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Development of techniques for the chemical induction of mutations in vegetative plant material

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DEVELOPMENT OF TECHNIQUES FOR THE CHEMICAL INDUCTION
OF MUTATIONS IN VEGETATIVE PLANT MATERIAL

by

Harold Melvin Pellett

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INTRODUCTION

In the last few decades the need for woody ornamental plant materials has changed greatly. The increase of public interest in ornamental plants has resulted in a need for new and different types, as well as a need for increased production of the present plant inventory. Several factors have contributed to this increased interest in ornamental plant materials. The present generation has more leisure time and a higher standard of living than was known by earlier generations. This increase in leisure time, coupled with a higher standard of living, has resulted in increased recreational activity. Home gardening has been one of these activities.

The public today is better informed about the care and maintenance of ornamental plants than in the past. This has been the result of effective distribution of information by extension groups, through gardening magazines, and through the promotion of the nursery industry. Most communities today have established garden clubs and many of the larger cities have arboretums or botanical gardens. Many of the large urban areas now have extension personnel to advise the public on problems concerned with the care of lawns and ornamental plant materials. The establishment of these groups and organizations is an indication of the rapidly growing interest in ornamental plants. Activities of these organizations will project this trend into the future.
Changes in architectural design of homes have created a need for a different type of plant inventory for landscape planting. The trend to the ranch style or split level home has increased the need for more dwarf plants. Many of the ornamentals that are used with two story homes have growth habits which do not permit their use in the landscape scheme of many of the homes currently under construction.

The increased need for greater numbers of plant materials having a different habit of growth has challenged the nursery industry. The industry as well as ornamental research personnel has placed emphasis on the economic aspects of increased production, and therefore, has neglected the need for a larger plant inventory to satisfy present demands.

Although there are many woody ornamental plants available throughout the United States and the Northern Hemisphere, many are adapted to milder climates and would not survive the severe Iowa winters. There are many however, that have not been adequately tested in areas outside of their natural habitat. Many arboretums are now growing plants in various locations in order to determine specific cultural requirements. By expanding this type of work, the plant material inventory suitable for a given locality will be greatly expanded.

Breeding work with woody ornamental plants has been limited primarily to those genera having outstanding flowering characteristics, such as roses and azaleas. Very few attempts
have been made to improve many of the woody plants that are grown for other ornamental attributes, such as habit of growth, texture, foliage, fall color, or fruiting habit. New introductions that have been added to the plant inventory have resulted from the selection of chance seedlings or spontaneous mutations.

The common breeding methods that have been successfully used to improve agronomic crops, small fruits, and vegetable crops have many limitations in woody ornamental plant improvement programs. Most woody ornamentals are heterozygous clones that are propagated solely by asexual means. These clones have been selected for some specific characteristic which is generally lost when the plant is propagated by means of seed. Another factor limiting the success of ornamental breeding is introduced by the long life cycle of most woody plants. Several years are necessary for observation of a plant before selections can be made. Thus a breeding plan requiring several generations is not practical. Many woody plants do not produce viable seeds or their seeds are extremely difficult to germinate.

A mutation breeding program might be used to great advantage with woody ornamental clones since new traits could be added without loss of the existing desirable characteristics. Secondly, the disadvantages encountered with the use of mutation breeding techniques in crop plants would not be important
with ornamental plants. Sterility, which is often encountered, would not be a problem, since the plants are propagated asexually. Sterility would actually be desirable with many trees, such as elms and maples, that seed heavily and with crabapples and other plants that produce large fleshy fruits. Dwarfness, another characteristic often resulting from mutation breeding methods, would also be a benefit in improving woody ornamentals. The production of dwarf plant types is one of the primary goals of ornamental breeding. Mutations resulting in a modification of foliar characters of the plant are of no benefit in crop plants, but may be of value for ornamental purposes. Thus the rate of mutants which could be used commercially would be much higher in ornamentals than in crop plants.
PURPOSE OF THE STUDY

The need for a larger inventory of woody ornamental plants places emphasis on increased numbers of dwarf forms, as well as plant materials with improved aesthetic characteristics. This study was designed to develop methods that could be used to induce mutations in vegetative material of woody ornamental plants by use of chemical mutagens. As a result of the many problems encountered when ordinary breeding techniques are applied to woody plants, a mutation breeding project was initiated. Mutations induced by the treatment of vegetative material would be more likely to result in improvement than mutations produced by treatment of seeds. Most woody ornaments are highly heterozygous and therefore are asexually propagated to preserve certain desired characteristics that would be lost by sexual propagation. Thus, if a mutation having an improved character were induced by seed treatment, other desirable qualities may be lost. Mutations possessing desirable ornamental attributes induced in vegetative tissue may be added to the qualities already present.

Chemical mutagens, theoretically, would be more adapted for use in the treatment of vegetative parts than would irradiation techniques which require the use of complex equipment. The irradiation equipment commonly available limits the volume of material that can be treated. Highly effective chemical mutagens are now available. In many instances these
chemicals are more effective than irradiation treatments in inducing mutations. Several of these mutagens have been used to treat seeds of barley and other crop plants. Treatment techniques must be developed before they can be used successfully to treat vegetative material of woody plants. Treatment of vegetative plant parts is often complicated by the morphology of the plant. For example, many woody ornamentals have tightly enclosed waxy buds. When stems containing buds of this type are soaked, air may be trapped inside the scales which interferes with the intake of solution.

The purpose of this study was to evaluate techniques for inducing mutations in vegetative tissue by treatment with chemical mutagens. The ultimate goal of the overall project is to develop techniques for successful production of dwarf and/or improved types of woody ornamental plants. Since woody plants are relatively slow growing and require a relatively large growing area, initial studies consisted of the treatment of vegetative parts of herbaceous plants. By using herbaceous materials for the preliminary studies, considerable time and effort was conserved in the initial evaluation of the techniques tested. Most herbaceous plants mature in a single growing season. The same amount of information was gained in one year that may have required eight to ten years if woody plants had been used in these studies. The information derived from these initial studies will be used to adjust treatment procedures that will be applied to woody ornamental plants.
In addition to determining satisfactory methods for applying chemical mutagens to vegetative plant material, a second objective of these studies was to establish an effective concentration range over which these chemicals could be successfully applied. An integral portion of this study was the determination of the cytological effects of the chemical mutagens on chromosome behavior and cell division.
REVIEW OF LITERATURE

Types of Mutations

Induced mutations can be classified as structural mutations, point or gene mutations, and genome mutations. Structural mutations are microscopically visible and result from fragmentation or breakage of chromosomes. These fragments give rise to deficiency or deletions of chromosome sections, duplication of chromosome segments, inversions, and translocations. The gene or point mutations are undetectable by microscopic means and result in mendelian segregation. Until recently these point mutations were thought to be very small structural mutations, too small to be detected by microscopic studies and therefore not true gene mutations (Stadler, 1941). In the last few years several scientists have demonstrated that induced gene mutations exist. This phenomena was shown by using mutagenic agents to produce back mutations (the reversion of a mutation to its original form). Giles (1955) working with Neurospora, accomplished this with X-rays. Zetterberg (1960) reported similar results.

