

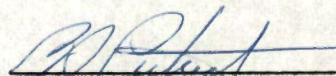
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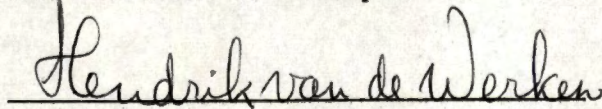
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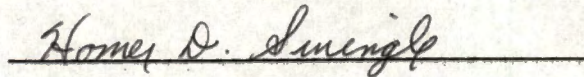
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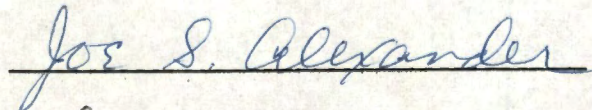
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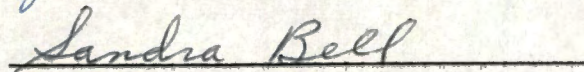

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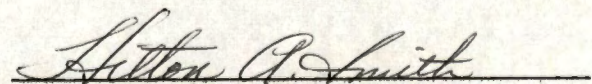

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INDUCTION OF ADVENTITIOUS BUDS ON CHRYSANTHEMUM MORIFOLIUM AND
PYRUS MALUS AND THEIR RESPONSE TO SOME MUTAGENIC CHEMICALS

A Dissertation
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Byoung Moon Choo

August, 1971

TO MY WIFE, SOONJA

who, together with (and in spite of) our two little angels J. Minyoung
and S. Haeyoung, has been continually patient and encouraging.

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ABSTRACT

The use of mutagenic chemicals applied to induced adventitious buds as means of increasing mutation rates in horticultural crops was tried. Large numbers of adventitious buds were encouraged by application of 6-furfurylamino purine plus indole-3-acetic acid on the Chrysanthemum morifolium cv. "Mandalay" and Pyrus Malus cv. "Triple Red Delicious." Some adventitious buds appeared on Pyrus Malus treated with kinetin plus IAA were apparently in response to extended life span rather than to chemical stimulation. With Chrysanthemum morifolium cv. "Mandalay," however, the treatment of the combination of these chemicals increased the number of adventitious buds formed. Adventitious buds seemed to originate in pericycle, outer phloem and cambium in the case of Pyrus Malus, and from endodermis, pericycle, or wound callus in the case of Chrysanthemum morifolium.

Application of colchicine to adventitious buds of chrysanthemum increased color mutations, and increased size mutations, whereas EMS induced lightening of colors without significant size changes. Changes in Malus were noted.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. REVIEW OF THE LITERATURE	3
Spontaneous Somatic Mutations in Horticultural Crops . . .	3
History	3
Ontogenic property of the shoot apex of angiosperms . .	5
Occurrence of spontaneous vegetative mutations	6
Application of theories in horticultural crops	8
Techniques of adventitious bud induction	10
Use of Growth Regulating Substances	11
Action of plant hormones (auxins and cytokinins)	11
Auxins	12
Cytokinins	12
Combinations of cytokinins and auxins	12
Artificial Induction of Mutation	13
History	13
Chemical mutagens	14
Ethyl methanesulfonate (EMS)	15
Colchicine	16
Chlorflurenol	18
Technique of Mutagenic Treatment	19
Anatomical considerations	19

CHAPTER

PAGE

Mutagenic agents	20
Plant parts	20
Propagation and Evaluation of Bud Sports	21
Time for treatment	22
The isolation technique	22
General screening methods	22
Microscopic detection	23
Progeny test	23
III. MATERIALS	25
Plant Materials	25
Chemical Preparations	25
Growth regulating substances	25
Chemical mutagenic agents	25
IV. METHODS AND TECHNIQUES	27
Induction of Adventitious Buds	27
<u>Chrysanthemum morifolium</u> cv. "Mandalay"	27
<u>Pyrus Malus</u> cv. "Triple Red Delicious"	28
Application of Mutagenic Agents	29
<u>Chrysanthemum morifolium</u> cv. "Mandalay"	29
<u>Pyrus Malus</u> cv. "Triple Red Delicious"	31
Microscopic Examinations	32
Analyses of Data	32
V. RESULTS	34
Induction of Adventitious Buds	34
<u>Chrysanthemum morifolium</u> cv. "Mandalay"	34

CHAPTER	PAGE
<u>Pyrus Malus</u> cv. "Triple Red Delicious"	38
Somatic Variations	47
<u>Chrysanthemum morifolium</u> cv. "Mandalay"	47
Effects of mutagenic chemicals on normal buds	47
Microscopic observation of buds recovering from mutagenic chemical application	47
Macroscopic observation	50
Microscopic examination	54
<u>Pyrus Malus</u> cv. "Triple Red Delicious"	57
Effect of mutagenic chemicals on apical meristems . .	57
VI. DISCUSSION	65
Induction of Adventitious Buds	65
<u>Chrysanthemum morifolium</u> cv. "Mandalay"	66
<u>Pyrus Malus</u> cv. "Triple Red Delicious"	73
Somatic Variations	77
<u>Chrysanthemum morifolium</u> cv. "Mandalay"	77
<u>Pyrus Malus</u> cv. "Triple Red Delicious"	80
VII. SUMMARY	82
LITERATURE CITED	85
APPENDIXES	92
Appendix I	93
Appendix II	94
VITA	95

LIST OF TABLES

TABLE	PAGE
1. Some Bud Sports in Horticultural Crops	4
2. Callus Formation on Disbudding Wounds of <u>Chrysanthemum</u> <u>morifolium</u> cv. "Mandalay"	35
3. Effects of Growth Regulating Substances on the Production of Adventitious Shoots from Callus on <u>Chrysanthemum</u> <u>morifolium</u> cv. "Mandalay"	37
4. Effects of Growth Regulating Substances on Both Number of Prosphaeroblast and Adventitious Shoot Induction on Dis- budded <u>Pyrus Malus</u> cv. "Triple Red Delicious"	40
5. Effect of Growth Regulating Substances on Prosphaeroblast and Adventitious Shoot Production in <u>Pyrus Malus</u> cv. "Triple Red Delicious"	41
6. Effects of IAA Plus Kinetin on Survival, and Prosphaeroblasts and Adventitious Shoot Production of <u>Pyrus Malus</u> cv. "Triple Red Delicious"	43
7. Effects of Growth Regulating Substances on the Formation of Prosphaeroblast on Disbudded <u>Pyrus Malus</u> cv. "Triple Red Delicious"	45
8. Effects of Growth Regulating Substances on the Formation of Prosphaeroblasts and Life Span of Disbudded <u>Pyrus Malus</u> cv. "Triple Red Delicious"	46

TABLE

PAGE

9.	Somatic Mutations Obtained in <u>Chrysanthemum morifolium</u> cv. "Mandalay" after Treatment of Adventitious Callus Buds and Normal Buds with Several Mutagenic Chemicals . . .	51
10.	Number of Somatic Mutations Obtained in <u>Chrysanthemum</u> <u>morifolium</u> cv. "Mandalay" from Normal Buds and Adventitious Buds, and Upon Treatment with Colchicine, Colchicine Plus Chlorflurenol, and EMS	52
11.	Comparison of Induced Complex Polyploid and Normal Plants of <u>Chrysanthemum morifolium</u> cv. "Mandalay"	58
12.	Leaf Thickness, Leaf Shape Index, and Stomatal Length of Leaves of Normal and Adventitious Shoots of <u>Pyrus Malus</u> cv. "Triple Red Delicious" which Developed from Buds Treated with Mutagenic Chemicals	62
13.	Leaf Thickness, Leaf Shape Index, and Stomatal Length of Two Adventitious Shoots Produced from a Single Prosphaeroblast on Disbudded <u>Pyrus Malus</u> cv. "Triple Red Delicious" . . .	93
14.	Leaf Thickness, Leaf Shape Index, and Stomatal Length of Two Adventitious Shoots Produced from Two Different Prosphaeroblast on One Disbudded <u>Pyrus Malus</u> cv. "Triple Red Delicious"	94

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LIST OF FIGURES

FIGURE	PAGE
1. Number of Callus Formations on <u>Chrysanthemum morifolium</u> cv. "Mandalay"	36
2. Multiple Adventitious Shoots Produced from a Single Wound Callus on <u>Chrysanthemum morifolium</u> cv. "Mandalay"	39
3. Recovery of Chrysanthemum Apical Meristem after Treatment with Mutagenic Chemicals (x35)	49
4. Color Deviations within Treatments in <u>Chrysanthemum morifolium</u> cv. "Mandalay." Left: Lightest Color, Center; Average Color, and Right; Darkest Color within Each Treatment. 1; Normal Bud-No Chemical, 2; Adventitious Bud-No Chemical, 3; Normal Bud-EMS Treatment, 4; Adventitious Bud-EMS, 5; Normal Bud-Colchicine, 6; Adventitious Bud-Colchicine, 7; Normal Bud-Colchicine Plus Chlorflurenol, 8; Adventitious Bud- Chlorflurenol Plus Colchicine	53
5. Distribution and Concentration of Anthocyanin in Enlarged Upper Surface Cells of Red Flowering Chrysanthemum Ligules	55
6. Upper Surface Cells of Yellow Flowering Chrysanthemum Ligules	56
7. Histological Differences in Various Organs Between Yellow (Left) and Red (Right) Flowering <u>Chrysanthemum morifolium</u> cv. "Mandalay"	59

FIGURE

PAGE

8. Histological Differences in Various Organs Between Diploid
(Left) and Polyploid (Right) Flowering Chrysanthemum
morifolium cv. "Mandalay" 60
9. Histological Differences in Various Organs Between Triploid
(Left) and Polyploid (Right) Pyrus Malus cv. "Triple Red
Delicious" 64
10. Development of Adventitious Callus Buds from Disbudded
Chrysanthemum morifolium cv. "Mandalay" (x35) 68
11. Two Different Origins of Adventitious Buds on Pyrus Malus
cv. "Triple Red Delicious" 75

CHAPTER I

INTRODUCTION

Horticulturists have used bud sports as means for expanding the diversity of cultivars for more than 2,000 years. Changes that are rather frequent include modification of color, form, and size of fruits, flowers, foliages and plant structure. The changes are abrupt and not difficult to transmit through the genetic apparatus. Equally abrupt changes, however, are brought about by the modification of chromosomes, and of genes.

At present systematic means of bringing about mutations of these kinds are not available. Attempts to provide means that will permit exploitation of the advantages offered by these types of change have included the use of high energy radiation and mutagenic chemicals which have proved capable of bringing about equally interesting changes.

The objective of this study was to suggest schemes which would increase the number and range of mutations with some possibility of control.

