mean	Dose (%)
A	5.77	0.001
A A B	4.50	0.1
B B	4.23	0.01
C	1.87	Control

TABLE 25. Analysis of variance for M_4I EMS aborted pollen rate

Source	DF	SS	MS	F	PR.GR.F
Model	5	40.39	8.08	3.38	0.0851
Error	6	14.33	2.39		
Total	11	54.73			
Rep.	2	0.41		0.09	0.9186
Treatment (T^3)	1	30.52		12.77	0.0117
R^2 = 0.7381		C.V. = 17.63	Mean = 8.77		SD = 1.55

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 6 MS = 2.39

Grouping	Mean	Dose (%)
A	11.83	0.001
B	8.47	0.01
B		
B	7.43	Control
B		
B	7.33	0.1

TABLE 26. Analysis of variance for M_2X X-ray-treated HCA line

Source	DF	SS	MS	F	PR.GR.F
Model	38	125112.25	3292.42	1.13	0.3368
Error	56	163566.04	2920.82		
Total	94	288678.19			
Rep.	34	56686.08		0.57	0.9589
Treatment	4	69426.07		5.86	0.0005

R^2 = 0.4334 C.V. = 68.94 Mean = 78.39 SD = 54.04

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 56 MS = 2920.82

Grouping	Mean	Dose (kR)
A	131.34	3
B	91.45	9
B B	76.03	6
B B	61.85	Control
B	50.67	12

TABLE 27. Analysis of variance for M_2X EMS-treated HCA line

Source	DF	SS	MS	F	PR.GR.F
Model	38	50525.25	1329.61	1.84	0.0190
Error	56	40570.22	724.47		
Total	94	91095.46			
Rep.	34	35452.42		1.44	0.1122
Treatment	4	15099.82		5.21	0.0012

R^2 = 0.5546 C.V. = 55.45 Mean = 48.54 SD = 26.92

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 56 MS = 724.47

Grouping	Mean	Dose (%)
A	61.85	Control
A		
A B	55.08	1.0
B		
B C	42.09	0.001
B C		
B C	36.39	0.01
C		
C	29.53	0.1

TABLE 28. Analysis of variance for M_2X AP-treated HCA line

Source	DF	SS	MS	F	PR.GR.F
Model	38	89104.47	2344.85	1.56	0.0637
Error	56	84128.80	1502.30		
Total	94	173233.27			
Rep.	34	50324.15		0.99	0.5091
Treatment	4	38780.32		6.45	0.0002

R^2 = 0.5144 C.V. = 44.21 Mean = 87.66 SD = 38.76

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 56 MS = 1502.30

Grouping	Mean	Dose (%)
A	110.57	0.1
A		
A	104.40	0.01
A		
A	99.29	0.001
A		
A	96.76	1.0
B	61.79	Control

TABLE 29. Analysis of variance for $M_3$0 EC-treated HCA line

Source	DF	SS	MS	F	PR.GR.F
Model	5	152088.03	30417.61	5.20	0.0161
Error	9	52636.57	5848.51		
Total	14	204724.60			
Rep.	2	8503.89		0.73	0.5097
Treatment	1	57988.83		9.92	0.0118

R^2 = 0.7429 C.V. = 60.24 Mean = 126.95 SD = 76.48

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 9 MS = 5848.51

Grouping	Mean	Dose (%)
A	279.87	0.001
A		
A B	208.20	Control
B		
B C	95.57	0.01
C		
C	36.40	0.1
C		
C	14.70	1.0

TABLE 30. Analysis of variance for M_3I HU-treated HCA line

Source	DF	SS	MS	F	PR.GR.F
Model	5	112115.07	22431.01	15.55	0.0046
Error	5	7210.52	1142.10		
Total	10	119365.59			
Rep.	2	14800.21		5.13	0.0614
Treatment (T^3)	1	37426.14		25.95	0.0038
R^2 = 0.9396		C.V. = 18.64	Mean = 203.69		SD = 37.98

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 5 MS = 1442.10

Grouping	Mean	Dose (%)
A	325.95	0.001
A		
A	301.47	0.01
B	114.77	Control
B		
B	113.33	0.1