Radiation Effects

The use of induced mutations as a breeding tool became of interest after the discovery by H. J. Muller (1927) that X-rays induce mutations. Interest in this new breeding technique remained high for several years, but due to the high
frequency of sterility and dwarfness induced, and the low frequency of favorable mutations, this optimism soon faded (Stadler, 1930). European scientists, especially the Swedish group, continued work on radiation induced mutations as a technique for plant improvement. It has been only recently that this technique has been reevaluated in this country because of the successes of the European workers.

Radiation acts in several different ways to induce mutations. These can be described as: (1) direct effects, (2) primary radiochemical reactions, and (3) chemical reaction chains. The extent of the direct effects is dependent on the type of radiation. Such factors as the charge of the ionizing particles, the energy of the particles, and the mass of the particles are also important (Ehrenberg and Nybom, 1954). Sparsely ionizing radiations act primarily by indirect effects, whereas the densely ionizing radiations act mainly by direct effects. The secondary effects are caused by unstable compounds or radioactive substances induced by the radiation treatment in the cellular material surrounding the nucleus. Dry seeds are more resistant to radiation treatments than are germinating seeds or vegetative plant parts.

Non-ionizing radiations, such as ultraviolet light can also induce mutations. Hollaender and Emmons (1941) determined that monochromatic ultraviolet light at 260 m. u. gives
greater mutagenic effects than other ultraviolet wavelengths. This wavelength corresponds to the range of greatest absorption of ultraviolet light by nucleic acid. Ultraviolet light has low penetrating power, and therefore is only of use in treating pollen or other small masses of tissue. Ultraviolet light can induce both gene mutations and structural changes. Faberge (1951) and Lovelace (1954) demonstrated the production of chromosomal aberrations as a result of exposure to ultraviolet light. Stadler (1941) showed that, with dosages of ultraviolet light and X-rays that give an equal amount of gene mutations, the ultraviolet rays produce less structural changes than do X-rays.

There have been many attempts to compare the effectiveness of the different types of radiation (Giles, 1943, Ehrenberg and Nybom, 1954). The limitations of these studies lie in the inability to accurately measure the amount of radiation energy received by the material under study. Neutrons, however, are usually considered to be more efficient than X-rays of equal energy (Ehrenberg and Nybom, 1954).

Sensitivity to Radiation Treatments

Since mutations, produced under the most effective methods, occur at a low frequency, there has been considerable study as to the most effective doses of application of the various mutagens. The optimum doses vary widely, depending
on the type of plant material and the stage of growth of the material. In general, the more rapid the growth, the greater the sensitivity of the material. Bauer (1957) reported that the lethal dose of X-rays in *Ribes nigrum* cuttings was 4000 - 6000 r., whereas 3000 r. was not lethal and produced several mutations. Bishop and Aalders (1955) reported that X-rays of 4000 - 5000 r. was the optimum dose that was not lethal in Cortland apple scions. Granhall, Gustafsson, Nilsson, and Olden (1949) reported that 5000 r. X-ray radiation was optimum for scions of apple and pear, and 2500 r. for cherry. All scions were killed at exposures over 10,000 r. Seeds appear to be much less sensitive to irradiation than growing plants or plant parts. Levels of 50,000 to 60,000 r. X-rays or gamma rays have been reported to be without a high degree of lethality to seeds (Davis, and Hammons, 1956). Lower levels of radiation have been reported to improve the germination percentage and to speed up the germination process (Sax, 1955, Sparrow, 1954, Kersten, Miller, and Smith, 1943).

Plant materials vary in their sensitivity to radiation. There have been several attempts to explain the underlying causes responsible for these differences. The identity of these factors is not definitely established at present. Closely related plants of similar chromosome number usually have a similar sensitivity. Chromosome size appears to be an important factor. Sparrow (1956) reported that, of the plants
studied, those with large chromosomes had a high radiosensitivity. Those with a high tolerance had relatively small chromosomes.

Dose rate is also considered to be an important factor in mutation breeding. Arnason and Morrison (1955) report that low rates of application are more effective in producing chromosome breakage than are higher rates of radiation of the same total dosage. They attribute this partially to the rejoining of the chromosomes. Breaks occurring over a longer period of time are less likely to rejoin at the same location.

Many studies have been conducted to determine what influence the state of the cell has on the number of chromosomal aberrations. These experiments have been performed during the various stages of meiosis, since many plants have an adequate supply of nuclei in the same stage of division at any given time. By collecting material at different stages of meiosis, the related sensitivity of the different stages can easily be compared. The results of these studies indicate that the sensitivity to chromosome breakage is partially correlated with total DNA content. Some workers report a close relationship between DNA duplication and sensitivity (Sparrow, 1944). Others report that, although sensitivity is correlated with the amount of DNA, a strong correlation may not exist. Sparrow, Moses, and Steale (1952) report that *Trillium erectum* sensitivity to X irradiation increases during meiotic prophase to a
peak at or near diplotene. Sensitivity then decreases to a point at early interphase. Thus, there is an increase in sensitivity as DNA content increases, but the highest point of DNA content, which is at interphase, is the period of lowest sensitivity. This sensitivity trend is closely associated with the contraction of the chromosomes. The most sensitive stage is at diplotene, when the chromonemata are shortest. The least sensitive stage is interphase, a point at which the chromonemata are most relaxed. Brumfield (1943) states that chromatid aberrations appear to be more frequent during metaphase than during the resting stage. This would agree with the theory that sensitivity may be correlated with the contraction of the chromosomes. Brumfield, however, believes that the sensitivity is more closely related to chromosomal movement.

Accompanying environmental and physiological conditions before, during, or shortly after irradiation treatments have been demonstrated to be of importance in the sensitivity of plant tissues to radiation. Nybom, Lundqvist, Gustafsson, and Ehrenberg (1953) demonstrated that seeds treated at the temperature of liquid air (−190°C) were less affected by radiation treatments than were seeds irradiated with equal dose rates at room temperature. The effect of minor changes in temperature has not been clearly established. Conflicting reports have resulted from various studies of temperature influence
within the range from 0°C to 30°C (Caldecott and Smith, 1952).

The nutritional status of the plant material at the time of treatment is another factor governing the sensitivity of plant materials to radiation treatments. Steffenson (1957) found that calcium deficient cultures had a higher occurrence of interchanges and deletions after X-ray treatments than control cultures. Manganese, iron, and magnesium deficiencies had no effect on the sensitivity to X-ray treatments.

