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STUDIES OF TOLUIDINE BLUE FOR INDUCING HAPLOIDY IN LYCOPERSICON ESCULENTUM AND ZEA MAYS

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STUDIES OF TOLUIDINE BLUE FOR INDUCING HAPLOIDY IN LYCOPERSICON ESCULENTUM AND ZEA MAYS

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INTRODUCTION

Haploidy was first proven cytologically and reported in Datura stramonium in 1922 (Blakeslee, Belling, Farnham, and Bergner, 1922). Haploid plants have since been recognized in many plant species (Kimber and Riley, 1963). One of the most practical uses of haploids is in the production of homozygous forms by doubling the single genome. This has been accomplished by decapitation (Burdick, 1951), colchicine treatment (Blakeslee and Avery, 1939; Newcomer, 1941; Toole and Bamford, 1945; Morgan and Rappleye, 1950), the occasional self fertilization (Cook, 1936) or spontaneous somatic diploidization (Chase, 1949b; 1952a, 1952b). In genetic and cytogenetic studies, haploid plants have provided valuable information regarding the phytogenetic relationship of plant species (Kostoff, 1942), the establishment of the basic chromosome number of species (Thompson, 1956; Levan, 1945; Humphrey, 1934), the amount of chromosome structural duplication and translocation in species from which the haploid was derived (Riley and Chapman, 1957), and induction of monosomic and trisomic lines to locate the genes of a given chromosome (Sears, 1939).

The major problem involved in the utilization of haploids has been the low frequency of spontaneous haploid production. Several methods have been tried to increase this frequency, such as hybridization (Gains and Aase, 1926; Jorgensen, 1928; Clausen and Lammerts, 1929; Gates, 1929; Kostoff, 1942; Hougas, Peloquin and Ross, 1952a, 1952b; Peloquin and Hougas, 1961), delayed pollination (Kihara, 1940; Smith, 1946), X-ray (Katayama, 1934; Ivanov, 1938; Swaminathan and Singe, 1958; Campos and Morgan, 1960; Jacob and Sen, 1961), alien

cytoplasm (Kihara and Tsunewaki, 1962), temperature shocks (Randolph, 1932; Muntzing, 1937; Nordenskiold, 1939), and chemical treatments (Van Overbeck, Conklin and Blakeslee, 1941; Levan, 1945; Deanon, 1957). As yet no method has proven effective for a range of species or has increased the frequency of haploids to an appreciable degree in a particular species. The effective use of the haploid, therefore, demands a technique by which a high frequency of haploids can be induced in a variety of plant species.

Chemical induction of haploidy has been reported in frogs (Briggs, 1952) and mice (Edwards, 1954) from treatment of the male sperm with the vital dye, toluidine blue. The dye appeared to inactivate the sperm nucleus without disrupting other functions of the cell. The treated sperms apparently stimulated the egg to develop without a genetic contribution to the developing embryo. Toluidine blue prevented the division of the generative nucleus of Vinca rosea (Ellis, 1964) and Tradescantia paludosa (Gearhart, 1966) in pollen cultured in vitro. The purpose of this study was to investigate the potential use of toluidine blue for induction of haploidy in species having binucleate pollen, such as Lycopersicon esculentum, and in species having trinucleate pollen, as Zea mays. The results of dye-induced haploidy could be compared with natural haploidy reported in the literature for the two species.

LITERATURE REVIEW

Even though haploidy was first described cytologically and reported in Datura stramonium in 1922 (Blakeslee, Belling, Farnham, and Bergner, 1922), haploid Gossypium barbadense was described in 1920 but was not proven until 1932 (Harland, 1955). The literature on haploidy has been reviewed by Gates and Goodwin (1930), Ivanov (1938), Kostoff (1942) and more recently by Kimber and Riley (1963) who listed by authors the species, genera and families from which haploids have been recorded. This list consisted of 71 species representing 39 genera in 16 families, clear evidence that haploids are not restricted to one particular taxonomic group but can be obtained from a variety of plant species under favorable conditions and proper technique.

The information concerning the origin of haploids that has been reported are discussed herein as spontaneous, or induced.

Spontaneous Haploids. Since the initial discovery in Datura, haploids have been found in fields of many cultivated plants. Lindstrom (1929) observed a haploid plant which arose spontaneously in F_2 population from varietal crosses of Lycopersicon esculentum. Also, in Lycopersicon, Morrison (1932) isolated six haploids from breeding cultures of commercial varieties. Three of the haploids were from the variety Marglobe, two from Gulf State Market, and one from Earliana. No reference to their origin was made. Spontaneous haploids also have been found in fields of Gossypium (Harland, 1955), Nicotiana (Goodspeed and Avery, 1929) and Medicago (Stranford and Clement, 1955).

Most of the spontaneous haploids reported in the literature arose from polyembryomic seeds. This phenomenon was first reported by Ramiah, Parthasarthi, and Ramanujan (1933) in Oryza sativa. Muntzing (1938)

examined 2201 twin seedlings in which he recognized 95 haploids representative of the genera Triticum, Secale, Herdeum, Phleum, Dactylis, Poa and Solanum. The extensive literature in reference to polyembryony has been summarized by Webber (1940) and recently by Kimber and Riley (1963). Only a few examples will be mentioned here to indicate the frequency and nature of occurrence.

The frequency of polyembryony has been reported to vary from species to species and from one variety to another within the same species. In Lycopersicon esculentum, the frequency of twin seedlings was estimated to be one in 50,000 to one in 100,000 (Cook, 1936). In Nicotiana tabaccum it was found to be one in 10,000 seeds (Nettancourt, 1960) and from 0.04 to 0.25 percent by Cameron (1949). Cameron also reported an increase in the frequency of plural seedlings by using monosomic varieties. In Gossypium barbadense, the frequency of twinning in one strain of Sea Island cotton was approximately one in 300 seeds. In another strain it was found to be one in 500 seeds (Beasley, 1940). Similarly Christensen and Bamford (1943) and Morgan and Rappleye (1950) observed varietal differences in the frequency of polyembryonic seeds of Capsicum frutescens. This was attributed to differences in varietal genotype. However, Campos and Morgan (1960) presented evidence to show that the female genotype rather than the genotype of the pollen parent determined the incidence of twinning.