TABLE 31. Analysis of variance for forage yield of M_3O EC-treated line

Source	DF	SS	MS	F	PR.GR.F
Model	6	2045.85	340.97	1.76	0.2053
Error	10	1939.71	193.97		
Total	16	3985.56			
Rep.	3	333.37		0.57	0.6456
Treatment (T^2)	1	1641.75		8.46	0.0156

R^2 = 0.5133 C.V. = 70.11 Mean = 19.86 SD = 13.93

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 10 MS = 193.97

Grouping	Mean	Dose (%)
A	41.30	0.1
B	16.03	Control
B		
B	15.63	0.001
B		
B	15.03	0.01
B		
B	14.03	1.0

TABLE 32. Analysis of variance for seed yield in M_3S EMS-treated line

Source	DF	SS	MS	F	PR.GR.F
Model	24	247.99	10.33	1.83	0.0407
Error	44	248.65	5.65		
Total	68	496.64			
Rep.	2	16.61		1.47	0.2410
Treatment	22	231.37		1.86	0.0395

R^2 = 0.4993 C.V. = 41.92 Mean = 5.67 SD = 2.38

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 44 MS = 5.65

Grouping	Mean	Dose (%)
A	9.55	0.01
B	4.69	0.001
B		
B	4.61	Control
B		
B	1.39	0.1

TABLE 33. Analysis of variance for seed yield in M_2X EC-treated line

Source	DF	SS	MS	F	PR.GR.F
Model	3	24.31	8.10	15.94	0.0001
Errof	17	8.64	0.51		
Total	20	32.95			
Rep.	2	0.28		0.55	0.4667
Treatment (T^3)	1	22.16		43.60	0.001
R^2 = 0.7378	C.V. = 10.82		Mean = 6.59		SD = 0.71

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 17 MS = 0.51

Grouping	Mean	Dose (%)
A	7.60	0.1
A A	7.60	0.001
A A	6.88	Control
B B	6.23	1.0
C	4.04	0.01

TABLE 34. Analysis of variance for seed yield in M_3X EMS-treated lines

Source	DF	SS	MS	F	PR.GR.F
Model	2	4145112.02	2072556.01	6.08	0.0458
Error	5	1703056.36	340611.27		
Total	7	5848168.38			
Rep.	2	1138521.90		3.34	0.1270
Treatment (T^2)	1	3006590.13		8.83	0.0311

R^2 = 0.7088 C.V. = 53.24 Mean = 1096.2 SD = 583.62

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 5 MS = 340611

Grouping	Mean	Dose (%)
A	2324.75	0.001
B	828.23	Control
B		
B	545.13	0.01

TABLE 35. Analysis of variance for 50% flowering frequency - M_3O EC

Source	DF	SS	MS	F	PR.GR.F
Model	6	101.44	16.91	3.38	0.0496
Error	9	44.95	4.99		
Total	15	146.40			
Rep.	3	4.66		0.31	0.8171
Treatment (T^2)	1	80.53		16.12	0.0030

R^2 = 0.6930 C.V. = 17.76 Mean = 12.59 SD = 2.23

DF Degrees of freedom
MS Mean square
PR.GR.F Probability of a greater F
C.V. Coefficient of variability
SS Sum of the squares
F Calculated F statistic
R^2 Residual mean square
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 9 MS = 4.99

Grouping	Mean	Dose (%)
A	18.75	0.1
B	12.68	0.001
B		
B	11.93	Control
B		
B	11.60	0.01
B		
B	10.23	1.0

TABLE 36. Analysis of variance for peak flowering frequency - M_4O EMS

Source	DF	SS	MS	F	PR.GR.F
Model	5	0.13	0.03	7.24	0.0159
Error	6	0.02	0.003		
Total	11	0.15			
Rep.	3	0.08		7.13	0.0210
Treatment (T^2)	1	0.05		12.74	0.0118

R^2 = 0.8579 C.V. = 16.45 Mean = 0.37 SD = 0.06

DF Degrees of freedom
SS Sum of the squares
MS Mean square
F Calculated F statistic
PR.GR.F Probability of a greater F
R^2 Residual mean square
C.V. Coefficient of variability
SD Standard deviation

Duncan's Multiple Range Test on the means of the above data

Means with the same letter are not significantly different

Alpha level = 0.05 DF = 6 MS = 0.004

Grouping	Mean	Dose (%)
A	0.46	Control
B	0.34	0.01
B		
B	0.30	0.001

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