Thoday and Read (1947) discovered that X-ray treatment of *Vicia* produced fewer chromosomal aberrations when the material was treated in an atmosphere of pure nitrogen than when irradiated in air. Kihlman (1955) studied the influence of oxygen content of the surrounding atmosphere on radiosensitivity. He found the maximum chromosome breakage by X-ray treatments to occur between 10 and 21 per cent with no increase at 100 per cent oxygen. These results can be explained by the secondary effects of radiation treatment. The lack of oxygen prevents the formation of $\text{H}_2\text{O}_2$ or $\text{HO}_2$ which are mutagenic in action. Since X-rays and other sparsely ionizing radiations act primarily by these indirect effects, the amount of oxygen available for such chemical reactions can be expected to be of importance. With the use of alpha particles and other densely ionizing radiations, the amount of oxygen available is not important, since these types of radiations act primarily by direct effects.
Chemical Mutagens

The use of chemical compounds as mutagenic agents became of interest after the discovery (Auerbach and Robson, 1946) that exposure to mustard gases may induce mutations. In the last decade, it has been established that many chemicals are mutagenic in action to varying degrees. These chemicals range from simple compounds, such as H$_2$O$_2$, to very complex organic compounds. The frequency of mutations produced by the mustard gases are usually low in comparison to radiation treatments. Recently, some of the newly discovered chemical mutagens have been demonstrated to produce a high frequency of mutations even exceeding those frequencies induced by radiation treatments (Ehrenberg, Gustafsson, and Lundqvist, 1959, 1961) (Heslot, Ferrary, Levy, and Monard, 1961). Among these chemicals are ethylenimine, ethylene oxide, diethyl sulphate, and ethyl methanesulphonate. Ehrenberg, Lundqvist and Strom, (1958) state that, "Ethylenimine with respect to barley is the most efficient mutagen agent hitherto known".

Although the mechanisms by which these various chemicals act are not clearly understood, many investigations have been conducted to determine their mode of action. Since these mutagenic chemicals consist of a wide range of unrelated chemical structures, their modes of action are likewise quite diverse. Auerbach (1951), discussed possible mechanisms of action of chemical mutagens. The latter author stated that
the structure of the chromosomes and the chemical specificity of the genes might be affected in various ways by chemical mutagens, such as chemical reactions with the proteins or nucleic acids, by release of energy near the chromosomes, by inactivation of enzymes for chromosome metabolism, or by disturbances of the reduplication processes of the genes by competing substances of similar composition.

Many of the more active chemical mutagens, can be classified as alkylating agents. These chemicals are believed to act primarily by reacting with the phosphate groups of the DNA molecules (Gustafsson and Ehrenberg, 1959). Loveless and Howarth (1959) discuss the efficiency of the ethylating agents compared to other alkylating substances. The greater efficiency of the ethylating group is attributed to their lower toxicity to living tissues. Therefore the ethylating chemicals can be used in higher doses.

Chemically related compounds may not produce similar results when applied to the same plant material, although they might be expected to do so on the basis of their structural similarities. Kihlman (1952) discovered that the purine compounds can be divided into those that act only during mitosis and those that can also act during the resting stage of cell division. He discovered that the former group could not permeate the nuclear membrane and therefore could only be functional during the division stages when the nuclear membrane breaks.
down. Similarly, a given compound may react differently with various plant materials because of differences in rate of penetration, and differences in reaction with the cytoplasm. Because of these differences, chemicals give a less uniform effect than do the radiation treatments. This non uniformity does not indicate specific differences between the genes and chromosomes of the different organisms treated (Prakken 1959).

Westergaard (1957) has provided an excellent description of the principles that are known about the mechanisms of action of chemical mutagens. The author established the following theories regarding these chemicals: (1) Point mutations are induced only by chemicals which are very unstable and reactive, (2) release of free energy in the reaction with cellular components may be the decisive factor in the mutational event, (3) probably all mutagens which induce point mutations also break chromosomes, but not all chromosome mutagens are able to induce gene (back) mutations, and (4) stable materials can cause chromosome breakage.

Westergaard (1957) also presented a theory relating to a system of balanced mutagen-antimutagen products of metabolism. Hydrogen peroxide which is frequently a product of metabolism is a mutagen of the first order. It acts directly. Catalase which also is involved in metabolism is an antimutagen. It lowers the mutation rate. Potassium cyanide is a mutagen of the second order. It does not act directly but destroys or
inactivates catalase and other antimutagens. Therefore catalase no longer holds the first order mutagens in check and the mutation frequency increases. These natural mutagens which occur in metabolism may account for the expression of spontaneous mutations. They are held in check by antimutagens such as catalase.

The cytological activity of a substance is often measured by the frequency of detectable breaks induced in root tip chromosomes of Allium or Vicia (Levan, 1951). Chromosomes have a tendency to rejoin at the broken location either in the original configuration or with other broken ends. The breaks that rejoin with other broken ends can be detected in microscopic studies. Those that heal are not detectable. Wolff and Luippold (1955) demonstrated that oxidative metabolism is necessary for the rejoining process. In the latter study material was irradiated in a vacuum and then put into water at 0 degrees C. to stop enzyme activity. This treatment prevented chromosome breaks from rejoining. The detectable breaks induced in root tips are not a good criterion for comparing the amount of breakage induced by different substances. These substances may also interfere with the rejoining capacity and bias conclusions drawn on the basis of observable breaks.

Use of Induced Mutations in Plant Breeding

The value of mutation breeding techniques is a source of controversy among plant breeders. The breeders who believe
the technique to be of little value, point to the extremely low rate of favorable mutants. Under the most optimum conditions known at present, the rate of mutation is relatively low. These men also point out that the high degree of sterility often associated with mutagenic treatment further limits the value of the technique. The proponents of mutation breeding point out the many valuable achievements that have resulted from such work. Reviews by Mac Key (1956), Gaul (1961b), and Nybom (1961) list these accomplishments.

The value of mutation breeding could be greatly enhanced if any or all of the following three goals could be accomplished: (1) By increasing the mutation rate which can be induced, (2) by directing the mutation process to give a higher frequency of desirable mutations induced, and (3) by improving selection techniques. Various studies have indicated that all of these objectives might be realized as more information becomes available on the subject of mutation breeding. The first objective can be reached by the development of more efficient mutagenic agents or treatments. The recent discovery of the efficiency of some of the ethylating chemicals has resulted in much optimism in this respect. Sax and Sax (1961) found that aging of seed greatly increased their sensitivity to gamma radiation. Gaul (1957) found that combined treatments of CO₂ and heat could be used with X-ray treatments to increase the frequency of survival without altering the mutation rate.
Nilan (1954) reported similar results. Konzak, Nilan, Legault, and Heiner (1961) reported that a heat shock immediately following gamma radiation treatment resulted in higher survival in barley. These accompanying treatments would allow irradiation with higher dosages to increase the mutation frequency.

There is considerable evidence to support the possibility of directed mutations. With radiation treatments, point mutations and chromosome mutations appear to occur at random. Oehlkers (1953) treated *Vicia* with uretane, salts of heavy metals, and nucleoproteids and found a concentration of breaks in the zone of the secondary constrictions of the satellite-chromosome. Kihlman and Levan (1951) reported similar results with ethoxycaffein. Ford (1948) found that nitrogen mustard produced breaks more commonly in the smaller chromosomes in *Vicia* than in an equal area of the larger chromosomes. Different chemicals also produce varying ratios of point mutations to structural mutations. Von Wettstein (1957) observed that nebularine increased the frequency of gene mutations but only rarely produced structural mutations. Ethoxycaffein gave opposite results. It produced numerous structural mutations without gene mutations. The use of mustard gas and ethylene oxide resulted in about the same ratio of point to structural mutations as X-rays.

Heiner, Konzak, Nilan, and Legault (1960) found that diethyl sulphate gave a much higher rate of chlorophyl mutants
in barley than did gamma rays, but produced very little chromosone damage. The frequency of the various types of chlorophyll mutants varied considerably between the two treatments. Ehrenberg *et al.* (1959) found that ethylene oxide and ethylenimine produced a much higher frequency of the more rare types of chlorophyll mutants than X-rays and neutron radiation. D'Amato and Gustafsson (1948), and Gustafsson and Nybom (1949) found that a colchicine pretreatment prior to radiation produced a difference in the mutant spectra obtained.