Polyembryomic seeds do not necessarily give rise to haploid seedlings. Combinations of haploid-haploid, haploid-diploid, haploid-triploid, diploid-diploid, diploid-triploid, diploid-tetraploid, and triploid-triploid have been found (Campos and Morgan, 1960; Webber, 1940). According to Webber, only 5 percent of the twin seedlings were haploids in

the majority of the species studied. However, in certain strains of Triticum, the frequency of haploidy among twins was as high as 25 percent and up to 50 percent in some Gossypium strains. In Asparagus officinalis, Randall and Rick (1945) obtained 405 multiple seedlings among which 97 percent were twins and 3 percent higher multiples. Among the twin seedlings 93.2 percent were diploids, and the remaining consisted of triploids, trisomic, haploids and tetraploids.

Induced Haploids. Several workers have tried to induce haploids by using different agents and methods such as hybridization, delayed pollination, x-rays, alien cytoplasm, temperature shocks and chemical treatments. The success achieved by these efforts has been limited and often contradicted.

The development of haploids following hybridization has been reported by many authors. Jorgensen (1928) was first to give critical account of this phenomenon even though the existence of haploids after hybridization had been reported earlier (Gains, 1926). Jorgensen obtained seven haploids and 28 maternal diploids from 90 pollinations of Solanum nigrum x S. luteum. Histological examination revealed that the pollen of S. luteum germinated and the pollen tubes entered the embryo sac of S. nigrum. One of the male nuclei fused with the endosperm nuclei and the other degenerated. Apparently, the presence of the male nuclei stimulated the egg to develop without actual fertilization. The 28 diploids were believed to be developed in similar way but chromosome duplication had taken place in the early embryonic development. Also in Solanum sp., Hougas, Peloquin and Ross (1958a; 1958b) used interspecific hybridization as a tool for obtaining haploid Solanum tuberosum. Selections of commercial varieties were used as the female parent for mating with diploid Solanum

species. With the aid of marker genes, they isolated 28 haploids (24 chromosomes) out of 6041 pollinations. Similarly, Peloquin and Hougas (1961) reported approximately 1400 haploids from 19 commercial varieties and 21 breeding stocks of potato. In intergeneric hybridization, Kostoff (1942) recognized two haploid plants from 38 crosses of Nicotiana triplex x Petunia violacea and one haploid from 52 crosses involved N. triplex and P. hybrida.

Although hybridization may increase the frequency of haploidy in some crops, Satina, Blakeslee and Avery (1937) presented evidence to show this was not the case in Datura. From interspecific hybridization they obtained only 14 haploids among 77,356 F_1 plants (0.018%) in contrast to 13 haploids derived from 76,080 seeds produced by self-pollinated plants (0.017%).

Intraspecific hybridization also has been reported to increase the frequency of haploids. In Zea mays, Chase (1949) found some stocks to produce more haploids than others regardless of pollen parent. Similarly, some stocks when used as the pollen parent were more efficient in stimulating haploid development than others. The production of haploids in any particular cross, therefore, was influenced by both parents and could be increased by crosses of high haploid producing stocks. The production of haploids in maize was found to be heritable and could be transmitted from one stock to another (Coe, 1959). Coe isolated a line with high frequency (3.23%) and another line with low frequency. Following a cross of the two lines, the frequency decreased, suggesting that the dominant factors confer low frequency. The progeny segregation further suggested that two genes are involved.

Induction of haploids by means of delayed pollination was demon-

strated by Kihara (1940). Flowers of Triticum monococcum were emasculated from two to nine days prior to pollination. The highest percentage of haploids was obtained when pollination was delayed nine days. Similar results were reported by Smith (1946) using male sterile lines. The number of haploids increased as the time of pollination was delayed, with nine days being the optimum.

Haploids have been observed after temperature treatments intended for induction of chromosome duplication. Randolph (1932) applied high temperature treatment to zygotes and pre-embryos to induce tetraploid maize. The ear shoots were enclosed with a cylinder surrounded by an electrical heating pad. From ears subjected to 43° C at 22 to 24 hours after pollination, one haploid plant was found. One maternal diploid was observed following another treatment in which the entire plant was exposed to a 43° C constant temperature 22 to 24 hours after pollination. The maternal diploid was thought to be a result of parthenogenetic development of the egg followed by doubling of the chromosome number. Another incident in which haploid was induced by high temperature treatment was reported by Nordenskiold (1939). He found haploid Secale derived from an ear treated for 45 minutes at temperature of 41° C at 21 hours following pollination. Low temperature apparently has similar effect. Muntzing (1937) exposed five spikes of Secale cereale to -3° C for 30 minutes at 20 hours after pollination to induce polyploidy. Out of the 46 seedlings raised, one haploid was recognized. In attempts to induce chromosome aberration in Datura, Blakeslee et al (1922), obtained the first haploid reported by cold treatment.

The alien cytoplasm method for haploid induction was described by Kihara and Tsunewaki (1962). They developed two lines of wheat, one

of which had the nucleus Triticum vulgare in Aegilops caudata cytoplasm and produced 1.7 percent haploids. The other line had Tayler's Tricicale nucleus in Aegilops caudata cytoplasm and produced 52.9 percent haploids. It was assumed that the high frequency of haploids produced by the two lines was due to the effect of A. caudata cytoplasm, and the difference in frequency between the two lines was due to the effect of two different genotypes in the same cytoplasm on parthenogenetic development of the embryo.

Attempts have been made to study the effect of x-rays on haploid development. Katayama (1934) estimated the occurrence of haploids in a field of Triticum monococcum to be about 0.48 percent. This percentage was increased when young spikes were irradiated during meiotic division. Higher frequencies were induced when spikes with mature pollen grains were x-rayed and their pollen applied to untreated pistiles emasculated three days before pollination. Natarajan and Swaminathan (1958) also reported an increase in the frequency of haploid Triticum aestivum when the spikes were irradiated two to three days prior to anthesis. Smith (1946), on the other hand, observed a lower percentage of haploids from x-ray treated pollen grains of Triticum monococcum. Furthermore, a decrease in seed production was noticed from pollen irradiated with 1000r or higher.

In Nicotiana rustica, Ivanov (1938) applied x-rayed pollen to flowers which had been emasculated two to three days before pollination. One haploid was obtained from pollen exposed to 17000r and three others from pollen treated with 26000 to 30,000r. He also found seven maternal diploids from flowers receiving pollen treated with 15000r, 21000r, and 23000r. Similar stimulation effects of x-ray have been reported in

Capsicum frutescens. Campos and Morgan (1960) obtained 17 haploids in the progeny of varietal crosses followed treatment of the pollen with x-ray. Seven of the haploids were from pollen receiving 1000r and the other ten found in the 2000r treatment.