The economically important plants Pyrus Malus cv. "Triple Red Delicious" and Chrysanthemum morifolium cv. "Mandalay" were chosen for the investigation because bud sports of each are valuable economically and because both are genetically complex. Chrysanthemum morifolium is hexaploid and Pyrus Malus cv. "Triple Red Delicious" is triploid. Two mutagenic chemicals, ethyl methanesulfonate and colchicine, and one

growth retardant, chlorflurenol, were used. A combination of 6-furfurylamino purine and indole-3-acetic acid were used to encourage cell proliferation and adventitious bud development.



CHAPTER II

REVIEW OF THE LITERATURE

I. SPONTANEOUS SOMATIC MUTATIONS IN HORTICULTURAL CROPS

History

Many cultivars of flowers, fruits, and vegetables have arisen as somatic mutations. According to Sturtevant (76), Darwin discussed this phenomenon in a scientific manner for the first time in his book "The Variation in Animals and Plants under Domestication" of 1868. He stated that there are two more or less distinct types that came to be known as continuous and discontinuous variations, respectively. The latter, sometimes called "sports," are often transmitted unchanged through vegetative propagation.

In 1919, the Annual of the American Rose Society listed 429 varieties of American roses of which 116, or more than 27 percent, were known to have originated as bud mutations (39). Since this report, numerous vegetative variations have been recorded in a wide range of plant species. A few of these reports are listed in Table I.

Although these examples are only a minor portion of the efforts to discover sports, they give us good evidence of the occurrence of bud sports in a wide range of horticultural crops and the continuous effort to improve crops through the utilization of bud sports.

TABLE 1
SOME BUD SPORTS IN HORTICULTURAL CROPS

Plant Species	Reporter(s)	Year	Obtained Variations
Roses	Gersdorff (39)	1919	Flower color and forms
Boston Fern	Benedict (10)	1923	Plant form variation
Irish Potato	Asseyeva (5)	1927	Skin color changes
Blackberries	Darrow (18)	1928	Thornless sport
Apple	Shamel <u>et al.</u> (71)	1922*	Red Delicious from normal
Citrus	Frost <u>et al.</u> (36)	1942	Cytochimeral branch
Apple	Dermen (22)	1951	Giant fruit in McIntosh
Carnation	Mehlquist <u>et al.</u> (48)	1954	Flower color
Peach	Dermen (25)	1956	Fuzzless fruit
Grapefruit	Cameron <u>et al.</u> (15)	1964	Red-fleshed fruit
Grape	Ourecky <u>et al.</u> (56)	1967	Large-fruited Concord
Pyracantha	van de Werken (79)	1967	Thornless, sterile

*Published in 1932.



Ontogenic Property of the Shoot Apex of Angiosperms

Considering the frequency of bud sports in horticultural crops, little is understood about the nature of sports. Most sports have been observed in woody plants which generally require a rather long time from seed to maturity, or in plants which are highly heterozygous (46). Another difficulty that prohibits identification of the nature of bud sports is the chimeral structure of many horticultural plants. Such chimeras originate not by cross pollination which might produce heterozygous plants, but in somatic cells as a result of some natural process by which changed cells arise and give rise in turn to changed tissue (26).

In order to understand the occurrence of somatic variation it is necessary to know the development of higher organs.

The work of Satina et al. (66) in 1940 and Satina and Blakeslee (65) in 1941 established that the stems of perhaps all angiosperms are formed of three distinct histogenic layers of cells. Histological and cytological study of 68 periclinal chimeras of colchicine treated Datura demonstrated not only the presence of three independent germ layers in the shoot apex, but also demonstrated the origin of various organs from these layers. They believed that the first histogenic layer (L-I) formed the epidermis. The initiation and development of the leaf, sepal and petal are similar and depend primarily on the activity of the second layer (L-II). Cells beneath the third layer (L-III) formed the central core and the stamens (65).

One year earlier than Satina et al., Foster (32) also reported existence of germ layers in angiosperms and reported that in angiosperms

cell division follows a certain pattern, and that cells are arranged in layers in the shoot apex. But he was unable to demonstrate the function of individual layers.

According to Esau (29), the existence of germ layers in the shoot apex of angiosperms was recognized by Schmidt as early as 1924. According to the tunica-corpora theory of Schmidt, two tissue zones occur in the apical meristem; the tunica, consisting of one or more peripheral layers of cells, and the corpus, a mass of cells overarched by the tunica.

Later, Satina (64) revealed the origin of the ovule. According to her the ovule of angiosperms is initiated from the innermost layer, L-III. The nucellus develops from L-II and the integument arises from the outmost layer, L-I. This study was later confirmed by Blaster and Einset (12) who studied chimeral apples.

Occurrence of Spontaneous Vegetative Mutations

Vegetative plant sports are more or less permanent changes or variations from normal (26). They are usually brought about by changes in one or more genes or by chromosomal fragmentation and duplication occurring in the nucleus of a single cell of the apical meristem of a growing plant.

As pointed out earlier, the complex structure in the three germ layers of the shoot apex of a plant gives complex opportunity for many changes in plant organs. Normally the buds and lateral shoots of plants arise exogenously, therefore, if the interior of a plant differs

genetically from the exterior, any buds or shoots of endogenous origin will give rise to individuals which differ from the original type. In other instances, replacement of one of the three germ layers by another which is genetically distinct from the original layer may account for a bud sport (18). Dermen (26) reported another type of bud variation which occurred by cytoplasmic mutation.

A brief discussion of the chimeric nature of angiosperms is necessary to understand the previous statements. In addition to the usual physical classification of chimeras, sectorial, mericlinal, and periclinal chimeras, Tilney-Basset (78) classified chimeras into four genetically distinct classes. These are:

1. Species chimera (Graft chimera); e.g., Winkler's (1907) original graft chimera obtained between two species of Solanum.
2. Chromosomal chimera; chimeras in which the layers differ in their chromosomal number.
3. Gene-differential chimera; chimeras in which one or more genes in a layer have been changed.
4. Plastid-differential chimeras; chimeras which are of cytoplasmic origin.

The last three chimeras, chromosomal, gene-differential, and plastid-differential, are of special importance for vegetatively propagated horticultural crops because these are the main causes of spontaneous and induced vegetative variations.

Application of Theories in Horticultural Crops

In 1928, several years before the birth of the theory of histogenic layers of the shoot apex by Satina et al. (66), Darrow (18) reported the appearance of a thorny shoot on a branch of a thornless blackberry sport known as Cory Thornless blackberry. Cytological evidence showed that the thorns are developed from the epidermal tissue (L-I), and mutation in L-I had suppressed thorn development in the sport. This phenomenon was later explained by the assumption that the replacement of L-I by underlying cells (L-II or L-III) may have taken place to account for the appearance of thornless branches on thorny plants (26). In 1939, Foster (32) reported the significance of germ layers in the shoot apex of seed plants. He stated that all variegation resulted from mutation in L-I. This study was later resumed by Dermen (26) with leaves of Euonymus japonica and Euphorbia pulcherima.

In citrus, Frost and Krug (36) studied two cytochimeras, which they concluded were 2-4-4 and 2-4-2. The chromosome number in the epidermis of young leaves and stems was $2n$ in both plants. At least part of the cortex of these stems was $4n$. Chromosomes in the stem procambium and pith were $4n$ in the 2-4-4 plant and $2n$ in the 2-4-2 plant. Although the fruit was not studied in this case, they reported that microspores were $4n$ in 2-4-4 and 2-4-2 plants. Root tips from stem cuttings were $4n$ in the 2-4-4, and $2n$ in the 2-4-2 plant,

It was Dermen (20, 21, 22, 23, and 24) who really appreciated and applied this theory in the field of horticulture. In a study on leaves and stems of 4-2-4 and 2-4-2 chimeral peaches, Dermen (26) showed that

the epidermal tissue of both organs originated from L-I; L-II gave rise to part of the cortical tissue and sometimes even extended beyond the cortical tissue deep into the vascular tissue system of the stem, while the entire internal tissue of the leaf blade originated from L-II; L-III usually gave rise to the inner part of the cortical tissue, conductive tissue and pith of the stem and the bulk of the midrib tissue in the leaves. A similar situation with apple stems was studied under the microscope.

According to Dermen (26), the variegation in leaves of citrus and poinsettia is due to a naturally occurring genetic color mutation in L-II at the apex of a shoot or bud and this mutated L-II suppressed chlorophyll development of plastids in cells of tissues originating from L-II. In case the central area in the variegated leaves is green, L-III in the variegated plant is normal. This phenomenon was confirmed with variegated privet which was due to a genetic change in L-II. But the variegated leaves of Euonymus japonica cv. "Mediopictus" were different from those of poinsettia and privet (26). In the case of Euonymus, mutation of the colorless form in this plant seems to be in L-III. He reported that there is perhaps at least one layer of cells of palisade tissue derived from the genetically normal L-II located over the colorless area.

It is fairly well accepted point that most bud mutations are not desirable. One of many such examples are the navel orange orchards that were found in California. According to Shamel and Pomeroy (72), in navel orange orchards of California more than 25 percent of the trees

were found to be of undesirable strains. This was caused by unintentional propagation of inferior bud variations.

Techniques of Adventitious Bud Induction

A method that stimulates the occurrence of bud sports on horticultural crops, a technique of eye excision or disbudding, has been developed by Asseyeva (5). In 1927, she used this method on Irish potatoes and was able to unveil the genotypical constitution of the deeper lying tissue. She also found that most of the spontaneous mutants were periclinal chimeras.

In 1948, Dermen (21) reported a technique that produced several spontaneous bud sports on some apple cultivars. He used a dozen of two-year-old trees of the apple cultivar "Lady." They were trimmed back to the main stem and topped about two feet above the soil level. One of the trees did not produce axillary shoots, instead it produced an adventitious bud 68 days after it was topped. He suggested that adventitious bud was initiated in the outer phloem.

In 1951, Dermen (22) reported an important technique. In addition to topping of the trees, he disbudded the trees. He reported that one of two disbudded McIntosh apple trees produced four adventitious buds. Among these four, a cluster of shoots developed from one bud and one shoot arose from another bud 93 days after the tree was disbudded. No shoots arose from the other two. No adventitious buds were produced on the other trees.

According to Dayton (19), the formation of adventitious buds or prosphaeroblasts by disbudding is totally dependent on the cultivar that

is used in the experiment. He reported that Golden Delicious did not produce any prosphaeroblasts. The wood remained moist and green internally for over 10 weeks after disbudding, but by this time the root systems were almost dead. Prosphaeroblasts formed on all trees of the Red Delicious types, as well as on those of striped McIntosh (19). A tree might produce three or four adventitious buds simultaneously, or only one followed by one or two more one to five weeks later. The shortest time between disbudding and the emergence of a green growing point 2 mm long was 21 days with Bridgham Red Delicious while the longest time was almost 7 weeks on one tree of Redspur, according to Dayton (19).