Improvement of selection techniques employed could greatly enhance the value of mutation breeding. Gaul (1958) found that fertile plants, after irradiation treatment had equally as many mutant progeny as did the sterile or semisterile plants. The sterility problem could be eliminated by working only with the plants that maintain high fertility. Although the easily detected mutants are usually of low frequency, there are numerous micro or physiological mutants that go unnoticed. By improved screening processes, these physiological mutants could be selected and might result in an individual having improved quality, increased pest resistance, or other desirable characters. The review by Gaul (1958) lists many such achievements, including discoveries of disease resistance, improved baking qualities in wheat, and low cumarin content in *Melilotus alba*.

Diplontic or intrasomatic selection in the treated plants must also be considered. Since normal cells usually divide
faster than the mutant cells, the latter group are often eliminated. Gaul (1958, 1961a) concludes that surviving mutation percentages are higher if the material treated has only one or a few cells in the meristematic region. He suggests that this also holds true for plants treated as vegetative tissues. This would account for the low frequency of results reported in fruit trees and ornamentals which have rather extensive meristematic tissues in the primary buds. The favorable results reported by Bauer (1957) with Ribes nigrum can be attributed to the fact that the primary shoots were cut off in order to force the secondary buds. These secondary buds contain relatively few cells.
Methods and Materials

Two methods for inducing mutations in vegetative material were tested. Ethylenimine was used as the mutagenic chemical in this study. Tomato plants were selected for this preliminary study since they have a short life cycle and the $M_2$ generation could be observed in a reasonable length of time. The tomato also has many known characters that are single gene controlled.

The first method tested involved treating the tomato plants by aspirating in a .01 per cent ethylenimine solution. The plants were treated in the seedling stage when the first pair of true leaves had unfolded. The aspiration treatment was allowed to proceed until most of the air was removed from the plant tissue. The concentration was determined from the effective range used in studies on treatment of barley seeds as reported by Ehrenberg et al. (1959). Ehrenberg reported an effective range of .01 to .1 per cent with dry seeds. Since higher concentrations within this range proved to be 100 per cent lethal in preliminary studies with vegetative material, the low end of the range was selected for trial. The percentage of survival of treated plants was no different than that of a comparison treatment using only distilled water.
The second method was designed to take advantage of the high volatility of ethylenimine. Tomato seedlings of the Rutgers variety were treated by placing them under a bell jar (volume of approximately 540 cubic inches). Prior to treatment the seedling plants were transplanted into a shallow plastic container 3" by 6" in size. Each container, with twenty five plants, was placed under a bell jar with a small vial containing 3 ml. of 10 per cent ethylenimine solution. The bell jar was placed over a glass panel and sealed with lanolin. There was no control of temperature or other environmental factors since the experiment was conducted in the greenhouse. The initial exposure was five days in length. At the end of this time the seedling leaves were very chlorotic. Only seven of the fifty plants treated survived. The remainder of the plants from the atmospheric treatment were from a subsequent treatment for a four day period. Exposure of plants for a four day period resulted in 90 per cent survival.

Another group of plants was aspirated in distilled water. This group was grown as a check to compare with the growth habits of plants exposed to the ethylenimine treatments.

After treatment, the plants were transplanted into pots and grown in the greenhouse. The plants were observed for misshapen leaves, chlorophyll leaf chimeral sectors, and other possible signs of treatment effects. The date of the first mature fruit was recorded for each plant and seed was extracted.
The progeny produced from these seeds were grown under field conditions and observed for segregating populations.

The amount of material treated in this study was limited. Therefore the results and conclusions are based on a relatively small number of plants.

Results

Leaf abnormalities of treated plants

In the atmospheric treatment, thirteen of the thirty four plants observed had chlorophyll chimeral segments in some of the first leaves produced (Figure 1). Some of these plants also had misshapen leaves. Only one plant had misshapen leaves without accompanying chlorophyll chimeral segments (Figure 2). Twenty plants had no observable abnormalities. Of the seven plants that survived the five day treatment period, five had chlorophyll abnormalities in some leaf segments. One of these plants (designated as Atm.-7) had smaller leaves than normal. This condition persisted throughout the life of the plant. The aspiration treatment resulted in only two plants of the 45 tested that had chlorophyll segmental chimeras. One plant from this treatment group (Asp.-21), Figure 3, was noticeably different from normal plants. The foliage appeared to be flaccid and the leaves were without serration. The plant was weak, sparsely foliated, and had spindly stems. Double flowers were common. Very little pollen was produced and the fruits were catfaced.
Figure 1. Tomato leaf illustrating a segmental chimera expressed after atmospheric treatment with ethylenimine.

Figure 2. Malformed tomato leaf expressed after atmospheric treatment with ethylenimine.
Figure 3. Leaf from abnormal tomato plant after treatment with ethylenimine applied by the aspiration technique.

Figure 4. Leaf from a normal tomato plant of the Rutgers variety.
The control group had no abnormalities (Figure 4).

**Date of first mature fruit**

Plants from the atmospheric treatment required a much longer time to produce mature fruit than did the plants from either the control group or those which had been aspirated. The number of days required to produce the first mature fruit on each plant was recorded as the number of days after the first ripe fruit was found in the experiment. Plants from the aspiration treatment required an average of 25.0 days with a range of 0 to 42 days. This did not differ significantly from the control group which averaged 20.2 days. The range of the control group was from 8 days to 35 days after the first ripe fruit was recorded. Plants from the atmospheric treatment required an average of 41.8 days with a range from 21 to 56 days. Using the F test of significance, this was statistically different from the control group at the .01 percent level.

**Sterility**

Among the M1 plants, there were eight plants that did not set fruit in the greenhouse. Two of these were from the aspiration treatment, while the remaining six were from the atmospheric treatment. Four of these latter plants were from the seven that survived five days of atmospheric treatment. Cuttings of these sterile plants were made and grown in the
field for further observation. Under field conditions the two plants from the aspiration treatment and two from the atmospheric treatment produced abundant fruit. Two of the remaining four M1 atmosphere treated plants produced some fruit, but not the quantity of fruit that was produced by normal plants. Cuttings from the other two M1 plants, both from the 5 day treatment, were highly sterile. One of these designated as Atm-4, failed to produce fruit from either of two cuttings. The remaining plant (Atm-7) produced a single fruit on one cutting. The other cutting failed to bear.

Segregating populations observed

In this experiment three segregating populations were observed. All were from plants surviving the atmospheric treatment. Two of these populations were from the seven M1 plants that survived the 5 day treatment period. One of the populations (Atm-1) appeared as segregates of plants with sparse foliage and a gray green color as compared to the bushy, green foliage characteristic of the Rutgers variety. Five of the fifteen plants observed had these characteristics. A second segregating population (Atm-4) consisted of two dwarf compact plants that were sterile and two normal plants. The parent of this progeny approached complete sterility. It was necessary to hand pollinate it to obtain fruit. No fruits were set in the field. The third mutant population had segregates of weak plants with sparse foliage and an erect growth habit.
Three of the fifteen plants in the progeny of Atm-19 had this appearance. Of the fifteen progeny plants from the plant, Asp-21, which was morphologically different from the other M1 plants, two plants resembled the parent. The remaining thirteen plants were normal. Since the parent plant produced little pollen, most of the progeny may have resulted from cross pollination.