The possibility of increasing the frequency of haploids by means of chemicals has been investigated. Van Overbeck, Conklin and Blakeslee (1941) injected Napthaleneacetic, Indolcacetic, and Indolebutyric acids into the ovaries of Datura stramonium and Melandrium dioicum flowers after the styles and the anthers were removed prior to anthesis. The chemicals stimulated parthenocarpic fruits but failed to induce parthenogenetic development of the female gametes. Chase (1952) stated, in his communication with Emerson and Lindstron, that hormone applications of the ovules before or during fertilization failed to stimulate the division of the egg cell of maize.

In an experiment designed to induce polyploidy in Beta vulgaris, Levan (1945) reported a haploid seedling resulted from colchicine treatment of bolting plants. It was assumed that the colchicine treatment had affected the capability of one pollen grain to fertilize the egg but not its stimulation of embryonic development. Deanon (1957), however, found a decline in the frequency of haploid maize when silks had been treated 24 hours before pollination. No significant increase was found when maleic hydrazide was applied in the same manner.

Uses of Haploids. Perhaps the most practical breeding value of haploids is the development of homozygous lines by doubling the chromosome complement of the haploid form. If parthenogensis can be induced in heterozygous plant, complete homozygous lines could be obtained in one generation. As compared with inbreeding, the standard method for synthe-

sizing homozygous lines, use of haploids would save time and effort, especially in forest and fruit tree breeding. Another unique feature of this method is the fixation of the genetic system of individual gametes. The probability of obtaining a homozygous individual from a heterozygote with a pair of alleles is one-half by haploid method and only one-fourth by the classical selfing method. With n independent loci, the probability would thus be $(1/2)^n$ and $(1/4)^n$ respectively. The efficiency of selection, therefore, is greater in a plant, such as maize, where the frequency of haploids is relatively high, especially if the number of the genes is large and the frequency of the favorable alleles is small (Nei, 1963).

Kostoff (1942), in discussing the potential use of haploids in plant breeding, discredited this method as a means of securing homozygous strains. He based his criticism chiefly on two points. First, the difficulty in securing haploids from any one variety is such that many of the genetic characters would not be included at least once. The second problem is the difficulty of doubling the chromosome number of a haploid so that the resulting diploid would represent potential homozygous breeding material. The induction of chromosome doubling has not been necessary in maize. About 10 percent of the haploids yielded successful self progeny as a result of spontaneous somatic diploidization (Chase, 1952).

Haploid crop plants have already been employed in some breeding programs. Pure line Marglobe variety of tomato, which was developed from a haploid seedling, has been grown commercially (Morrison, 1932). In maize, over a hundred homozygous diploid lines have been developed to substitute for the inbred lines which are normally used for commercial

hybrid corn (Chase, 1952b). The haploid derived lines appeared as good as the inbred lines for general vigor, yield, and combining ability (Thompson, 1954). In rice, Hu (1959) found no differences between 30 doubled haploid lines and comparable pure lines of the same varieties in plant height, panicle length, panicle number and yield per plant.

The use of haploidy as a breeding tool in cotton has been suggested by Harland (1955) who indicated that pure lines from doubled haploids exhibited extreme uniformity equivalent to inbred lines which have been self-fertilized for more than 35 years. The lint quality and yield were found to be equal to that of the commercial varieties. The use of doubled haploids as recurrent parents in interspecific hybridization for purification and maintenance of the commercial varieties has increased (Meyer, 1960). The possible use of such lines for commercial hybrid seeds in cotton has been suggested (Harland; Meyer). Also, the totally homozygous lines of cotton can be used as an indicator of the magnitude of environmental heterogeneity as proposed by Silow and Stephens (1944).

To overcome the difficulties of breeding at the autotetraploid level in Solanum tuberosum, Hougas and Peloquin (1958) suggested that the work be carried out at the haploid (24 chromosomes) level and thus obtain all the advantages of disomic inheritance. Haploid S. tuberosum was found to be fertile and interfertile in crosses with natural diploid species. Thus the haploid offered a new approach for gene transfer from other tuber bearing diploid Solanum species to common potato (Hougas, Peloquin and Ross, 1958; Hougas and Peloquin, 1960).

Haploids are a useful device in genetic and cytogenetic work. The cytogenetic behavior of the haploid may serve as a basic tool to

determine the chromosome constitution of the species. Meiotic pairing and the amount of autosyndesis between chromosomes of a haploid measures the phytogenetic relationship of the species. If the chromosome pairing of a haploid is mostly autosyndetic, it indicates that the species from which the haploid arose was either autopolyploid or allopolyploid with closely related genomes. If, on the other hand, the haploid forms univalents or only occasional bivalents, the species would be allopolyploid with two distinct genomes (Kostoff, 1942).

The degree of homology in haploids has been used to establish the basic chromosome number of the species. From observation of bivalent formation of haploid Brassica oleracea var acephala (9 chromosomes), Thompson (1956) was led to believe that three of the six basic chromosomes of Brassica are duplicated and the other three are present only once.

Sears (1939) used haploid Triticum to study the immediate effects of deficiencies and duplication of chromosomes and chromosome parts on the plant and monosomics and trisomics to locate genes on specific chromosomes of the species.

MATERIALS AND METHODS

Two species, Lycopersicon esculentum and Zea mays were used in this study.

LYCOPERSICON ESCULENTUM

Experimental Plant Materials. A stock d a c l r y (dwarf, green stem, potato leaf, yellow foliage, yellow fruit, and colorless skin) was maintained in the greenhouse of the New Hampshire Experiment Station in Durham. This stock was crossed to Marglobe variety which possesses the dominant alleles D A C L R Y. Plants of the F_1 progeny were backcrossed to the recessive female parent. From the backcross segregation, seedlings with green stem (aa) and cut leaf (Cc) were selected and used as female parent for the experiment which was conducted in the field in summer 1965. For the greenhouse experiments the following spring, the line was maintained by vegetative propagation. The male parent for both field and greenhouse experiments was the variety Marglobe.

Pollen Treatments and Pollinations. Pollen from several freshly opened flowers was collected on a depression slide, moistened with drop of the aqueous solution of toluidine blue and then applied on the stigma of emasculated flower buds of the female parent. Preliminary trials revealed that crosses employing pollen treated with concentrations higher than 13 ppm prevented fruit set. Accordingly, concentrations from 0 ppm to 13 ppm in the field experiment and 5 ppm to 10 ppm plus a control with 0 ppm in the greenhouse experiment were used. For each treatment a minimum of 50 flowers was used. The pollinations were made in the morning during the month of July in the field and during the months of March and April in the greenhouse.