II. USE OF GROWTH REGULATING SUBSTANCES

Action of Plant Hormones (Auxins and Cytokinins)

Generally the total development of plants is classified as two distinct phenomena; those connected with growth and those with differentiation. Growth will include the processes of cell division and enlargement which result in increase in size and green weight of the plants, while differentiation can be thought of as including all of the irreversible or semi-irreversible changes which may occur in the cell wall, the protoplasm, or the inclusions of the undifferentiated cell (42). According to Sachs (76), cell division as well as expansion in plants are affected by the presence of various types of substances, including the auxins, gibberellins, kinins, and some other growth regulators.

Auxins. In 1880, Sachs launched the first theory of substances controlling plant growth (45). In 1933 K6gl and co-workers in the Netherlands found two materials, strongly active in Avena growth tests, which they named auxin a and auxin b. Auxin a was first isolated from human urine, and auxin b from corn germ oil (45). Further research by K6gl's group identified another auxin compound which is identical with indole-3-acetic acid (IAA) (45).

It was quickly found by Went (81) that different concentrations of auxin may have opposite effects on the growth of plants and this hormone could be isolated by allowing it to diffuse from the tip of Avena into an agar block. He also found that low concentration of auxin promote plant growth, higher concentrations inhibit it. This inhibitory concept soon led to the discovery by Thimann and Skoog (77) and later by Snow (74) that auxins which are formed at a plant apex inhibit the growth of lateral buds. Later, two very important functions of auxin, the formation of adventitious roots and the abscission of plant organs at low concentration of auxins were discovered by several workers (51).

Cytokinins. Function and activity of cytokinins are numerous. These are stimulation of cell mitosis (73), cell enlargement (80), morphogenesis (57), delay of senescence (61), overcoming dormancy (77), increased pigmentation (44), and possibly many more. Morphogenesis has a significant meaning in this study.

Combinations of cytokinins and auxins. Effects of auxins and cytokinins, independent of each other, on the differentiation of plants

have been reported by several workers. Jacobs (42) reported that auxin induced the differentiation of xylem in Coleus tissue below the buds. Application of benzyladenine to moss protonemata of the proper physiological age causes bud formation on specific cells (13). Cytokinin is not acting as a trigger but has to be present during a critical period of time, until differentiation is stabilized. Application of cytokinins to root segments of the fern Ophioglossum petiolatum at relatively high concentration induced considerable proliferation of root tissue and a concomitant organization of numerous apical meristems in this tissue (57).

According to Wickson and Thimann (82), in general the higher the auxin concentration the higher the kinetin concentration needed in vitro condition. Apparently the quantitative interaction between kinetin and IAA is less critical in vivo than in vitro, although the correct combination of the two chemicals is necessary in both cases.

III. ARTIFICIAL INDUCTION OF MUTATION

History

There was growing interest in discontinuous variations in the 1890's. In 1901 there appeared the first volume of de Vries' monumental "Die Mutationstheorie." de Vries wrote (cited by Gaul (37)):

"Knowledge of the laws of mutation will probably lead to the artificial production of mutations at will, and thus the creation of completely new properties of plants and animals. And if one can form improved, higher yielding and more beautiful cultivated strains by the selection method, then perhaps some day one will be able to produce permanently better species of cultivated plants and animals by mastery of mutation."

Artificial induction of mutations was not reported until 1926 when Muller reported what he called "artificial transmutation of the gene" (50). The major feature of proof was an increase in mutation rate by some 1,500 times in the progeny of Drosophilla melanogesta males exposed to a heavy dose of x-rays. Stadler (75) had begun his studies with barley at about the same time that Muller's work with x-ray began and he produced 14 chlorophyll mutants from 1,243 x-ray treated barley seedlings.

In 1936, Shamel and Pomeroy (72) predicted that with the increasing use of x-rays and other methods of stimulating somatic variations, it would be possible that the development of bud sports may eventually become as important a method in plant improvement as cross-fertilization is with plants which are commonly reproduced sexually.

Chemical Mutagens

In 1941 Auerbach and Robson obtained clear evidence that nitrogen mustard is a mutagenic agent (7). If induced polyploidy is regarded as a mutation, Blakeslee et al. (12) reported the use of colchicine earlier.

Mutagens may affect plants or plant parts by inducing: (1) changes in genes, (2) structural changes in chromosomes, and (3) physiological disturbances. These effects are not mutually exclusive, since breaks in chromosomes, involving structural changes, may cause gene mutation, and very small structural changes, such as inversions or deletions, may be interpreted as gene changes. Physiological disturbances include those effects on plants that result from treatment with mutagens but which disappear as the plant develops or do not carry over into the next generation or the next vegetative propagates (14).

The first mutagenic chemical agent, mustard urethane, was found in Germany in the early years of World War II. In recent years, many chemicals such as alkylating agents, urethane, some alkaloids and peroxides, formaldehyde, and substances related to nucleic acid and nitrous acid have been found to have mutagenic properties (6). Among these groups, alkylating agents are the most potent mutagenic agents for the higher plant species. This group includes dialkyl sulfate, sulfonic esters (including ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), iso-methylsulfonate, alkylmethane sulfonate, many other epoxides), myerlon, chloromethyl ester, and mustard (7).

Ethyl Methanesulfonate (EMS)

As a sulfonic ester (alkyl alkane sulfonate) which is one of the alkylating agents, EMS has proved one of the most interesting and promising chemical mutagens. It was realized that the alkylating agents are capable of inducing visible mutations which are different from those induced by radiation, both in expression and chromosomal position (30).

It is believed that EMS can be far more effective than ionizing radiation in inducing cytoplasmic mutations (43). According to Ehrenberg et al. (27), the maximum level of chlorophyll mutations generally amounts to 10-11 percent for both neutron and x-rays while EMS gives rates up to 50-60 percent. Jacob (41) reported that EMS induced up to 16 percent chlorophyll mutants in Arabidopsis thaliana.

Somatic variations that appeared upon EMS treatment are not limited to chlorophyll mutations. Ferwerda (31) reported that some 8 clearly distinct somatic aberrations were found on 128 individual EMS

treated Irish potato tubers. Somatic aberrations such as ivy leaf and variegated leaf, and color of tuber, e.g., yellow skin from a red skinned variety were found. According to Gaul et al. (38), in EMS-induced genetic variability in barley, the macromutants exhibited such characteristics as earliness, dwarfness, dense and lax spikes, gigas growth, small leaves, large seed, waxless culm and leaves, broad leaves, rolled leaves, etc. They also mentioned that both macro- and positive micro-mutations affecting yield were higher after EMS-treatment than after x-ray irradiation. However, Goud (40) reported that although EMS treatment produced a high frequency of visible mutations (macro-mutations) in hexaploids wheat, it failed to induce usable micro-mutations.

Freese (35) reported that many of the mutations induced in phage E. coli by alkylating agents arise only after a number of duplications. This delay is thought to be owing to the occurrence of copy-error mutations several generations after DNA has been exposed. These copy-errors during duplication of DNA are also found in spontaneous mutations along with direct gene changes (35).

Sato and Gaul (67) reported another mode of action of EMS as a mutagenic chemical. They stated that it was possible that the heritable change in barley occurs in the nucleus rather than in the cytoplasm. They assumed that small "invisible" deficiencies of chromosome segments are the main cause of variation.

Colchicine

Colchicine has been used successfully to produce chromosome doubled specimen in many cultivars. In experiments performed with peach,

Dermen (23) reported that only rarely did this occur in all tissues. Instead, he usually obtained periclinal chimeras. Similar results were obtained by Pyror et al. (59) in an experiment performed with azaleas.

The efficiency of colchicine as a tetraploid inducer in plants is so great that hardly any other method has been used since this chemical was found. According to Beasley (9), one out of 600 cotton plants treated by "heat-shock" became polyploid (1:600), but colchicine produced ploidy in 50 of 100 (1:2) of the cotton plants surviving the chemical treatment. Nebel and Ruttle (53) reported that in many floricultural species colchicine was effective in inducing tetraploidy.

Arisumi (3) reported that with the exception of small numbers of complete tetraploids and mericlinal chimeras, sixteen out of twenty four cultivars of daylilies treated with colchicine produced chimeras. In azaleas, some induced polyploids were complete, but many were chimeral (59). These results reflect that shoots originating from two or more cells are likely to be chimeras (3). However, interesting results were reported by Arisumi and Frazier (4) on experiments with Saintpaulia. According to them 28 complete tetraploids and a periclinal chimera were obtained from 29 colchicine-treated leaf cuttings of Saintpaulia. The results were considered good evidence of the origin of Saintpaulia shoots from a single cell.

Colchicine doubles chromosome numbers at the metaphase stage of cell division through metaphasic arrest (11, 28). The chemical paralyzes the spindle mechanism in mitosis, which prevents the migration of the divided chromosomes to the pole at anaphase. Without chromosome migration

to the poles, cell division does not take place. As a result, the daughter cell becomes a tetraploid.

An important property of colchicine as a mutagenic chemical was revealed by Chen and Rose (16) in 1963 when three distinct grass type mutants in sorghum were induced after colchicine treatment and established to be true gene mutations. Since this report was published, numerous studies have proven the mutagenic effect of colchicine on a range of plant materials, on sorghum (34, 62) through chromosome structure substitution, on Decatur barley (69) including albino seedlings, awnlessness, erectoides, reduced height, earlier and later maturity, partial sterility, and resistance to powdery mildew, on ryegrass (1) including chlorotic plants, chimeras with floral abnormality, and two ovary abnormalities, and on tobacco when Apparao and Popinath (2) found a mutated branch which was slender and produced linear, undulated leaves.

Reduction of chromosomal level from tetraploid to diploid in sorghum was reported by Sanders and Franzke (63). According to them, following colchicine treatment, complex diploid mutants were obtained from tetraploid seedlings of four sorghum lines. Since untreated seedlings remained at the original chromosomal level, reduction of tetraploid cells must have occurred in colchicine-treated tetraploid seedlings. They considered the diploid cells must have proliferated and organized into apical meristems which gave rise to diploid plants.

Chlorflurenol

Chlorflurenol (Methyl-2-chloro-9-hydroxyfluorene-(9)-carboxylate), known as Morphactin, was first reported as a new growth regulator in 1964

(68). According to Schneider (68) the action of the chemical is systemic, which means that this compound is transported and distributed in the plant not strictly polarly (as IAA) but basi- and acro-petally (as gibberellic acid). Whether chlorflurenol is a mutagenic agent is not established yet. However, the response of many different species of ornamental shrubs to treatment with chlorflurenol at The University of Tennessee Agricultural Experiment Station, Knoxville, Tennessee, indicates that this chemical affected severe disorganization of meristems and may have contributed to the development of some somatic mutants. Schneider (68) suggested that it affects inhibition of mitotic activity in apical meristems, and of mitosis in apical cells.

IV. TECHNIQUE OF MUTAGENIC TREATMENT

Anatomical Considerations

As was mentioned earlier in the review, the formation of plant organs is dependent upon complicated developmental processes of various origins, i.e., L-I, L-II, and L-III. For this reason the appearance of mutations in the form of sectorial chimeras are naturally more difficult to detect (54). The multitude of possible phenotypical expressions is increased not only by the combination of tissues of different genotypes, but also through biochemical interaction between tissues.