Discussion

The results of these studies with tomato plants indicate that the atmospheric method of treatment with ethylenimine may be of value in inducing changes in vegetative plant parts. Before the method can be utilized efficiently many aspects of treatment application must be standardized. Since the material in this study was treated in the greenhouse, there was no control of temperature. Differences in temperature at time of treatment could cause inconsistent results. The activity of the chemical would be related to the temperature. Secondly, the sensitivity of the plant might vary under different ranges of temperatures. Temperature extremes could effect metabolic activities changing the resistance of the plant to the chemical.

Another aspect to be considered is the amount of chemical required for treatment. Even with an equal atmospheric volume and standardized environmental conditions the amount of ethylenimine required to produce similar effects would vary. Un-
related plants differ in their sensitivity, and plants of the same clone would also have varying degrees of sensitivity, depending on the stage of growth or development. The amount of plant material exposed in the closed atmosphere might also be an important factor. The plant may absorb practically all of the chemical from the atmosphere. Therefore, a larger volume of plant material would require more chemical to produce an equal concentration within the plant tissue.

The results from this experiment indicate a close relationship between induced genetic changes and lethality. The aspiration treatment technique was not effective in inducing genetic changes. Associated with this treatment was a high percentage of survival. The atmospheric method which had a low frequency of survival was effective in inducing changes as exhibited by leaf segmental chimeras and three segregating M2 populations. The five day treatment which was highly lethal was much more effective than the four day treatment which resulted in 90 per cent survival of treated plants.

Although the results indicate no effect from the aspiration treatment the method should not be discarded without further tests. The cytological studies of effects on root tip chromosomes of Allium, Pisum, and Vicia indicate that the concentration used in the study was not sufficient. Since ethylenimine is highly volatile it is possible that the chemical is lost from the plant before it has time to induce the desired effects. Therefore, a soak period in the solution following
the aspiration treatment may be necessary.

The method should be an effective means of application considering the favorable results obtained in barley with solution soak treatments. The aspiration treatment is used only to assure the contact of the solution with the meristemetic regions and therefore is, in reality, a modified soak technique.

In order to evaluate the conclusion that the concentration of the chemical mutagen used in the initial study was too low, tomato seedlings were treated by the aspiration method with .02 per cent ethylenimine, followed by soak periods of 3½ and 4½ hours. The treatment caused considerable lethality (exceeding 50 per cent). Following treatment many plants had chlorophyll leaf chimeral segments.

Another factor requiring consideration in treatments involving chemical mutagens is the type and rate of reactions of the chemical with water. If the chemical undergoes rapid hydrolysis, the solution should be prepared immediately prior to treatment or possibly should be renewed during treatment to insure uniform concentrations of the chemical. Froese-Gertzen et al. (1963) studied the hydrolysis rates of ethyl methanesulphonate at various temperatures. They found that the concentration of ethyl methanesulphonate decreased by 50 per cent in 93.2 hours at 20°C and in 25.9 hours at 30°C. The rate of hydrolysis of diethyl sulphate is several times faster than ethyl methanesulphonate (Heiner et al., 1960). The hydrolysis
products also influence the types of effects produced by the treatments. The alcohol produced in the hydrolysis of ethyl methanesulphonate might act as a surface active agent, aiding penetration and the ethyl sulfuric acid may affect the permeability of the cell membrane (Nilan and Konzak, 1961). The extremes in pH produced by these hydrolysis reactions may also influence the results. Thus the effects of buffering should be considered.

From the preceding discussion it is apparent that there is much to be learned about the activity of chemical mutagens. Knowledge of these factors as well as the chemistry involved in the actual mutation process is necessary before these methods can be employed to their greatest efficiency.
Plants of *Vicia faba*, *Allium cepa* and *Pisum sativum*, were used to study the effects of various concentrations of ethylenimine and ethyl methanesulphonate on root tip chromosomes. By using representatives of three genera, a comparison of the differences and similarities in action and doses required to induce changes could be made. The material was treated by soaking root tips in various concentrations of hydrolyzed ethylenimine and ethyl methanesulphonate solution. The root tips were then fixed in acetic-alcohol fixitive and prepared as feulgen squashes. *Vicia* and *Pisum* were grown as seedlings and *Allium* roots were produced from small bulbs. All plant materials were grown in a perlite medium in an intermittent mist propagation bed. After the roots were \( \frac{1}{2} \) to 1" long, the material was transferred to a dilute nutrient solution to standardize the rate of division. This was performed 24 hours prior to treatment. The seedlings were supported on a \( \frac{1}{4} \)" mesh screen above the solution in which the roots were immersed. Aeration was supplied by bubbling air through capillary tubing connected to a pressure tank. This arrangement was set up in a laboratory having a constant temperature of 70°F (Figure 5). All treatments were conducted under these conditions. Since ethylenimine is extremely
volatile, treatment consisted of soaking the root tips in solutions in airtight containers so the concentration of the solution would not decrease during the treatment period. This precaution was not necessary with ethyl methanesulphonate. Root tips that were not fixed directly after the treatment period were placed back into the nutrient solution. This allowed the chemical to diffuse out of the cells.

In the study, four concentrations of ethylenimine solution, .01, .02, .04, and .10 per cent were used. Ethyl methanesulphonate was applied at concentrations of .2, .4, .5 and 1 per cent. Different lengths of treatment application were also studied at the four concentrations. Various combinations of treatment time plus a lapse period after the treatment prior to fixation were also studied. A number of these treatment period-concentration variables were repeated to assure accurate results. Tables 1, 2, 3 and 4 summarize combinations of concentrations and treatment periods observed.

Results of Ethylenimine Treatments

**Allium cepa observations**

In the root tips treated at .02 per cent ethylenimine, some evidence of chromosome stickiness and breakage was evident after two hours of treatment. Division of most cells was normal. After three hours of treatment, no early anaphase nuclei were observed and stickiness was more apparent. Stickiness was exhibited by sticky bridges in late anaphase cells
Table 1. Ethylenimine treatments observed in *Allium cepa* root tips

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Table 2. Ethylenimine treatments in *Vicia faba*

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Table 3. Ethylenimine treatments observed in *Pisum sativum*

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Table 4. Ethyl methanesulphonate treatments observed

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and by slight clumping of chromosomes in late prophase. Cells observed after 5\textsuperscript{1/2} hours of treatment did not appear noticeably different from the material treated for 3 hours, except that there were now only telophase cells that were in late stages of division. Some of these telophase cells were bridged. Observations of material treated for 5\textsuperscript{1/2} hours and fixed 5\textsuperscript{4/4} and 18 hours after the end of the treatment showed normal cell division taking place. After the 18 hour lapse period, the rate of division was of low frequency. Some of the cells had deteriorated.