Seed Extract and Germination. The individual fruits were cut in halves and placed in a 250 ml beaker containing tap water and covered with sheet of glass. After three days for fermentation, the seeds were extracted in a strainer by washing out pulp and skin and then were dried on absorbant paper. Seeds from each fruit were sown in sterilized mixture of soil, peat moss and sand in 2 x 2 jiffy pots. The pots were labeled and placed in trays, 35 pots in each tray. The trays were arranged on greenhouse benches where supplemental lighting was provided.

Classification of Haploids and Diploids. The first screening criterion was based on the stem color of the seedlings. The purple stem seedlings were assumed to be diploids since they carried the marker gene of the male parent. Consequently, all the purple stem plants were removed as they germinated.

The second screening was based on the segregation of the leaf marker, cut leaf vs. potato leaf, as calculated by X^2 values. If the green stem seedlings from each fruit were to segregate 3 cut leaf to one potato leaf, it would indicate that the seedlings were a result of accidental self pollination. If, on the other hand, the ratio was one to one, it would suggest haploid and/or maternal diploid population.

A third screening was based on plant fertility. All green stem seedlings were grown to maturity in 6-inch clay pots in the greenhouse. Seed set was checked periodically. Plants which produced abundant seeds were diploid since the haploids of this species are sterile. To investigate the origin of the diploid plants, seeds from fertile cut leaf plants were extracted and sown in rows two inches apart in 18 x 12 inch wooden trays containing sterilized mixture of soil, peat moss and sand. If the seedlings from the cut leaf plant segregate for cut leaf and potato leaf,

the plant was then heterozygous for the leaf marker. This would rule out the possibility of having maternal diploid origin.

The final criterion for haploid recognition was the cytological examination by means of root tip squash. Root tips were treated with 0.2 percent colchicine solution for two hours and fixed in Farmer's fluid (95 percent alcohol, 3 parts, and glacial acetic acid, one part). After 24 hours fixation, they were placed for five to ten minutes in a mixture of equal proportions of 95 percent alcohol and concentrated hydrochloric acid. They were then transferred to Carnoy's solution (95 percent alcohol, six parts, and glacial acetic acid, one part; chloroform, three parts) for five minutes. The root tips were then squashed in drop of proprionic carmin solution and heated gently before observation for chromosome counts.

To estimate the frequency of haploids as a result of polyembryony, approximately 4000 seeds of the male and the female lines used in this study were germinated. The seeds were oriented in petri dishes containing filter paper, which was moistened with 100 ppm potassium nitrate solution. The dishes were placed on trays and incubated in germinator with alternating 20-30° C and 100 percent humidity. Three counts were made in five day intervals.

ZEA MAYS

Plant Materials and Plant Growth. The female line used in this investigation was the hybrid variety Pa 602A, which was obtained from Agway Company. It is a double cross hybrid derived from (WF 9 X Ohio 51A) X (Pa 54 X W 22) and is characterized by white embryo and yellow endosperm. The male line PEM (purple embryo marker) was kindly supplied by Dr. Chase of the Dekalb Agricultural Association. The line carries a

set of genes (b pI A C Rnj:Cu du pr Pwr), which produces deep purple pigment in the embryo and the endosperm of the mature kernel. Seeds from both lines were sown in the greenhouse in 2 x 2 inch jiffy pots containing jiffy mix. About four weeks after germination they were transplanted in the field 18 inches apart in rows spaced 5 feet apart. In another plot, the seeds were sown directly in the field.

Pollen Treatment and Pollination. The female parent line was detasseled as soon as the tassels appeared. The upper ear shoot was covered with a 3 x 7.5 inch glassine bag two to three days before the silks emerged. The silks were cut back with scissors to about half inch below the tip of the husk one or two days prior to pollination. Only the top ear was used. The remaining ears were removed from the plant to eliminate competition. The tassels of the plant from which pollen was collected were bagged late in the afternoon. To collect the pollen the following day, the bags were shaken and removed. The pollen was poured from the bag into a wire mesh screen on a sheet of paper. The debris which passed through the screen was picked up with a spear needle. One gram of pollen was measured in 5 ml graduate cylinder several times and found to be equal to 1.4 ml. To each ear shoot 1.4 ml (one gram) of pollen was treated and applied. Pollen was treated with toluidine blue in both dry and solution form. In the dry treatments the dye was mixed with talc. In the solution form the dye was incorporated in Cook and Walden's (1965) pollen germination medium which consisted of 12 percent sugar, 100 ppm boric acid and 300 ppm calcium chloride. In the dry applications, 1.4 ml of pollen was mixed thoroughly with 2, 1, 0.5, 0.25, and 0.1 grams of 0.001 percent toluidine blue. Pollen for the control ears was mixed in the same way, but with talc only. A minimum

of ten ears was used for each of the five treatments with three ears for each of the five controls. In the treatments in which liquid toluidine blue was applied, 1.4 ml of pollen was placed in 5 ml beaker. To this, 1 ml of the dye was added, stirred well, and applied to the silks with a camel hair brush. In another set of treatments, the pollen was suspended in 2 ml of the dye solution and applied to the silk in similar manner. Concentrations between 0 to 10 ppm were used. Ten ears were used for each of the eleven treatments including the control. The ears were bagged with No. 6 kraft bags and stapled to the stock after pollination. The pollination was completed between 2 to 4 pm. A randomized complete block design was used in which the block consisted of one ear per treatment.

Harvesting of Ears and Isolation of Haploids. The pollinated ears reamined in the field from the time of pollination, July 14 to August 7, until they were harvested September 24. Kernels of each ear were counted and separated into two classes based on the color of the embryo. The purple embryo class represents the hybrid embryos and white embryo class represents the maternal embryos. Samples consisting of ten white embryo kernels from each ear were germinated for chromosome count. The preparation of root tips and the staining procedures were similar to that described by Brown (1966). Kernels were oriented on moistened filter papers in petri dishes with embryo facing up. The dishes were placed under constant fluorescent light at room temperature. After 24 hours, the roots were cut off, placed in 0.2 percent colchicine solution for two hours, then fixed in Carnoy's. After a few minutes, they were transferred to fresh Carnoy's and incubated for 24 hours at 60^{o} C, then washed thoroughly with distilled water and hydrolized in one normal hydrochloric acid for 20 to 15 minutes

at 60° C. After hydrolization, the root tips were washed thoroughly with distilled water and stained in 0.25 percent aqueous solution of basic fuchsin for 15 to 20 minutes. The excess stain was removed by washing the roots with two changes of distilled water for one hour and the roots were digested in 5% cellulase-5% pectinase solution at pH 4.2 for four hours or longer. The deeply stained tips were cut off and macerated in a drop of iron-propionocarmin, heated gently, and examined for chromosome count.