Ideally, the treated tissue should consist of single cells such as pollen grains (52) or egg cells (49), or a few, undifferentiated cells such as is the case with Saintpaulia (4).

Mutagenic Agents

Several mutagenic chemicals may be advantageous for treating plants growing in the field or in the greenhouse (54). Radiation treatment may be advantageous when used on plant segments such as the dormant scions of fruit trees (71). Other advantages of chemical treatment are the simple facilities required, less time and less expense. The main problem with mutagenic chemicals seems to be that of absorption by the treated materials (54). The vital parts of the growing portions are often well protected. This problem can be overcome to some extent by using agents such as glycerin (59) and lanolin (53).

There are different optimum concentrations of mutagenic chemicals for different plant species and there is a negative relation between radiation dose and time of exposure (28, 41, and 60).

Plant Parts

Vegetatively propagated plants present problems and prospects very different from those of seed-propagated plants with regard to the artificial induction of mutations and their use for plant breeding purposes. A problem with vegetatively propagated plants is the heterozygous nature of the plants (54).

Of primary concern is the selection of suitable parts of the plant species in question. In most cases it seems preferable to use the various plant parts commonly used in commercial plant propagation. Some of these are young shoots of carnation (48), petunia (53), and azalea (59), leaf peticle of African violet (4), eyes of the potato tuber (31),

dormant buds of apple, peach and pear (58), and other organs by which the plant to be used is most easily propagated.

Treatment should be limited to the buds or parts of the plants from which the mutations are to be isolated later.

V. PROPAGATION AND EVALUATION OF BUD SPORTS

When a single cell mutation occurs in a plant it affects only those tissues derived from it. If the changes occur in a central cell of a growing point, it affects only one layer or sector of a layer and consequently it involves only one tissue or a region of that tissue. The other tissues or areas remain normal. These facts must be taken into consideration when attempts are made to maintain or propagate sports of chimeral nature (26).

Methods of propagation vary with the nature of the plants (26); these can be distinguished as follows: (a) bud grafting, (b) shoot grafting, (c) rooting of stem cuttings, (d) topworking, (e) rooting of leaves or leaf segments, and (f) growing of shoots from root cuttings. By use of a, b, c, and d which permit a bud of the sport to develop into the new plant, the chimeral form of a sport may be maintained. Under the method d, somewhat quicker results are to be obtained than through a, b, and c (72). For woody plants in general, the topworking method is often desirable and has the advantages of economy of operation and an early conclusion. Budding on seedlings is satisfactory, especially when quantitative as well as qualitative characteristics are being studied (72).

Time for Treatment

Usually, treatment at the beginning of the vegetative period is most convenient and suitable (67). Treatment just at the beginning of sprouting or bud swelling generally seems to be preferred, compared with treatment of material in deep dormancy or in a more advanced stage of growth (54). The best time might be just as the new buds begin to develop, perhaps in mid-summer.

Arisumi and Franzier (4) reported that in Saintpaulia treated with colchicine, leaf cuttings treated 6 days after cutting produced more tetraploids than those treated immediately after cutting.

The Isolation Technique

General screening methods. Guidelines for the screening of induced mutations are given by Gaul (38) by listing valuable induced characteristics for different crop plants such as: growth forms, fungus resistance, insect resistance, time of maturity, self-fertility, germination temperature, quality and yield. Shamel and Pomeroy (72) suggested that improved vegetative variations are likely to be obtained from healthy fully developed specimens under favorable environmental conditions. Coyne (17) found that unfavorable environmental conditions such as low or high temperatures or a dry environment have to be provided to discover positive variations. In these ways we are able to evaluate the adaptability of new mutants to wider environmental conditions.

According to Gaul (37), there are two distinct positive types of mutations called macro- and micro-mutations. Deviations which can be

reliably recognized in a single plant are macro-mutation. In contrast to the macro-mutations, micro-mutations cannot be reliably determined in a single plant, but only in a stand of thirty or more vegetative propagates of the treated plant (37). There are two types of micro-mutations: these are manifest micro-mutations and cryptic micro-mutations. Manifest micro-mutations are easily recognized if appropriate methods are used such as measuring and weighing. On the other hand, cryptic micro-mutations are not easily recognized. Only drastic changes in the environment make this type of mutation manifest. Often a mutation is difficult to find because the mutated sector is too small to be easily detected (54).

Microscopic detection. This method is especially important when one is trying to determine the level of ploidy. According to Pratt and Ourecky (58) the ploidy of a tissue was identifiable in growing buds by the relative sizes of cells, resting nuclei and metaphase plates and by chromosome counts in apple. A similar observation was made on grapes by Ourecky et al. (56). According to them ploidy of sports was identified by the examination of shoot tips, root tips or pollen mother cells, the rate of pollen germination, and size of stomata.

According to Semenluk and Arisumi (70), leaves and stems of the colchicine treated lateral buds of roses were different from the controls in size, texture, color, shape, and thickness due to increasing ploidy.

Progeny test. It is necessary to test the result of mutagenic treatment through several vegetative generations since the chance of having an immediately identifiable mutation is low (37). On vegetatively

propagated species the shoot may have to be pruned back to the original treated area to stimulate shoot growth from the point where mutagenic agents were applied (46). The most often induced somatic mutations are sectorial chimeras and the buds on that portion must be stimulated in order to eliminate possible intrasomic competition.

According to Bauer (8), studies on black currants showed that the first primary shoots coming from mutagenic treated buds only rarely revealed any mutational changes. By cutting back to the originally mutagen treated stock five consecutive years, he was able to isolate 324 aberrant plants from the original 343 treated shoots.

CHAPTER III

MATERIALS

Plant Materials

Two different species of plant materials were used in these experiments.

Rooted cuttings of Chrysanthemum morifolium cv. "Mandalay" were purchased from Yoder Brothers, Inc., Barberton, Ohio, and arrived on April 24, 1970.

Two to three foot tall, one-year old Pyrus Malus cv. "Triple Red Delicious" on standard rootstocks were purchased from Adams County Nursery and Fruit Farm, Aspers, Pennsylvania, and received on April 27, 1970.

Chemical Preparations

Growth regulating substances. Twenty mg. of 6-furfurylamino purine and 3 mg. of indole 3-acetic acid (IAA) were each dissolved in 100 ml of 95 percent ethyl alcohol as stock solutions. These solutions were shaken on a mechanical shaker for two hours and stored in dark bottles at 40° F.

Chemical mutagenic agents. A 2 percent stock solution of ethyl-methanesulfonate (EMS) in distilled water and a 5 percent stock solution of colchicine in distilled water were prepared and stored in the dark

at 4° C. A 20 ppm stock solution of chlorflurenol was prepared and stored in the same way.

A 10 percent glycerol solution was prepared to be used as a wetting agent with the above solutions.

CHAPTER IV

METHODS AND TECHNIQUES

I. INDUCTION OF ADVENTITIOUS BUDS

Chrysanthemum morifolium cv. "Mandalay"

Three hundred rooted cuttings of Chrysanthemum morifolium cv. "Mandalay" were placed under intermittent mist upon arrival, April 24, 1970. On April 25, 60 six-inch pots were planted with five cuttings each and placed on a bench in a greenhouse. The plants received artificial illumination for five hours from 10:00 p.m. to 3:00 a.m. daily with 300 W incandescent light bulbs spaced 80 cm apart and 80 cm above the bench in order to prevent flower bud initiation. On May 12, 1970, the chrysanthemums were topped in order to obtain two main branches per plant. The chrysanthemums were irrigated with a solution containing 175 ppm of 20-20-20 fertilizer daily.

On June 8, 1970, the new shoots were topped again and treated with growth regulating substances. Each experimental unit consisted of five pots, containing five plants each. Twelve units were disbudded and defoliated manually before they were treated with chemicals. The treatments were: (a) a spray application of 60 ml aqueous solution containing 20 ppm kinetin plus 3 ppm IAA, (b) the same chemicals in lanolin, (c) lanolin on another unit, and (d) disbudded and nontreated unit. In addition, there was a unit of nondisbudded plants without chemical treatment. These were arranged in three randomized blocks.

The number of adventitious callus formed, the number of days required for emergence of adventitious shoots, and the number of adventitious shoots produced from these buds were recorded. In order to have samples to determine the histogenesis of adventitious buds, ten extra plants in two pots were disbudded and these were sprayed with the growth regulating substances just as were the other plants. Sample collections began one day later, June 25, 1970, and five additional samples were collected at two day intervals. Shoots emerged from adventitious callus buds about nine days after treatment.

Pyrus Malus cv. "Triple Red Delicious"

On April 28, 1970, two hundred sixteen Pyrus Malus cv. "Triple Red Delicious" were potted individually in 10 1/2-inch pots and placed on a gravel surface out doors. Twenty-four apple trees were used per treatment and there were three experiments. Experiment I used disbudded trees treated with 20 ppm kinetin plus 3 ppm IAA in lanolin paste, experiment II used disbudded trees treated with plain lanolin paste, and experiment III used trees which were not disbudded or treated. All trees were topped above the 15th bud above the union immediately after they were potted. All of the existing buds except the top three buds were cut out with a sharp grafting knife as described by Dermen (22). The three buds at the top of the trees were allowed to produce shoots to indicate physiological activity of the tree. On May 15, 1970, the trees were topped above the twelfth node above the union so that no normal buds remained. Sprouts that developed below the union were removed as soon as they appeared.

The following data were collected throughout the summer of 1970: number of days required to develop visible prosphaeroblast (19), number of days required to produce adventitious shoots from the prosphaeroblast, number of prosphaeroblasts per tree, number of adventitious shoots per tree, number of adventitious shoots produced per prosphaeroblast, number of trees that died between April 29 and July 1, 1970, number of trees that died between July 1 and August 30, 1970, number of trees that died without producing prosphaeroblasts, number of bifurcated adventitious shoots, and number of adventitious shoots originated from wound tissue at the former bud site. Twelve prosphaeroblasts were collected in order to study the histogenesis of adventitious buds microscopically at various stages of development.

II. APPLICATION OF MUTAGENIC AGENTS

Chrysanthemum morifolium cv. "Mandalay"

The experiment was started with five plants per pot, four pots per unit, five treatments, and with three replications (total of three hundred plants). After adventitious callus buds emerged from the dis-budding wounds, the plants were arranged into two groups, plants with normal buds and those with adventitious buds. A total of two hundred sixty adventitious shoots, on two hundred forty disbudded chrysanthemum plants, were treated with mutagenic chemicals. Two hundred forty normal shoots on sixty control plants were used. Treatments were: 65 adventitious shoots not treated, 65 adventitious shoots treated with 2000 ppm EMS in a 7.5 percent glycerol solution, 65 adventitious shoots

treated with 5000 ppm colchicine in a 7.5 percent glycerol solution, and 5000 ppm colchicine plus 20 ppm chlorflurenol in a 7.5 percent glycerol solution was applied to 65 adventitious shoots. Two hundred and forty normal shoots were divided into four groups of 60 and they were treated with mutagenic chemicals as described above. Each shoot was treated four times. Retreatment took place upon recovery from the previous treatment. Treatments were given during a period between June 20 and July 24, 1970. Three samples per treatment were collected at three-day intervals six times (from July 24 to August 11, 1970) for microscopic observation.