Root tips treated with .04 per cent ethylenimine for two hours showed evidence of considerable breakage and stickiness. Some bridges were observed and the frequency of anaphase cells was low. After 5\textsuperscript{1/2} hours of treatment at .04 per cent ethylenimine, the rate of division was less than normal and considerable stickiness was apparent. There were no anaphase cells and many cells had died. Root tips treated for 5\textsuperscript{1/2} hours had a very low rate of division after a 5\textsuperscript{4/4} hour lapse. Deteriorated cells were common (Figure 15). The few cells that exhibited division eighteen hours after the end of treatment had chromosomes which were tightly clumped. After a 66 hour lapse period, a few of the roots treated with .04 per cent ethylenimine for 5\textsuperscript{1/2} hours had recovered. Deteriorated cells were present but not common. A few micronuclei were observed.

Root tips treated with .1 per cent ethylenimine showed
much the same effects as those treated at .04 per cent after 2 and 3½ hours of treatment. These effects were more pronounced than they were at the lower concentrations. After 5½ hours of treatment the rate of division was low with only a few cells with tightly clumped chromosomes. Many interphase nuclei had micronuclei attached by chromatin material (Figure 14). In addition, many of the cells had a large amount of chromatin material distributed throughout the cell, while the nucleus was in the interphase condition. This chromatin material appeared much the same as chromatin in an early prophase configuration (Figure 14). Material treated for 5½ hours and given a lapse period before fixation had more deteriorated cells with a longer lapse period. The root tips studied after a 6½ hour lapse period had more cells with dispersed chromatin material than did the root tips with no lapse period after the 5½ hour soak.

The same treatments repeated at later dates gave similar results. Root tips collected at the same time also exhibited similar behavior, although there was a slight difference in the rate in which effects were demonstrated in material treated for the shorter periods of time.

**Vicia faba** observations

All of the material treated at .01 per cent ethylenimine had mostly normal divisions with a few rare cases of micro-
nuclei and two observations of bridged anaphase cells. The root tips treated for 3 hours at .02 per cent had a few bridged telophase cells after a 5 hour lapse. Stickiness was also demonstrated by clumping of the chromosomes. No anaphase cells were present. After an 18 hour lapse, cell division was normal although a few chromosome bridges were observed.

Root tips treated at .04 per cent ethylenimine for a 2\(\frac{1}{2}\) hour period had a low frequency of anaphase cells. Some of these had bridges present. Stickiness was quite evident. Material treated for 4\(\frac{1}{2}\) hours had some anaphase cells present after a 6\(\frac{1}{2}\) hour lapse. Micronuclei were present but less common than material collected at the end of the 4\(\frac{1}{2}\) hour treatment. After a 17\(\frac{1}{2}\) hour lapse the rate of cell division was extremely low and micronuclei were more common than they were 6\(\frac{1}{2}\) hours after treatment.

Material treated at a concentration of .1 per cent ethylenimine had a few micronuclei after 2\(\frac{1}{2}\) hours of treatment. No anaphase cells were present in this material and the chromosomes were tightly clumped. Root tips treated for 4\(\frac{1}{2}\) hours at .1 per cent ethylenimine had cells with many micronuclei 6\(\frac{1}{2}\) hours after the end of the treatment period. Chromosome fragments were abundant and extreme stickiness was evident. After a 17\(\frac{1}{2}\) hour lapse there was no cell division as the roots had apparently died.
Pisum sativum observations

All root tips treated with .01 per cent ethylenimine exhibited only normal cell division, regardless of the length of treatment. There was no reduction in rate of division in any of the treatments at this concentration.

The root tips treated with .02 per cent ethylenimine had only normal cell division after \(\frac{1}{2}\) hour and 1 hour of treatment. Root tips soaked for 2 hours in .02 per cent ethylenimine had cells with chromosome fragments in late prophase. Bridges were common and metaphase chromosomes showed signs of stickiness. No early anaphase cells were present. Cells from root tips treated for \(3\frac{1}{2}\) hours showed similar effects. In addition, a few tetraploid cells were observed. A low frequency of normal metaphase cells was noted. After \(5\frac{1}{2}\) hours of treatment at .02 per cent ethylenimine, there were no normal metaphase cells. Many chromatid arms showed signs of being repelled by their sister chromatids. Although there were no normal anaphase configurations, there were some cells exhibiting a type of separation similar to that illustrated by Figure 11. Root tips collected \(8\frac{1}{2}\) hours after the end of a 2 hour treatment at .02 per cent ethylenimine had normal divisions. The number of dividing cells was less frequent than in untreated material. Cells from root tips treated for 4 hours at .02 per cent ethylenimine and observed after a \(6\frac{1}{2}\) hour lapse had many examples of chromatid repulsion. Some
clumped chromosomes were reverting to interphase. No orderly anaphase cells were present. Chromosome fragments were observed in cells in late prophase. Cells of root tips treated at a .02 per cent concentration of ethylenimine for 12 hours had many small micronuclei. Chromatids were repelling each other. There were no cells in the normal anaphase condition.

Root tips treated at .04 per cent ethylenimine exhibited effects similar to those of material treated at .02 per cent for a comparable length of time. The effects, however, were more pronounced. Material treated for 4 and 5½ hours had low rates of division following lapse periods of 5 hours or longer. Micronuclei were present after 5½ hours of treatment.

Material treated with .1 per cent ethylenimine showed evidence of effectiveness in shorter periods of treatment than did the treatments with lower concentrations. Cell division was normal after ½ hour of treatment. A few bridges and chromosome fragments were observed after treatment for 1 hour. After 1½ hours of treatment no anaphase cells or normal metaphase cells were present. After 3 hours of treatment at .1 per cent ethylenimine, the rate of cell division had decreased. Root tips treated for 4½ hours had many deteriorated cells and the chromosomes in the few cells that were in division were tightly clumped. The cells had many micronuclei. Repeated treatments using the same treatment combinations showed similar results.
Discussion of Ethylenimine Treatments

The range of concentrations of ethylenimine used in this study includes the effective range for inducing chromosomal aberrations. A treatment concentration of .01 per cent ethylenimine did not show any evidence of effectiveness in the treatment periods tested using Pisum, but did show a few isolated instances of induced aberrations with Vicia. A concentration of .02 per cent ethylenimine had a considerable effect on all three plant materials used in this study. Aberrations were first observed approximately 2 hours after initiating treatment. These early symptoms included bridges and a lack of early anaphase configurations. Pisum root tip cells had chromosomal fragments apparent in late prophase cells. Concentrations of .04 and .1 per cent ethylenimine produced similar effects, with the exception that the material treated with higher concentrations exhibited abnormalities in shorter treatment periods.

The three plant materials used, Pisum, Vicia, and Allium, responded similarly in many ways to the ethylenimine treatments but showed some differences in behavior. Pisum root tip cells exhibited a considerable amount of chromosome fragmentation in late prophase within two hours after initiating treatment at concentrations of .02, .04, and .1 per cent (Figure 10). The Allium and Vicia root tip cells did not have any observable fragments under comparable treatments. However, this does not
exclude the possibility that chromosome fragments existed under these conditions. Since *Vicia* and *Allium* have much larger chromosomes than does *Pisum*, the amount of chromatin material in the cells makes detection of fragments difficult in these plant materials. The ethylenimine treatments caused clumping of chromosomes at the metaphase stage and prohibited anaphase configurations. Because of this, chromosome fragments could not be detected readily in these stages. Considerable fragmentation did occur under the treatments studied. This was demonstrated by the large number of micronuclei present after treatment. Cells of all plant materials had micronuclei present after 4½ or 5½ hour treatments at .1 per cent ethylenimine.