EXPERIMENTAL RESULTS

LYCOPERSICON ESCULENTUM

Seed Set. In general, there was a relationship between the number of seeds set and the concentration of the dye. The higher the concentration of toluidine blue, the lower the number of seeds per fruit. This relationship was more noticeable in the greenhouse experiment where self and cross contamination was more rigidly controlled. The control in both field and greenhouse experiments yielded more seeds per fruit than any of the treatment pollinations. Table 1 shows the number of seeds obtained at different levels of concentration. Although the table indicates no definite trend, there was a pronounced reduction in seeds per fruit at 8 ppm or higher concentrations with only one exception (treatment 12 ppm). The analysis of variance (Table 2) of these data shows a highly significant difference. The lack of consistency of the data in Table 1 was a result of self contamination which was detected by the gene markers in the resulting seedlings. The effect of toluidine blue treatment on seed set from the greenhouse experiment is shown in Table 3. A similar relationship between dye concentration and seed set was found, except that the reduction in number of seeds per fruit was much higher than that shown in Table 1. Here again pollinations from 8 ppm or higher concentrations yielded fewer seeds per fruit than those of the lower concentrations. The analysis of variance (Table 4) of these data again shows a significant difference in the mean square for treatments. The LSD (Table 3) reveals that the means of treatments 8, 9 and 10 ppm significantly differ from the mean of the control.

Table 1 - Effect of toluidine blue on seed set in field grown tomatoes.

Treatments ppm	Fruits	Seeds	Seeds/fruit
0	9	603	67.00
1	9	363	40.33
2	6	261	43.50
3	8	329	41.12
4	6	155	25.83*
5	11	560	50.90
6	8	459	57.38
7	5	280	56.00
8	5	121	24.20*
9	6	206	34.33
10	6	153	25.5 *
11	2	52	26.00*
12	4	251	62.75
LSD			40.17

*significantly below the value of the control

Table 2 - Variance analysis of the effect of toluidine blue on seed set in field grown tomatoes.

S.V	d.f	MS	F
treatment	12	3947.60	2.751**
error	72	1434.89	

**significant at 0.01 level

Table 3 - Effect of toluidine blue on seed set in greenhouse grown tomatoes.

Treatments ppm	Fruits	Seeds	Seeds/fruit
0	21	408	19.43
5	17	250	14.71
6	14	261	18.64
7	14	200	14.29
8	15	153	10.20*
9	18	146	8.11*
10	14	152	10.86*
LSD			7.67

*significantly below the value of the control

Table 4 - Variance analysis of effect of toluidine blue on seed set in greenhouse grown tomatoes.

S.V	d.f	MS	F
treatment	6	313.367	2.353*
error	106	133.167	

*significant at .05

Classification of Seedlings and the Isolation of Haploid. In the field experiment, there were 229 green stem seedlings among a total of 3593 seedlings. The frequency of green stem seedlings for each concentration is shown in Table 5. There was no green stem seedling found in treatments below 5 ppm nor above 11 ppm. The highest frequency was found in 6 ppm treatment. The X^2 value and the heterogeneity test for the segregation of the leaf marker is present in Table 6. The segregation was found to be 1:1 (potato leaf vs cut leaf) ratio, suggesting that the green stem seedlings were either haploids and/or maternal diploids, with one exception. One fruit was found in which the seedlings segregated 3 cut leaf to 1 potato leaf, indicating possible self contamination. The heterogeneity test indicated homogeneity of the population for 1:1 segregation. At maturity, these green stem, cut leaf plants were found to be fertile and set seeds upon selfing. Since the haploid plants of tomato are known to be sterile, the high fertility ruled out the possibility of induction of haploidy in this experiment. In order to investigate the origin of the diploid, seeds from 95 plants were germinated. Fifty-four plants were found to be heterozygous for the leaf marker; the remaining 41 plants were homozygous. The high frequency of heterozygous plants clearly indicates the green stem seedlings were results of self pollination.

The greenhouse experiment yielded only five green stem seedlings out of 1234 germinated seeds (Table 7). The green stem seedlings were found in the 8 and 9 ppm treatments. Cytological examination of the root tips showed that two of these seedlings were haploids, one haploid from each of the two treatments.

Table 5 - Frequency of green stem seedlings appearing after treatment of pollen in field grown tomatoes.

Treatment ppm	Purple Stem	Green Stem	Percentage
0	255	--	--
1	279	--	--
2	233	--	--
3	277	--	--
4	129	--	--
5	456	48	10.52
6	292	128	43.84
7	103	3	2.91
8	106	8	7.55
9	113	23	20.35
10	73	--	--
11	148	19	12.83
12	139	--	--

Table 6 - X^2 values and heterogeneity test for leaf marker segregation in seedlings from field grown tomatoes.

Fruits No.	Leaf Marker Cut Leaf	Segregation Data	x^2
1	2	3	.20
2	17	23	.90
3	1	2	.33
4	7	1	4.50*
5	62	50	1.28
6	1	1	0
7	0	1	.33
8	3	3	0
9	1	1	0
10	3	5	.5
11	10	4	2.57
12	3	8	2.27
13	7	16	3.52
14	2	7	2.77
15	11	8	.42

heterogeneity test X^2 = 26.30, not significant at .05 level

*fits 3:1 ratio

Table 7 - Frequency of green stem seedlings appearing after treatment of pollen in greenhouse grown tomatoes.

Treatment ppm	Purple Stem	Green Stem	Ratio
0	328	--	--
5	126	--	--
6	140	--	--
7	97	--	--
8	184	2	0.91
9	216	3	1.39
10	138	--	--

Even though haploid tomatoes were not observed in approximately 4000 seeds from both parents which were germinated in the laboratory to estimate the frequency of natural haploidy resulting from polyembroyony, it is possible that the two haploid seedlings obtained from the greenhouse experiment were not produced by treatment of tomato pollen with 8 and 9 ppm. Considering the number of seeds examined in both field and greenhouse experiments, two haploid seedlings out of 4827 is not above spontaneous levels.