On August 10, 1970, forty shoots from each treatment were harvested and placed under intermittent mist in a silica sand medium. On September 14, 1970, the rooted cuttings were planted in six-inch pots, five plants per pot, and eight pots per treatment. The pots were placed on a greenhouse bench and received artificial illumination to prevent bud set. On October 20, 1970, the chrysanthemums were pinched once to obtain two laterals. On November 10, 1970, artificial illumination was discontinued. The shoots which produced flowers were laterals of the treated shoots.

Flowers started to open at the end of December, 1970, and evaluation of these blooms was begun on January 7, 1971. Screening for any type of macro-mutations was done by visual inspection: (a) leaves and stems of the treated laterals were compared for differences in size, texture, color, shape, and thickness; (b) ligules, disks, and whole flowers of treated plants were compared for possible deviation in color, shape, size, and texture; (c) changes in flowering pattern, plant shape, plant height, and number of flowers per inflorescence were noted.

Ligules and leaf samples were collected from aberrant plants for microscopic examination. Color as well as black and white photographs were taken on January 12, 1971.

Pyrus Malus cv. "Triple Red Delicious"

Treatment of normal buds of Triple Red Delicious with mutagenic chemicals started on May 7, 1970. Treatments were: 2000 ppm EMS in a 10 percent glycerol solution, and 20 ppm chlorflurenol plus 5000 ppm colchicine in a 10 percent glycerol solution applied to the apex of each growing shoot twice per day until May 27, 1970. At that time the shoots started to show considerable damage due to treatment. The first six trees of each group were not treated with mutagenic chemicals, the buds of six trees were treated with EMS, the buds of six were treated with colchicine, and those of the remaining six received colchicine plus chlorflurenol. Treatment of adventitious buds began on June 8, 1970, and continued as shoots emerged.

Treatment with mutagenic chemicals was resumed on an individual bud basis as the buds recovered from the previous treatment. The last application of mutagenic chemicals took place on August 15, 1970, when most shoots had stopped growing.

On July 27, 1970, apices of 90 actively growing untreated summer shoots were selected and divided into three similar groups. Similar chemical treatments were made on July 27, 1970, and five samples from each treatment were collected six times at three-day intervals for microscopic observations. Buds were sliced off longitudinally from each side.

After the hard outer scales were removed, the buds were dropped in a killing and fixing solution.

On August 30, 1970, leaf thickness, leaf shape index, and size of stomata were recorded from ten leaves per tree. Portions of the lower epidermis from the collected leaves were peeled off and mounted in water, and stomatal size was recorded. The largest diameter, including the guard cells, was used as a measure of stomatal size. Leaf samples were collected and preserved for further microscopic examination.

III. MICROSCOPIC EXAMINATIONS

The samples collected for microscopic observations were immediately tagged for identification and placed in Farmer's fixing and killing solution. Methods, kind and time of sample collection were recorded in detail.

Samples were dehydrated and embedded in paraffin. Duration of the tissue submergence in the various solutions was dependent upon the nature of the samples. Samples were sectioned from 8 to 15 microns. The thickness of the sections was dependent on the nature of the materials. Sections were stained using a modification of the safranin-fast green counter stain schedule.

IV. ANALYSES OF DATA

The data from this experiment were subjected to an analysis of variance. The $p > .05$ level of significance was used. If significant differences were detected, means were separated by Duncan's Multiple

Range Test. The standard deviations at 26 were calculated manually when needed. All analyses of variance were done using an IBM 360/65 computer at The University of Tennessee Computing Center.

CHAPTER V

RESULTS

The results of these experiments are divided into two major portions, induction of adventitious bud formation and development of somatic variations. The results obtained with the different species are described separately.

I. INDUCTION OF ADVENTITIOUS BUDS

Chrysanthemum morifolium cv. "Mandalay"

Application of an aqueous solution of kinetin plus IAA to disbudded chrysanthemums increased the formation of adventitious callus significantly (Table 2 and Figure 1). Application of these chemicals in lanolin paste also increased adventitious callus formation significantly but to a lesser degree than when the aqueous solution was used. The number of adventitious callus obtained from plants treated with plain lanolin was not significantly different from that of the control plants.

The number of adventitious shoots developed from the wound tissue is presented in Table 3. Total number of adventitious shoots produced from the plants treated with an aqueous solution of kinetin plus IAA was the highest (182 adventitious shoots from 767 disbudding wounds) followed by IAA plus kinetin in lanolin paste (143 from 696), control (100 from 693), and lanolin alone (87 from 712).

TABLE 2
CALLUS FORMATION ON DISBUDDING WOUNDS OF CHRYSANTHEMUM
MORIFOLIUM cv. "MANDALAY"

Treatment	Number of Disbudding Wounds*	Number of Wounds Forming Callus	% of Wounds Showing Callus
Control	11.55	2.92	25.25 c**
IAA + Kinetin (Aqueous)	12.78	8.21	64.28 a
IAA + Kinetin (Lanolin)	11.60	3.68	31.75 b
Lanolin Alone	11.87	2.73	23.03 c

*Number of wounds per plant.

**Means followed by a common letter are not significantly different at $p > .05$.

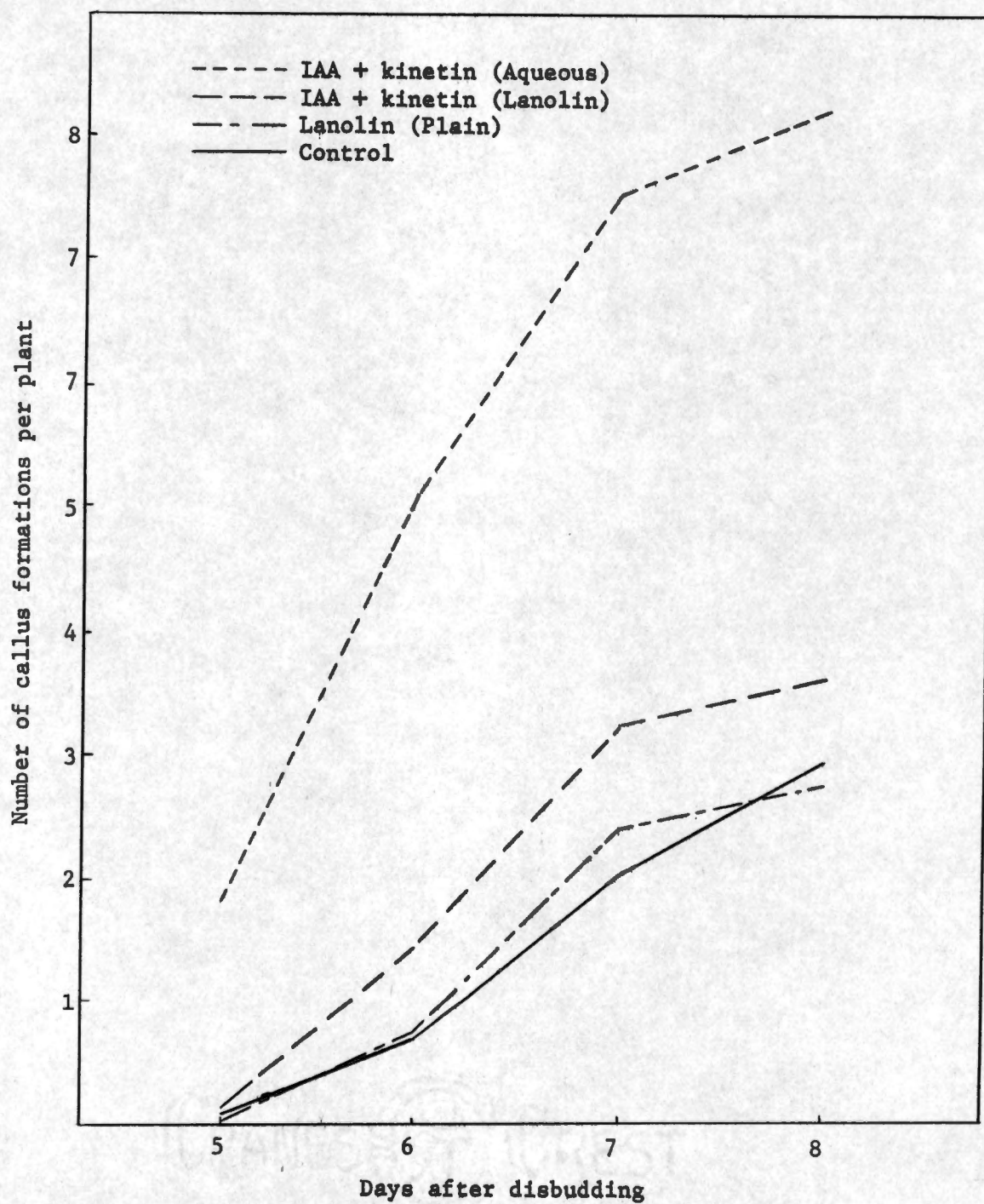


Figure 1. Number of callus formations on Chrysanthemum morifolium cv. "Mandalay."

TABLE 3

EFFECTS OF GROWTH REGULATING SUBSTANCES ON THE PRODUCTION OF
ADVENTITIOUS SHOOTS FROM CALLUS ON CHRYSANTHEMUM
MORIFOLIUM cv. "MANDALAY"

Treatment	No. of Shoots Produced	% Shoot Production	
		Based on No. of Callus	Based on No. of Disbudding Wounds
Control	100	57.14 ab*	14.43 c
IAA + Kinetin (Aqueous)	182	36.92 c	23.73 a
IAA + Kinetin (Lanolin)	143	64.71 a	20.55 b
Lanolin Alone	87	53.05 b	12.22 c

* Means followed by a common letter are not significantly different
at $p > 05$.

Adventitious shoot production per callus varied. Many produced several shoots (Figure 2), others produced a single shoot, and some produced none.

Pyrus Malus cv. "Triple Red Delicious"

The number of prosphaeroblasts and the number of adventitious buds producing shoots were significantly increased by kinetin plus IAA in lanolin paste (Table 4). Figures are computed on the basis of the initial number of trees in each treatment.