The presence of micronuclei indicates that breakage caused by ethylenimine can occur late in the division cycle. *Vicia* cells had a few micronuclei present after 2½ hours of treatment with .1 per cent ethylenimine. Lower concentrations evidently do not cause breakage of chromosomes in the later stages, since longer periods of time were required before the appearance of micronuclei under the lower concentrations of ethylenimine.

The three plant materials used in this study had about the same degree of sensitivity to ethylenimine treatments. All three exhibited a lower frequency of division than normal after approximately 5 hours of treatment at .04 per cent and
an extremely low rate after the same length of treatment at .1 per cent. There was no recovery of the material after treatments at the latter concentration and considerable damage to the tissue was caused by the .04 per cent treatments. Treatments with .02 per cent ethylenimine did not show any reduction in rate of division under the conditions of this experiment.

The type of micronuclei observed in Allium tissue was unusual. The micronuclei were often attached as knobs on the sides of the macronuclei (Figure 14). Many cells also exhibited small ribbons of chromatin material dispersed throughout the cell. This material was of the configuration of chromatin material in early prophase. These same cells had intact nuclei in the interphase stage.

Observation of a few tetraploid cells indicates some impairment of spindle formation. Lack of anaphase configurations and proper alignment at metaphase might have been caused by lack of spindle formation or by the tendency of the chromosomes to stick together.

Results of Ethyl Methanesulphonate Treatments

Pisum sativum observations

Pisum root tips treated in a .2 per cent solution of ethyl methanesulphonate first exhibited effects of the treatment by the formation of many small micronuclei after 3 hours
of treatment. The division process remained normal after 21 hours of treatment at this concentration. There was very little evidence of stickiness or of rejoining of chromosomes, although there was considerable breakage of chromosomes, as exhibited by fragmentation and the formation of micronuclei. Treatment with concentrations of .4 per cent and 1 per cent produced similar results. Micronuclei were observed in some root tips treated at the 1 per cent concentration after 1 hour of treatment and were very thick (several per cell) after 1½ hours of treatment. Normal cell division continued after 8 hours at the 1 per cent ethyl methanesulphonate treatment and after 24 hours at a .4 per cent concentration.

Vicia faba observations

Vicia root tip cells were studied under the microscope after various lengths of treatment in a .5 per cent solution of ethyl methanesulphonate. After 2 hours of treatment a few micronuclei were observed. Cell division was normal after 3½ hours of treatment. Some micronuclei were observed but there was no evidence of stickiness or rejoining of chromosomes. After 19 hours of treatment, micronuclei were abundant. Cell division followed normal patterns, but there was considerable evidence of stickiness and rejoining of chromosome breaks (Figures 18, 19, 20, and 21). This treatment proved to be lethal as none of the treated seedlings recovered.
The .5 per cent ethyl methanesulphonate treatment was repeated at a later date. After a 16 hour treatment period, there were some fragments and micronuclei present but there was little evidence of the rejoining of broken chromosomes.

Discussion of Ethyl Methanesulphonate Treatments

Solutions prepared by hydrolyzing ethyl methanesulphonate in water caused considerable breakage of chromosomes without evidence of stickiness or rejoining that often accompanies breakage by mutagenic chemicals. Only when the treatment exceeded a lethal dose, was there any evidence of chromosome rejoining. Ethyl methanesulphonate treatments did not act as mitotic poisons as did ethylenimine treatments.

The presence of micronuclei in cells that had not passed through a telophase stage is difficult to explain. Micronuclei were present in cells in early division stages and in nearly all metaphase cells within 2 hours after beginning of treatment (Figure 16). Due to the short time interval after beginning treatment, these micronuclei must have originated from chromosome breaks during the resting stage and by some means, the broken fragments were passed through the nuclear envelope into the cell.

Summary of Cytological Studies

Root tip cells of *Vicia*, *Pisum*, and *Allium* were studied after treatment in solutions of ethylenimine and ethyl methane-
sulphonate for various treatment periods. Ethylenimine was applied at concentrations of .01, .02, .04 and .10 per cent. Ethyl methanesulphonate was studied at concentrations of .2, .4, .5 and 1.0 per cent. The following results were observed:

1. Ethylenimine had very little effect at a concentration of .01 per cent.
2. Higher concentrations of ethylenimine caused cell division to stall at metaphase after treatments of 2 hours duration. Chromosome bridges were present after short periods of treatment.
3. A high degree of stickiness was observed. This was indicated by clumped chromosomes and sticky chromosome bridges.
4. Ethylenimine had some effect on spindle formation. A few tetraploid cells were observed.
5. Breakage can occur in late stages of division under treatments of high concentrations of ethylenimine. Micronuclei were observed after 2½ hours of treatment at .1 per cent ethylenimine.
6. Ethyl methanesulphonate caused considerable chromosome breakage at the concentrations studied.
7. Ethyl methanesulphonate did not interrupt the division cycle as did ethylenimine.
8. Very little stickiness or rejoining of chromosomes occurred after non-lethal ethyl methanesulphonate treatments.
9. Ethyl methanesulphonate treatments resulted in an abundance of micronuclei in cells which had not completed a division cycle.
Figure 5. Apparatus used to standardize growth rate of root tips prior to treatment with ethylenimine and ethyl methanesulphonate.

Figure 6. Pisum cells treated with .02 per cent ethylenimine for 2 hours. A sticky chromosome bridge is illustrated in the center of the photomicrograph.

Figure 7. Allium cells treated with .04 per cent ethylenimine for 2 hours. Cell at the right has a chromatid bridge, cell on the left has a chromosome bridge.
Figure 8. *Allium* cells treated with .04 per cent ethylenimine for 3 hours. Cell in the center has both chromosome and chromatid bridges present.

Figure 9. *Pisum* cells treated with .02 per cent ethylenimine for 4 hours. Note doubled chromosomes exhibiting extreme stickiness 6 1/2 hours after termination of treatment.

Figure 10. *Pisum* cells 2 hours after termination of 1 hour soak in .1 per cent ethylenimine solution. Cell in lower center has 14 chromosomes and 3 fragments clearly visible.
Figure 11. **Vicia** cells 5 hours after termination of 3 hour treatment with .02 per cent ethylenimine. Cells in center illustrate type of separation that occasionally occurs after a lapse period when treated with ethylenimine.

Figure 12. **Allium** cells treated with .04 per cent ethylenimine for 5 1/2 hours. Extensive stickiness and joining of chromosomes is evident.

Figure 13. **Vicia** cells 5 hours after termination of a 3 hour treatment with .02 per cent ethylenimine. Two acrocentric chromosomes at lower left are joined by a narrow thread of chromatin material.
Figure 14. *Allium* cells treated for 5 1/2 hours with .1 per cent ethylenimine. Interphase nuclei have attached micronuclei. Chromatin material in early prophase type of configuration is dispersed throughout the cells.