ZEA MAYS

Kernel Set. In general, there was no definite relationship between the number of kernels set and the concentration of the dye. There was, however, a considerable difference in kernels set between dry and solution treatments. There were many more kernels per ear in

the dry treatments than in the solution treatments. In the dry treatments, there were more kernels per ear in the controls than the treatments, except for one treatment in which the number of kernels exceeded its control (Table 8). The highest number of kernels per ear, 678, was found in the control in which 1.0 gram of pollen was mixed with 0.5 gram of talc. Similarly the highest number among the treatments was found in the same pollen-talc combination. Such a peculiarity is difficult to interpret. It may suggest, however, that the combination of pollen and talc in this proportion brought the pollen population to the optimum. The statistical analysis of the data is presented in Table 9. The method was suggested by Urban (1966). To each observation a constant number (500) was added. The average of the corresponding control was subtracted from each observation in the treatment. The purpose of adding a constant number is to avoid having negative numbers. The data were then analyzed as a completely randomized block design with one missing block (ear). The analysis of variance, as shown in Table 9, shows no difference among treatment means.

For treatments in which the dye was incorporated in Cook and Walder's medium, the number of kernels was reduced in all treatments (Table 10). Column 6 represents the number of kernels per ear for treatments in which one gram of pollen was moistened in one ml of the medium. Examination of this column indicates reduction in kernel set as the concentration of the dye increases. Treatment of pollen with 10 ppm yielded approximately half of the kernels obtained from the control. A similar relationship is shown in column 7, which shows the kernels set per ear from pollen suspended in 2 mls of the media. The number of kernels from the 10 ppm treatment also was found to be half of that in the control.

The analysis of variance (Table 11) shows no significant difference in the treatment means. There was, however, a high significant difference between the one ml and two ml treatments. Although the amount of dye available to pollen was doubled when the medium containing the dye was increased from one ml to two mls per gram of pollen, the reduction in kernels set could not be attributed to the increase of the amount of the dye alone since a reduction in the control was also found. It may be possible that the osmotic pressure with the excess of liquid had been altered. Coe et al (1966) found better kernels set in corn when the water was replaced by paraffine oil in Cook and Walder's medium. The discrepancy in data from dry treatments and solution treatments regarding kernels set also can be attributed to the change in the osmotic pressure. The average number of kernels in any of the dry treatments was higher than the control of the solution treatments. It is probable, then, that water itself is responsible for the discrepancy in the two types of application.

Classification of Seeds for Haploidy. Kernels from each ear were classified into two groups, purple embryo and white embryo. The purple embryo kernels represent the diploid (hybrid) class and the white embryo seeds should represent the possible haploids, maternal diploids and diploids with mutant color gene (Chase, 1965). The frequency of white embryo seeds was very high in both dry and solution treatments. Table 12 shows the frequency of white embryo kernels in the dry treatments. The frequency was found to be higher in the control, column 7, than the treatments, column 6. The same result was obtained from the solution treatment, as shown in Table 13. These results indicate lack of relationship between the frequency of white embryo kernels and the

dye treatments. Samples of at least ten white embryo kernels from each ear were germinated for cytological analysis. In at least 1500 root tips that were examined, no haploid seedling was found. The high frequency of white embryo apparently does not reflect the frequency of haploidy, and it is rather difficult to explain this high frequency. Contamination in the field was prevented. Aside from bagging the ear shoots before and after pollination and the female detasseled before the tassel emerged, the plants were flowered earlier than any corn plants in the area. If contamination had occurred, it would be from the purple embryo marker line and the frequency of white embryo would have been smaller. Color mutation at such high frequency is not likely. The only realistic explanation, then, is that the purple pigment failed to develop in an appreciable degree to be visible. This may relate to environmental factors, such as temperature during seed development which was not high enough to bring about pigment development.

Table 8 - Effect of dry applications of toluidine blue on seed set in corn.

Treatments Pollen/Talc in Grams	No. of Ears		No. of Kernels		Kernels/Ear	
	Treatment[o]	Control[oo]	Treatment[o]	Control[oo]	Treatment[o]	Control[oo]
1*:2**	9	2	4802	1057	533.56	528.50
1 :1	9	2	4960	1183	551.11	591.50
1 :0.5	8	2	4731	1356	591.38	678.00
1 :0.25	9	2	4623	1250	513.67	625.00
1 :0.1	9	2	4423	1317	491.44	658.50

*Pollen in grams

**Talc in grams with or without toluidine blue

[o]With .001 percent toluidine blue

[oo]Without toluidine blue

Table 9 - Variance analysis of effect of dry application of toluidine blue on seed set.

S.V	d.f	MS	F
block	8	63364.7	2.188
treatment	4	41644.24	
error	31*	19029.79	

*one degree of freedom subtracted to compensate for one missing date

Table 10 - Effect of liquid applications of toluidine blue on seed set in corn.

Treatment ppm	No. of Ears		No. of Kernels		Kernels/ear		Total	Average
	1 ml*	2 ml**	1 ml	2 ml	1 ml	2 ml		
0	7	6	1430	493	204.28	82.17	1923	147.92
1	7	3	1241	187	177.20	62.33	1428	142.80
2	7	7	1164	422	166.28	60.29	1586	113.28
3	7	5	1172	346	167.28	69.20	1518	126.5
4	7	3	1167	163	116.71	54.33	1330	133.00
5	7	6	1154	310	164.86	51.67	1464	112.62
6	7	5	1085	270	155.00	54.00	1355	112.92
7	8	7	1171	363	146.38	51.86	1534	102.27
8	8	8	1257	344	157.12	43.00	1601	100.10
9	7	7	820	225	117.14	32.14	1045	74.64
10	8	7	884	295	110.50	42.14	1179	78.60

*1 gram of pollen moistened in 1 ml solution

**1 gram of pollen suspended in 2 ml solution

Table 11 - Variance analysis of liquid applications of toluidine blue on seed set.

S.V	d.f	MS	F
block (day)	6	442392.47	3.279**
treatment	10	114063.51	0.845
concentration	1	12322261.77	91.338**
treat. x conc.	10	26537.21	.196
error	116	134907.50	

**significant at 0.01

Table 12 - Frequency of white embryo marker in corn derived from pollinations treated with dry toluidine blue.

Treatments Pollen/Talc in Grams	Purple Embryo		White Embryo		White in Percent of Total	
	Treatments[o]	Control[oo]	Treatments[o]	Control[oo]	Treatments[o]	Control[00]
1*:2**	3922	806	880	251	18.33	23.75
1 :1	4156	883	804	300	16.21	25.36
1 :0.5	4189	1200	542	347	11.46	22.43
1 :0.25	3764	1021	859	229	18.58	18.32
1 :0.1	3713	1248	710	69	16.05	5.24

*Pollen in grams

**Talc with or without toluidine blue

[o]With 0.001 percent toluidine blue

[oo]Without toluidine blue

Table 13 - Frequency of white embryo marker in corn derived from pollinations treated with solution toluidine blue.