The total number of prosphaeroblasts was not increased by application of growth regulating substances but the formation of prosphaeroblasts appeared to be hastened by these chemicals as shown in Table 5. During the month of May, disbudded apple trees treated with IAA plus kinetin produced 62 prosphaeroblasts (or 45.59 percent of the total produced within this treatment series) while those of the controls (disbudded and plain lanolin applied) produced 40 prosphaeroblasts (or 33.06 percent). During the month of June the treated series produced 72 prosphaeroblasts (or 53.95 percent of the total) while the control trees produced 81 prosphaeroblasts (or 66.94 percent). During the month of July, controls produced no prosphaeroblast and treated trees produced 2 (or 1.46 percent of the total) prosphaeroblasts. For both the control and the treated trees, the number of prosphaeroblasts that produced adventitious shoots was highest during June, 57 (or 81.42 percent) for the control and 67 (or 77.01 percent) for the treated trees. In one particular apple tree, which was treated with kinetin plus IAA, a prosphaeroblast was first observed on June 3, 1970, and it produced an



Figure 2. Multiple adventitious shoots produced from a single wound callus on Chrysanthemum morifolium cv. "Mandalay."

TABLE 4

EFFECTS OF GROWTH REGULATING SUBSTANCES ON BOTH NUMBER OF
PROSPHAEROBLAST AND ADVENTITIOUS SHOOT INDUCTION ON
DISBUDDED PYRUS MALUS cv. "TRIPLE RED DELICIOUS"

Treatment	No. of Prosphaero- blast per Tree	No. of Prosphaero- blast That Produced Adventitious Shoots
Disbudded + Plain Lanolin Paste	1.68	.97
Disbudded + IAA + Kinetin in Lanolin	1.88	1.21
L.S.D. @ 5%	.16	.19

TABLE 5

EFFECT OF GROWTH REGULATING SUBSTANCES ON PROSPHAEROBLAST AND
ADVENTITIOUS SHOOT PRODUCTION IN PYRUS MALUS
cv. "TRIPLE RED DELICIOUS"^{*}

	Plain Lanolin	IAA + Kinetin (in Lanolin)
No. of prosphaeroblast obtained:		
in May	40	62
in June	81	72
in July	0	2
Total	<u>121</u>	<u>136</u>
No. of prosphaeroblast that produced adventitious shoots:		
in May	3	13
in June	57	67
in July	10	6
in August	0	1
Total	<u>70</u>	<u>87</u>
First day of prosphaeroblast formation	May 25	May 25
First day of ad. shoot emergence	May 28	May 27
Last day of prosphaeroblast formation	June 26	July 10
Last day of ad. shoot emergence	July 8	August 12

^{*} Data obtained from 72 disbudded apple trees per treatment.

adventitious shoot on August 12, 1970, 71 days later or 105 days after the tree was disbudded.

The apple trees which were treated with growth regulating substances showed similar tendencies. Treated trees showed a tendency toward earlier and longer periods of adventitious shoot production than controls (Table 6). The number of days required for development of the first visible prosphaeroblast and the first adventitious shoot was not different between the controls and the treated trees. However, the last new prosphaeroblasts that became visible on treated trees were two weeks later than the last one in the control (June 26 vs. July 10). The last day of adventitious shoot emergence on the treated trees was almost 5 weeks later than that on the controls. Survival of disbudded apple trees was increased by the application of kinetin plus IAA. Fifty-one percent of the control trees survived as compared with 65 percent of the treated trees. As presented in Table 6, it was observed that about 67 percent of the trees in both the control and treated group produced adventitious shoots from prosphaeroblasts. An average of 1.33 adventitious shoots were produced per prosphaeroblast. When only prosphaeroblasts that actually produced adventitious shoots are considered, the figure is 2.06 shoots.

No significant differences were found between control trees and the treated ones in respect to the largest number of prosphaeroblasts produced per single tree (6 on control vs. 6 on the treated tree), the largest number of prosphaeroblast per tree that produced adventitious shoots (3 on control vs. 3 on treated trees), largest number of

TABLE 6

EFFECTS OF IAA PLUS KINETIN ON SURVIVAL, AND PROSPHAEROBLASTS AND
ADVENTITIOUS SHOOT PRODUCTION OF PYRUS MALUS cv.
"TRIPLE RED DELICIOUS"

	Plain Lanolin	IAA + Kinetin (in Lanolin)
Total number of trees	72	72
Number of live trees on August 15, 1970	37	47
Total number of prosphaeroblast on all living trees	87	109
Average number of prosphaeroblast per living tree	2.35	2.32
Total number of prosphaeroblast that produced adventitious shoots	58	74
Average number of prosphaeroblasts that produced shoots per surviving tree	1.57	1.57
Total number of shoots produced on all living trees	116	156
Average number of adventitious shoots per living tree	3.31	3.32

adventitious shoots produced per single prosphaeroblast (7 on control vs. 6 on treated trees), largest number of adventitious shoots produced per single tree (8 on control vs. 9 on treated trees), the number of bifurcated adventitious shoots (1 vs. 1), and number of adventitious shoots which originated from wound tissues (2 vs. 2) (Table 7).

As mentioned earlier, survival of the trees increased through treatment with kinetin plus IAA. Forty-nine percent of the control and 34 percent of treated trees died during the experiments (Table 8). A smaller number of apple trees treated with growth regulating substances died in the first 9 weeks of experiments (11 trees or 44 percent of the total number that died) than in the later 9 weeks (14 trees or 56 percent of the total number that died). In the control group more trees died in the first 9 weeks (24 trees or 69 percent) than in the last 9 weeks (11 trees or 31 percent). As shown in Table 8, apple trees in both the control and treated group died not only after prosphaeroblast formation but also after the production of a considerable number of adventitious shoots. Leaf and stomata sizes of the adventitious shoots suggest that when two adventitious shoots arose from the same prosphaeroblast they usually showed similar characteristics (Appendix I) while characteristics generally appeared different when they arose from different prosphaeroblasts (Appendix II).

The first leaves to appear on the adventitious shoots were deeply serrated, misshaped and asymmetrical. One or more bifurcated leaves appeared on most adventitious shoots in the earlier stage of development. The leaf shape was usually normal at the 5th or 6th node.

TABLE 7

EFFECTS OF GROWTH REGULATING SUBSTANCES ON THE FORMATION OF
 PROSPHAEROBLAST ON DISBUDDED PYRUS MALUS cv.
 "TRIPLE RED DELICIOUS"

	Plain Lanolin	IAA + Kinetin (in Lanolin)
Largest number of prosphaeroblast per tree	6	6
Largest number of prosphaeroblast that produced ad. shoots per tree	3	3
Largest number of shoots produced per prosphaeroblast	7	6
Largest number of shoots produced per tree	8	9
Number of bifurcated adventitious shoots	1	1
Number of adventitious buds formed on wound tissue	2	2

TABLE 8

EFFECTS OF GROWTH REGULATING SUBSTANCES ON THE FORMATION OF
PROSPHAEROBLASTS AND LIFE SPAN OF DISBUDDED PYRUS
MALUS cv. "TRIPLE RED DELICIOUS"

	Plain Lanolin	IAA + Kinetin (in Lanolin)
Number of trees that died:		
between April 1 and July 1, 1970	24	11
between July 1 and August 15, 1970	<u>11</u>	<u>14</u>
Total	35	25
Number of trees that died:		
without prosphaeroblast	14	10
after prosphaeroblast formation	21	15
after adventitious shoot emergence	10	5
Number of prosphaeroblast on trees that died	34	37
Number of prosphaeroblast with adventitious shoot on trees that died	12	13
Number of adventitious shoots on trees that died	9	19

II. SOMATIC VARIATIONS

Chrysanthemum morifolium cv. "Mandalay"

Effects of mutagenic chemicals on normal buds. The first apparent effect of both colchicine and colchicine plus chlorflurenol on chrysanthemum buds was that growth stopped. The duration of this halt varied from seven days to two months. EMS treatment of normal buds of chrysanthemum did not cause a long stoppage of growth. In general within a week resumption of growth was observed from both adventitious and normal buds treated with EMS.

Microscopic examination revealed that only 40 percent of the EMS treated buds were physically affected by the chemical at the apical meristem, 77 percent of the colchicine treated buds were physically affected, and 60 percent of the colchicine plus chlorflurenol treated apical meristems were affected.

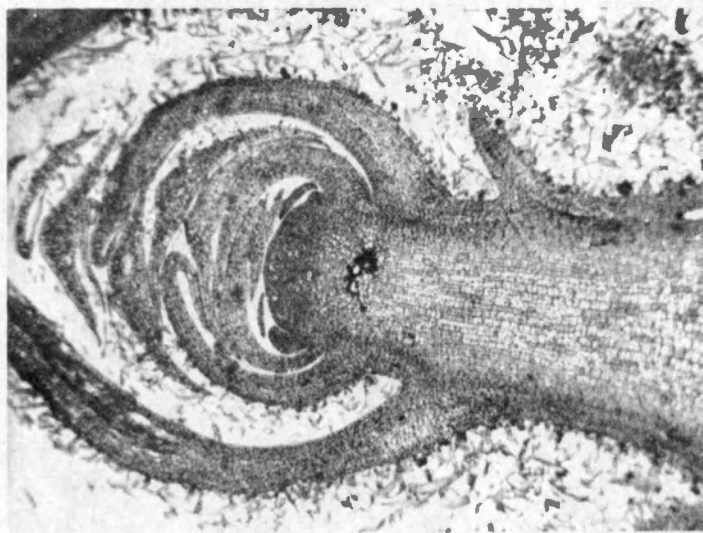
Microscopic observation of buds recovering from mutagenic chemical application. Chemical injury to the apical meristem of EMS treated chrysanthemum buds and axillary meristems was diverse. The most distinct injury caused by EMS treatment was physical destruction of L-I (and in one instance injury was observed through L-II) upon direct contact with the chemical solution. Vacuolation of cells, loss of cell contents, collapse of cells, and distortion of the apex were observed. Fifteen days after the chemical was applied the new tissue of affected layers appeared to be reorganized and became normal. In many instances, the

chemical residue remained inside the plant tissue at the place where the chemical was applied (Figure 3).