Figure 15. *Allium* cells 5 1/4" hours after termination of a 5 1/2 hour treatment with .04 per cent ethylenimine. Cells exhibit aggregation of feulgen positive material indicating deterioration of cells.
Figure 16. *Pisum* cells after 4 3/4 hours of treatment with .4 per cent ethyl methanesulphonate. Very small micronuclei are abundant and are dispersed throughout the entire cell.

Figure 17. *Pisum* cells after 4 3/4 hours of treatment with .4 per cent ethyl methanesulphonate. Center anaphase cell has a stranded chromosome.
Figure 18. *Vicia* cell after 19 hours of treatment with .5 per cent ethyl methanesulphonate. A ring chromosome is clearly visible.

Figure 19. *Vicia* cell after 19 hours of treatment with .5 per cent ethyl methanesulphonate. A small ring chromosome is stranded.

Figure 20. *Vicia* cell after 19 hours of treatment with .5 per cent ethyl methanesulphonate. Two chromosomes have bridged during the anaphase stage of division.
Figure 21. *Vicia* cell after 19 hours of treatment with .5 per cent ethyl methanesulphonate. Doubled chromosomes, with much evidence of stickiness and re-joining of chromosome arms are illustrated.

Figure 22. *Vicia* cell after 19 hours of treatment with .5 per cent ethyl methanesulphonate. Note doubled chromosome stranded at anaphase.
TREATMENT OF VEGETATIVE PARTS OF WOODY ORNAMENTAL PLANTS

Methods and Materials

Several different woody ornamental plant materials were treated by the aspiration method with ethylenimine and ethyl methanesulphonate. A range of concentrations and various periods of solution soak following aspiration were studied (Table 5). Both chemicals were prepared as stock solutions and stored prior to treatment in order to permit hydrolysis of the chemicals prior to application. Vegetative material was treated as seedlings, rooted cuttings and buds.

Results

Chlorophyll was leached from the leaves of growing materials as a result of treatment with ethyl methanesulphonate. The amount of leaching increased with an increase in concentrations. Higher concentrations also resulted in lower survival rates following equal soak periods. The mature leaves died as a result of treatment but some shoots developed from lateral buds. The ethylenimine treatments also resulted in the death of leaves but did not extract the chlorophyll. The survival of the treated plant material is recorded in Table 5. One plant of *Calycanthus floridus* treated by aspiration in a solution containing .02 per cent ethylenimine followed by a 2½ hour soak produced yellow foliage. Other than this one
plant, there has been little indication of effectiveness. Only a few instances of chlorophyll leaf segmental chimeras have been observed.

Discussion

Although the results of these studies are inconclusive there has not been sufficient time to evaluate the overall effects of these treatments. The yellow foliage characteristic induced in one plant of Calycanthus suggests that these treatments may later prove successful, but this might be only one isolated case. The survival percentages indicate what treatment combinations might be successful for inducing mutations in vegetative parts of woody ornamentals. In the studies on treatment methods with tomato plants, a high degree of lethality appears to be strongly associated with a higher rate of mutation. Based on this assumption and considering the results derived from all studies conducted in this investigation, an aspiration treatment with .5 per cent ethyl methane-sulphonate followed by a 1 or 2 hour soak period or a 1.0 per cent concentration followed by a 1 hour soak would be those most likely to succeed. An aspiration treatment with ethyleni-mine would most likely be effective at a concentration of .04 per cent followed by a 2 or 3 hour soak period.
Table 5. Summary of survival and mutagenic treatments applied to vegetative parts of woody ornamentals

<table>
<thead>
<tr>
<th>Plant</th>
<th>Part</th>
<th>Aspiration treatment</th>
<th>Length of No.</th>
<th>No. of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euonymus radicans</td>
<td>pyramidalis buds</td>
<td>.02 E.I. 4 hr. soak</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; .5% E.M.S. 1 hr. rinsed</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; .5% E.M.S. 1 hr. rinsed</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 1% E.M.S. 1 hr. rinsed</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 1% E.M.S. 1 hr. rinsed</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H2O 1 hr. rinsed</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Euonymus radicans</td>
<td>Cuttings .1% E.I. 0 hr.</td>
<td>15</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Physocarpos opulifolius</td>
<td>Cuttings .1% E.I. 0 hr.</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Kolkwitzia amabilis</td>
<td>Seedlings .5% E.M.S. 3 hr.</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; .2% E.M.S. 3 hr.</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; .02% E.I. 2 1/2 hr.</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Calycanthus floridus</td>
<td>Seedlings .02% E.I. 2 1/2 hr.</td>
<td>19</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Kolkwitzia amabilis</td>
<td>Seedlings .2% E.M.S. 2 hr.</td>
<td>30</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; .03% E.I. 2 1/2 hr.</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 1% E.M.S. 1 hr.</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 1% E.M.S. 1 hr. rinsed</td>
<td>25</td>
<td>16</td>
</tr>
</tbody>
</table>

*Rinsed in water following treatment.*
SUMMARY AND CONCLUSIONS

Successful use of chemical mutagens for the production of mutations in grain crops has involved the treatment of germinating seeds. Since most ornamental plants are heterozygous clones, vegetative parts must be employed as the treatment unit in order to retain certain desirable traits that would be lost by seed propagation. Different treatment techniques, therefore are necessary for mutation breeding of ornamental plants.

This study was designed to evaluate two methods of inducing mutations in vegetative plant parts by use of chemical mutagens. The treatment techniques evaluated included an aspiration treatment and an atmospheric vapor treatment. Tomato plants were used as the vegetative material for this study since a short life cycle is involved and results could be obtained in a much shorter time interval than possible if woody plants were utilized. The aspiration treatment involved aspirating tomato seedlings submerged in a .01 per cent solution of ethylenimine. This treatment resulted in a high survival of treated plants but was not effective in producing genetic changes. The atmospheric treatment was designed to take advantage of the volatility of ethylenimine. Three ml. of 10 per cent ethylenimine solution was placed under a sealed bell jar containing seedling tomatoes. The chemical was allowed to evaporate into the atmosphere. A five day period in this
atmosphere resulted in high mortality, but was also highly effective in inducing mutations in the surviving plants.

The effects caused by different concentrations of ethylenimine and ethyl methanesulphonate on root tip chromosomes of Vicia, Allium, and Pisum were studied. The concentration used in the aspiration treatment (.01 per cent ethylenimine) had little effect on the chromosomes of root tip cells. Higher concentrations caused considerable chromosome breakage and stalled the cell division sequence at metaphase. Ethyl methanesulphonate also caused considerable chromosome breakage but did not interrupt the division cycle.

Vegetative parts of several woody ornamentals were also treated. One chlorophyll mutant has been observed in a Calycanthus floridus seedling treated by aspiration with ethylenimine. Sufficient time has not elapsed since treatment to adequately evaluate the effects of treatment on these woody ornamentals.

The following conclusions were made based on the studies described: (1) The aspiration method although ineffective in the studies with tomato plants should not be discarded. The cytological studies indicate that the concentration used was too low. Modification of the treatment to include a soak period following aspiration may be effective. Higher concentrations should also be used. (2) The atmospheric method of
inducing mutations using ethylenimine was successful. However, before this method can be utilized to its fullest potential the treatment must be standardized. (3) The efficiency of a treatment in inducing mutations is highly correlated to a low frequency of survival of the treated plants.
BIBLIOGRAPHY


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