Treatment ppm	Purple Embryo		White Embryo		White in Percent of Total	
	1 ml*	2ml**	1 ml*	2ml**	1 ml *	2 ml**
0	1299	400	131	93	9.16	18.86
1	1127	170	114	17	9.19	9.09
2	979	319	188	103	15.16	24.41
3	1045	306	127	40	10.83	11.56
4	1078	125	89	38	7.62	23.31
5	1001	245	153	65	13.25	20.97
6	913	149	172	121	15.87	44.81
7	908	288	263	75	22.46	20.65
8	1041	252	216	92	17.18	26.74
9	669	164	151	61	18.41	27.2
10	676	253	208	42	25.26	14.14

*1 gram of pollen moistened with 1 ml solution

**1 gram of pollen suspended in 2 ml solution

DISCUSSION

Pollen treatments of tomato and corn with toluidine blue failed to induce haploidy. If treatments of toluidine blue actually prevented the division of the generative nucleus (Ellis, 1964; Gearhart, 1966) and rendered the nucleus of the frog's sperm genetically inactive but stimulated eggs to develop (Briggs, 1952), the failure of haploid development and the reduction of seeds set in tomato and corn could be either due to failure of the pollen tubes to reach the ovary and stimulate seed development or the failure of the endosperm to develop and support proliferation of the unfertilized egg.

Pollen tube growth is a phenomenon of rapid cell elongation. This growth appears to be mediated by the amount of RNA present in the pollen grain and the growing pollen tube. It has been demonstrated that a considerable amount of RNA which is required for pollen germination and pollen tube growth is synthesized prior to anthesis. Woodward (1958) studied the snythesis of nucleic acids during pollen formation in Tradescantia. His results indicated that DNA synthesis began in the interphase of the generative nucleus following the pollen mitotic division and was completed 44 hours afterward. The amount of DNA was then maintained until anthesis. As DNA duplication was completed, increase of the cytoplasmic RNA was noticed. Laroson (1965) also reported increased RNA prior to anthesis. The relationship of RNA and pollen tube growth was demonstrated by Mascarenhas (1965). When RNA synthesis in both generative and vegetative nuclei of Tradescantia germinating pollen was stopped by actinomycin-D, the tube growth was reduced to about 60 to 85 percent of the normal length. Similar relationships between cell elongation and the amount of RNA was found in root tip of corn seedlings.

Woodstock and Skoog (1960 and 1961) have shown a close correlation between the rate of cell elongation and the RNA content in root tips of six inbred lines of corn. The amount of RNA in the cell apparently determines the rate of its elongation and its final size.

Aside from the possible effect of toluidine blue on DNA and the inhibition of RNA synthesis, the dye could have a direct association with RNA per se. Korson (1951) developed a staining technique in which the distribution of nucleic acids could be readily seen in the cell. A triple stain consisting of orange G, methyl green and toluidine blue was used. The results (Table 14) showed that the chromatin DNA stained green, the nucleoli (RNA) stained blue and the cytoplasm (protein) stained orange with blue granules (RNA's). The interpretation of the results was that the orange G stain combined with the protein leaving the nucleic acids free for methyl green and toluidine blue. Similarly the methyl green dye stained DNA, leaving only RNA available for the toluidine blue. Another evidence for RNA stainability with toluidine blue was demonstrated by Love and Liles (1959), Love and Suskind (1961) and Love and Walish (1963). The staining procedure was based on removal of a protein-amino group to librate the phosphoryl group of the nucleic acid which will bind with the toluidine blue molecule. He was able to identify nine types of nucleo-proteins which reflect different types of RNA's (Table 15).

Although the staining procedures cited above are based on fixed material, it does show the affinity of toluidine blue to RNA. With in vitro cultured pollen of Tradescantia, Gearhart (1966) found suppression of the pollen tubes as the concentration of toluidine blue increased in spite of the fact that pollen tubes grown in vitro attained only a small

Table 14** - Effect of methods for the removal of nucleic acids on trichrome staining.

Procedure	Results		
	Chromatin	Nucleoli	Cytoplasm
Control	green	blue	orange with blue granules
Desoxyribonuclease	colorless	blue	orange with blue granules
Ribonuclease	green	colorless	orange with colorless granules
Desoxyribonuclease plus ribonuclease	colorless	colorless	orange* with colorless granules
Cold perchloric acid	green	colorless	orange* with colorless granules
Hot perchloric acid or hot trichloracetic acid	colorless	colorless	orange* with colorless granules

*This orange G staining was of the same intensity in slides treated with the combined enzymes, hot trichloracetic acid, hot perchloric acid and in controls stained with orange G only. If the orange G staining is taken as a measure of protein content, this would seem to indicate that the enzymes were relatively free of proteolytic activity and were splitting only their respective nucleic acid substrates.

**Taken directly from Roy Korson, Stain Tech. 21:268.1751

Table 15* - Characteristics of nine types of ribonucleoprotein (RNP) stained by the toluidine blue-molybdate method.

Type of RNP	Site	Morphology	Changes During Mitosis
(1) Nucleolini and granular parachromatin	Nucleolini lie in pars amorpha of nucleolus. Granular parachromatin in nucleoplasm.	Nucleolini are solid or occasionally hollow spheres, or groups of spheres.	Nucleolini extruded during prophase to give rise to prominent parachromatin granules which are extruded into cytoplasm at onset of metaphase. Nucleolini formed before pars amorpha in telophase.
(2) Chromosomal RNP (type A)	In late prophase, in metaphase, anaphase and early telophase chromosomes.	As chromosomes.	Detectable from late prophase to early telophase.
(3) Chromosomal RNP (type B)	In interphase chromatin and chromosomes.	As chromatin and chromosomes.	Unaltered.
(4) Diffuse cytoplasmic RNP (type A)	In cytoplasm of interphase, early prophase and late telophase cells.	Diffuse in cytoplasm, except in Golgi zone.	Disappears in late prophase and reappears in late telophase.