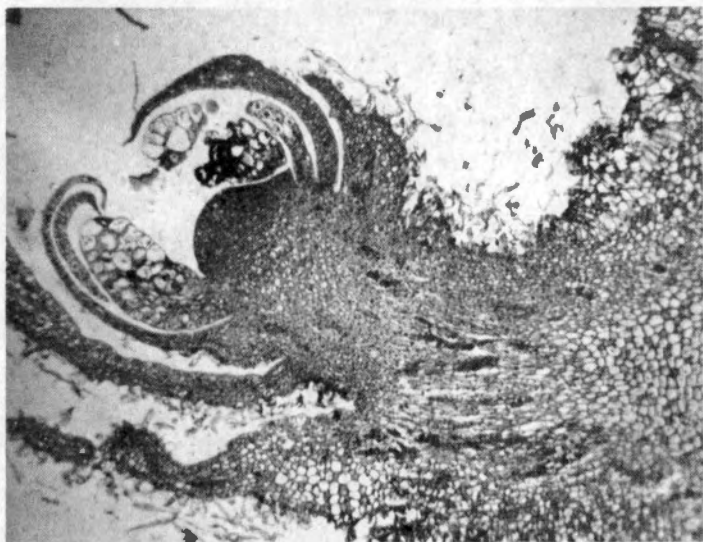
Chrysanthemum buds treated with colchicine showed a thickening of the apical meristem as an immediate response to the chemical. This was caused by cessation of mitosis and cell enlargement at the apex. Six days after the chemical was applied, the first two or three leaflets had extremely large cells and nuclei as shown in Figure 3. Between the 9th and 12th day after colchicine was applied, both nuclei and cells of the apical meristem appeared to become enlarged as well as their nuclei and during this period damage to the apical dome was observed. Between the 15th and 18th day after treatment, with most of the apical meristem still at rest, the base of the apical meristem had thickened considerably due to longitudinal cell division in the pro-cambial region. Apparently cambial activity was not inhibited by this chemical, or at least cessation of cambial activity was much shorter than that of the apical meristem.

Buds treated with chlorflurenol plus colchicine showed a similar response, but cessation of apical meristematic activity occurred about three days earlier than in buds treated with colchicine alone and there also was a complete cessation of cell mitoses.

In slightly injured apices, local or direct healing began by activation of individual cells. When the central zone of the apex was killed, regeneration began from the pith meristem. Sometimes complete healing of the damaged central zone of the apex was observed (Figure 3). The third pattern occurred when a complete destruction of the apical



EMS treated apical meristem.
Note chemical residues inside tissue.



Colchicine treated apical meristem.
Adaxial development of the damaged side is noted.



Colchicine plus chlorflurenol treated meristem. A complete cessation of apical meristematic activity and unusual thickening of longitudinal tissue is evident.

Figure 3. Recovery of chrysanthemum apical meristem after treatment with mutagenic chemicals (x35).

meristem had taken place. In this case the cells on the adaxial side of the damage became meristematic. This was especially observed on buds treated with colchicine (Figure 3).

Macroscopic observation. The first two or three leaves emerging after treatment with each of the three chemicals showed extensive deformity. The most severe deformity was observed on colchicine treated chrysanthemum plants and the least with EMS. Several buds which were treated with either colchicine or chlorflurenol plus colchicine produced leaves which were uniformly broader, and darker green than those of the control, also the leaf margins showed a tendency toward deeper serration as compared to the predominantly single serration on nontreated plants.

A total of 47 plants having distinct changes in flower color, flowering pattern, and flower form were isolated from four hundred eighty plants. These mutations included fourteen different changes in color, form, or flowering pattern (Tables 9 and 10).

The most drastic somatic mutations were five red-flowering chrysanthemums. Although intensity of color varied, formation and shape of ligules were distinct from other flowers. Ligules of these blooms were short, wide and semi-incurved while normal flowers had long, narrow, and flat ligules. Figure 4 shows typical forms and colors of both flowers and ligules. Two of these red-flowering plants were obtained from adventitious buds treated with colchicine and three of these were obtained from adventitious buds treated with colchicine plus chlorflurenol. No normal buds or adventitious buds treated with EMS produced red-flowering

TABLE 9

SOMATIC MUTATIONS OBTAINED IN CHRYSANTHEMUM MORIFOLIUM cv. "MANDALAY"
AFTER TREATMENT OF ADVENTITIOUS CALLUS BUDS AND NORMAL
BUDS WITH SEVERAL MUTAGENIC CHEMICALS

Bud Type Treatment		Flower Color	Other	No.	% Based on Total No.
Normal	None	No change	None	0	0.00
	Colchicine	Dark bronze		4	
		Dark center		2	
			Long ligules	$\frac{1}{7}$	11.67
	Chlorflurenol + colchicine	None	None	0	0.00
Adventi- tious	EMS	Yellow center		2	
			Few ligules	$\frac{2}{4}$	6.67
	None	Dark bronze		2	3.33
				2	
				1	
			Large bloom	1	
				1	
			Bunchiness	5	
			Dwarfness	2	
			Large bloom	1	
			Low vigour	1	
			Complex ploidy	$\frac{1}{15}$	25.00
	Chlorflurenol + colchicine	Red Light bronze		3	
				1	
			Large bloom	3	
			Bunchiness	3	
			Dwarfness	$\frac{1}{11}$	18.33
	EMS	Yellow Yellow center Yellow & bronze chimera		2	
				3	
				1	
			Long ligules	1	
			Bunchiness	$\frac{1}{8}$	13.33

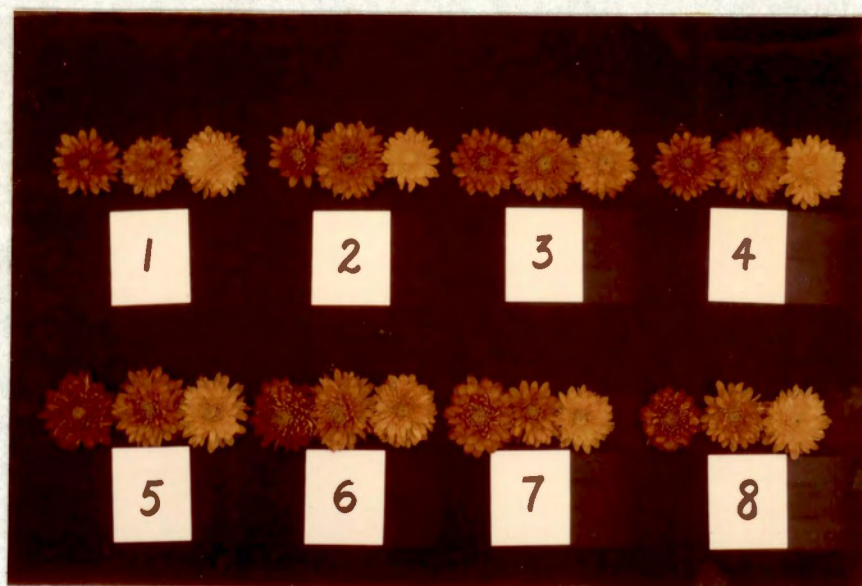
TABLE 10

NUMBER OF SOMATIC MUTATIONS OBTAINED IN CHRYSANTHEMUM MORIFOLIUM
 cv. "MANDALAY" FROM NORMAL BUDS AND ADVENTITIOUS BUDS, AND
 UPON TREATMENT WITH COLCHICINE, COLCHICINE PLUS
 CHLORFLURENOL, AND EMS

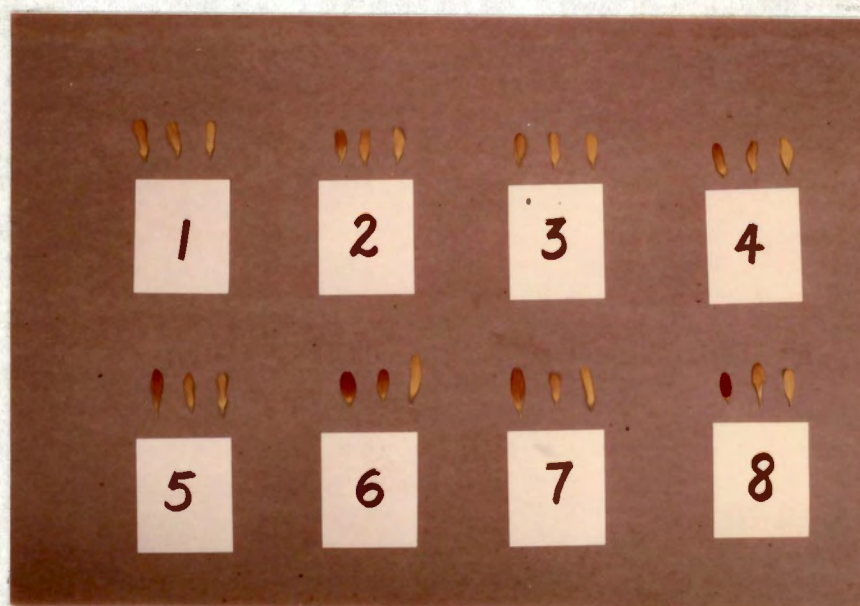
	No. of Mutations	% Based on Total
Normal buds *	11	4.58
Adventitious buds *	36	15.00
Colchicine treatment **	22	18.33
Colchicine plus chlorflurenol treatment **	11	9.17
EMS treatment **	12	10.00
Control **	2	1.67

* All treatments.

** All buds.



Chrysanthemum flowers of identical maturity.

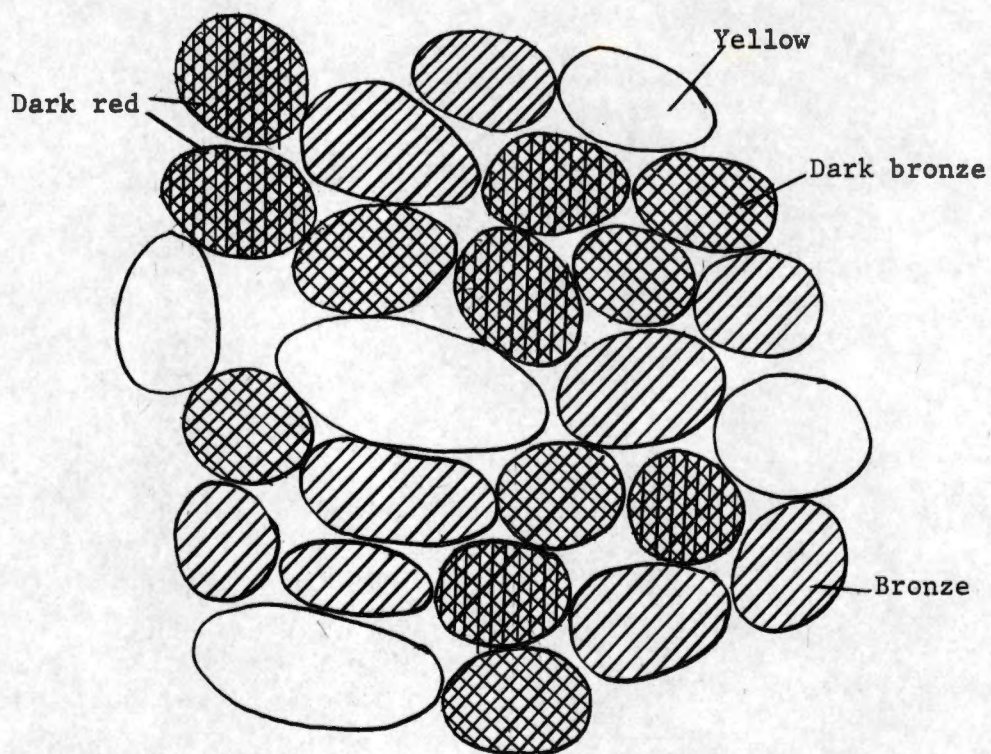


Chrysanthemum ligules with identical maturity.