Table 15 - continued

Type of RNP	Site	Morphology	Changes During Mitosis
(5) Diffuse cytoplasmic RNP (type B)	In cytoplasm.	Diffuse throughout cytoplasm.	Unaltered.
(6) Perichromosomal RNP	Nucleoplasm and around chromosomes.	Amorphous.	Condenses around chromosomes to form a sheath in metaphase.
(7) Cytoplasmic granular RNP	In cytoplasm.	Coarsely granular in cytoplasm, except in Golgi zone.	Disappears in late prophase and reappears in late telophase.
(8) Pars amorpha of nucleolus	Nucleolus.	Amorphous.	Disappears in late prophase, or is extruded into cytoplasm. Not included in telophase nucleus.
(9) Amorphous parachromatin	Minimal in interphase increases in prophase and is extruded into spindle zone in metaphase; also in anaphase spindle.	Amorphous.	See "site".

*Taken from R. Love and J. R. Walsh, Jour. Histochem. Cytochem. 11:191.1963

fraction of their length in vivo. It seems quite possible that toluidine treated pollen of tomato and corn failed to attain the normal tube length which they must achieve in the style to insure fertilization as a result of inactivation of RNA which is required for pollen tube growth.

In regard to embryo-endosperm relationship, Brink and Cooper (1940) reported that the ovules are very low in food reserves at the time of fertilization. This was shown in a survey of 36 species of angiospermae. Consequently, the embryo development at early stages is dependent upon the translocation of food materials. The endosperm is considered to function in the translocation of food materials. The endosperm is considered to function in the transfer of nutrients from the adjacent tissues to the developing embryo. Brink and Cooper (1947) further confirmed that, in many plant species, the endosperm is merely a medium for the growth of the young embryo and has no other function. If the endosperm does not succeed in its function, the embryo which is dependent upon it fails to develop also. Triple fusion of the polar nuclei is necessary for endosperm development. The possibility of haploid embryo with diploid or tetraploid endosperm has been investigated. Chase (1964) studied the endosperm genotype in corn utilizing gene dose effect on the color of the endosperm accompanied haploid embryo. A white endosperm inbred line (4C082) was crossed reciprocally with yellow endosperm inbred line (W22). No class other than triploid endosperm was found. Also in maize, Sarker and Coe (1966) investigated the origin of maternal haploidy and the genetic constitution of its endosperm using a gene marker in the aleurone. The results clearly demonstrated that the endosperm of kernels containing a haploid embryo has one set of chromo-

somes from the male and two sets from the female ruling out the possibility of having diploid or tetraploid endosperm. If the nucleus of toluidine treated pollen of tomato and corn was capable of stimulating the egg development but contributed no genetic material to the developing embryo, certainly it is not capable of contributing genetic material to the pre-endosperm nuclear fusion which is necessary for the endosperm proliferation.

The great majority of haploid sporophytes reported in the literature have occurred spontaneously. A variety of physical and chemical treatments have been unable to achieve a suitable method for induction of haploidy in plants. Although much is known about the origin of haploids and the techniques for their isolation, little, if any, is known concerning the stimulus involved. The development of spontaneous haploids apparently required a nonfunctional nucleus to stimulate the egg and functional sister nucleus to fuse with the endosperm nuclei. This phenomenon seems to be genetically controlled with only a few genes involved (Coe, 1959). Sarker and Coe (1966) in this connection concluded:

> From the foregoing results and discussion, maternal haploidy in maize is concluded to result primarily from failure of fertilization caused by abnormal condition, either inherent or induced, in a male or female gamete and subsequent development of the reduced egg into the embryo. The reciprocal event, failure of fusion of the male gamete with polar nuclei, is not expressed because fertilization of the endosperm abnormality is not simply an accident but is under genetic control is shown by inherited differences in haploid yielding potential for both male and female parents.

Differences between high and low haploid frequency inducing male lines may possibly be detected chemically by pollen analysis. Pollen analysis by paper chromatography has shown that the only differ-

ences between male sterile and male fertile pollen of maize was two free amino acids, proline and alanine. A large quantity of proline was found in the normal pollen and no proline was detectable in sterile pollen. The concentration of alanine, on the other hand, was much higher in the sterile pollen than that found in the fertile. The differences in the constitution of the two amino acids was detectable even before the pollen shed (Khoo and Stinson, 1957). Other workers have indicated that the protein and free amino acids constitution of the pollen are synthesized before anthesis. Woodard (1958) found the amount of protein was increased 120 percent at the completion of mitotic division of the pollen grain. Poddubnaya-Arnoldi et al (1959) found that the protein and the amino acids constitution of the pollen grain was higher than that in the germinated grains and pollen tubes. It seems, then, pollen analysis might well reveal the exact nature of the stimulus that causes spontaneous haploidy. Lines with high and low frequency of haploid corn would be the best plant material to be used for such study.

SUMMARY

The possible use of toluidine blue dye for induction of haploidy was investigated. Two plant species were used - Lycopersicon esculentum, having binucleate pollen grain, and Zea mays, having trinucleate pollen grain. The dye was applied to the pollen of tomato in aqueous solution at concentrations from 1 ppm to 13 ppm in the field experiment and from 5 ppm to 10 ppm in the greenhouse experiment. In the case of corn, the dye was mixed with talc at 0.001 percent, and the mixture was then mixed with pollen in five different proportions - one gram of pollen mixed with 2, 1, 0.5, 0.25 and 0.1 gram of the toluidine-talc mixture. In the controls, the pollen was mixed with talc alone at the same proportions. In another experiment, one gram of corn pollen was moistened with 1 and 2 mls of Cook and Walden's medium in which 1 to 10 ppm toluidine blue was incorporated.

Gene markers, green stem and potato leaf, in tomatoes, and a purple embryo marker in corn, were utilized for primary screening and isolating of the putative haploids. This was followed by cytological examination of the root tips.

The result yielded only two haploid seedlings in tomato which were found in 8 and 9 ppm treatments. In corn no haploids were obtained from any of the treatments even though the frequency of white embryo kernel was high. No relationship was found between the frequency of white embryo and the frequency of haploidy. The frequency of haploidy found in tomato was too small to be considered as a result of the dye treatment.

Seeds set in both species was reduced in all levels of concentration, but no definite trend was found. Probable explanations were

advanced regarding the failure of induction of haploidy and the reduction of seed set. Some pollen tubes affected by toluidine blue treatment of the pollen failed to reach the eggs and so failed to effect fertilization as a result of RNA inactivation. Another possibility is that the generative nuclei were inactivated by the dye treatment which caused failure of the endosperm development.

Chemical analysis of pollen from lines with high and low frequency of haploidy was suggested to help in understanding the mechanism involved in spontaneous haploidy.

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