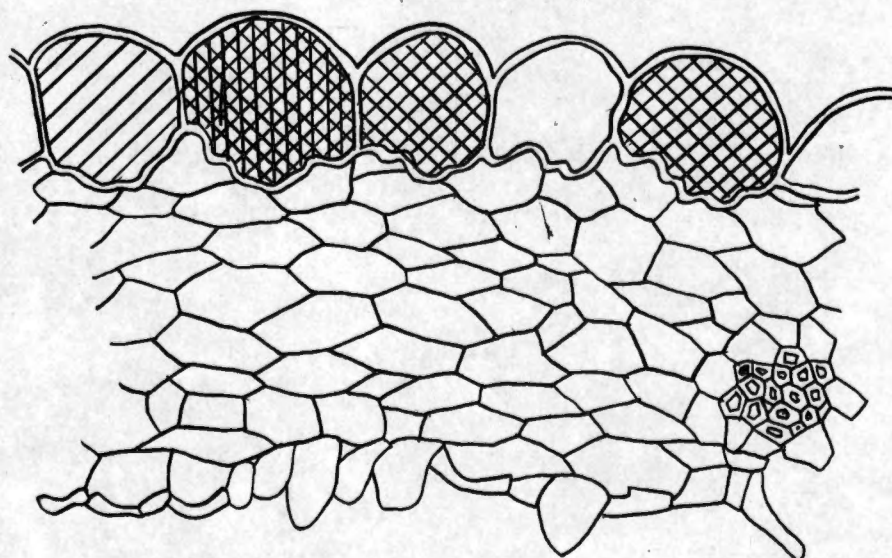
Figure 4. Color deviations within treatments in Chrysanthemum molifolium cv. "Mandalay." Left; lightest color, center; average color, and right; darkest color within each treatment. 1; normal bud-no chemical, 2; adventitious bud-no chemical, 3; normal bud-EMS treatment, 4; adventitious bud-EMS, 5; normal bud-colchicine, 6; adventitious bud-colchicine, 7; normal bud-colchicine plus chlorflurenol, 8; adventitious bud-chlorflurenol plus colchicine.

plants in this experiment. Moreover, two of the adventitious buds treated with EMS produced completely yellow flowers. Both adventitious buds without mutagenic treatment and normal buds treated with colchicine produced dark bronze (Figure 4). Two dwarf types with a large number of blooms were obtained from adventitious buds treated with colchicine. One similar variant appeared following treatment with colchicine plus chlorflurenol. Increased density of the inflorescence was observed in response to treatment of adventitious buds, five with colchicine, three with colchicine plus chlorflurenol, and one with EMS. No such flowering pattern was obtained from normal buds. Two distinct sectorial chimeras, affecting flower color, were obtained, one from an adventitious bud treated with colchicine and another from an adventitious bud treated with EMS. Three plants with flowers with a dark center were obtained by colchicine treatment and five yellow centered flowers were obtained by EMS treatment. One complex tetraploid plant was produced from an adventitious callus bud treated with colchicine.

Microscopic examination. Microscopic examination of ligules showed that the red pigmentation (anthocyanin) of the ligules was concentrated in the upper surface cells (Figure 5). The ligules of red flowers had larger surface cells as well as larger epidermal cells than the ligules of either normal or yellow flowers (Figures 5 and 6). Neither size nor thickness of the epidermal cells of the foliage was increased on red flowering plants but the degree of pubescence was. Increased pubescence was also evident macroscopically as mentioned

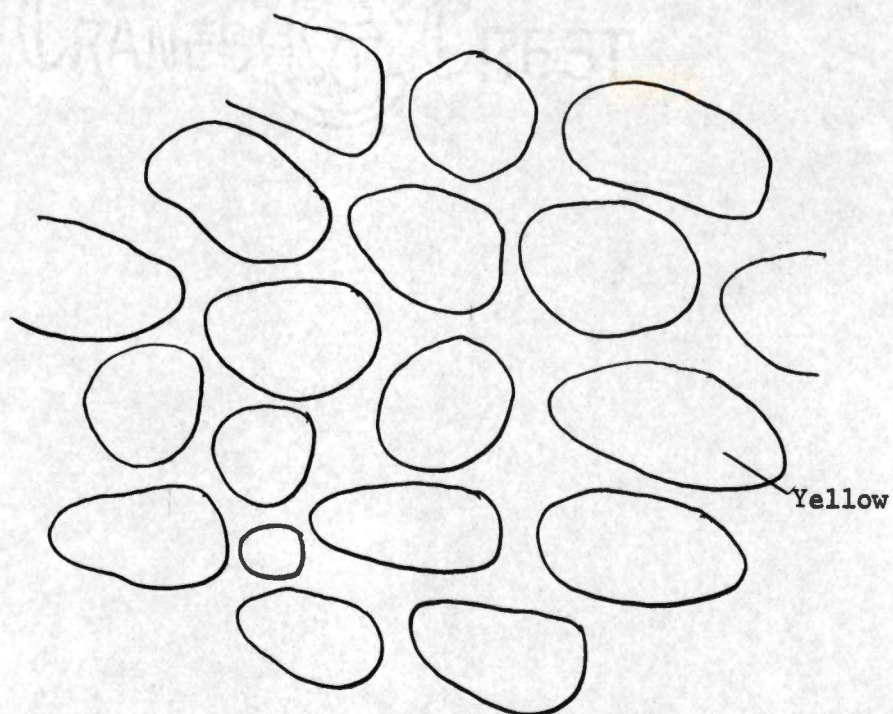


A. Surface view (x400)

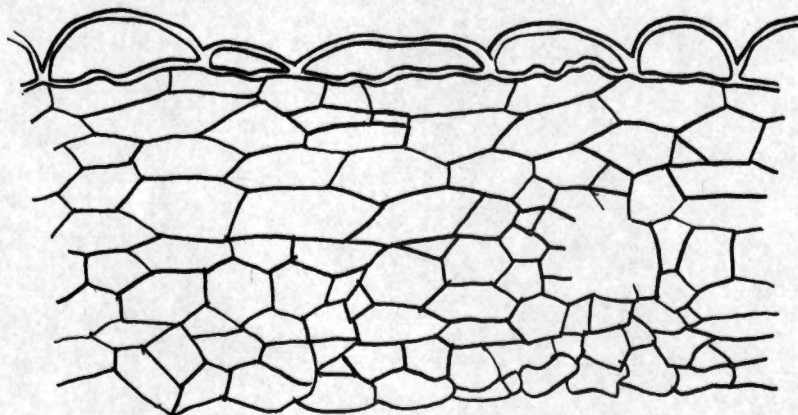


B. Cross section (x400)

Figure 5. Distribution and concentration of anthocyanin in enlarged upper surface cells of red flowering chrysanthemum ligules.



A. Surface view (x400)



B. Cross section (x400)

Figure 6. Upper surface cells of yellow flowering chrysanthemum ligules.

earlier. Leaves of red flowering plants were more pubescent on both the upper and lower epidermis while the control plants showed some hairs on the lower epidermis and none on the upper epidermis.

It was observed microscopically that both the pollen and stomata of red flowering plants were larger than those of yellow flowering plants.

One plant which originated from an adventitious callus bud treated with colchicine seemed to be a complex polyploid but did not produce a large bloom in terms of flower diameter. In all other aspects, such as number of rays, ligules, chaff and weight of bloom, this plant was significantly different from normal plants (Table 11). The inflorescence of this plant was a compound cyme as the laterals below the epical flower were branched also resulting in $3x-2$ flowers per inflorescence (x = normal).

Microscopic study of this induced complex polyploid and normal plants showed that the size of the epidermal cells and the size of the pollen were greater in the induced complex polyploid individual than in the normal plants. The induced complex polyploid individual also had larger pollen. The polyploid plant also had thicker epidermal cells on the leaves than normal plants (Figures 7 and 8).

Pyrus Malus cv. "Triple Red Delicious"

Effect of mutagenic chemicals on apical meristems. It was observed, under the microscope, that it was more difficult to effectively apply mutagenic chemicals to apple buds than to chrysanthemum buds.

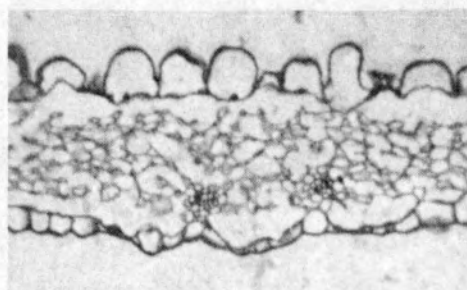
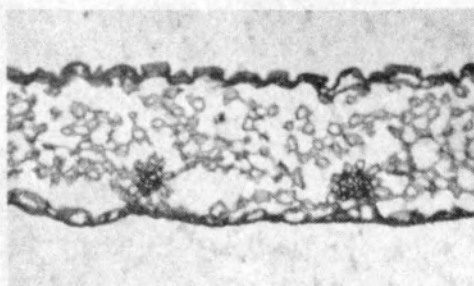
TABLE 11

COMPARISON OF INDUCED COMPLEX POLYPLOID AND NORMAL PLANTS OF
CHRYSANTHEMUM MORIFOLIUM cv. "MANDALAY"*

Recordings	Complex Polyploid	Normal Plants	F Value
Flower diameter (mm)	62.13	63.00	N.S.
Number of ligulates (outmost layer)	31.13	17.75	**
Number of ligules	13.75	11.25	N.S.
Total number of ligules	210.75	137.38	**
Total number of chaff	94.13	82.38	**
Size of disk (mm)	14.88	3.13	**
Petal shape index (length/width)	2.79	3.54	**
Total green weight of petals (gr)	3.35	1.81	**
Base weight (disk and chaff) (gr)	1.41	.21	**
Pollen size (μ)	44.51	30.23	**

* Figures were computed from ten flowers per treatment.

** Significantly different at $p > .05$.



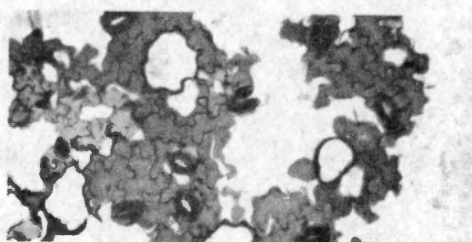
Cross section of a ligule (x100).



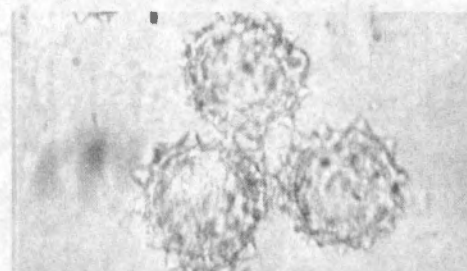
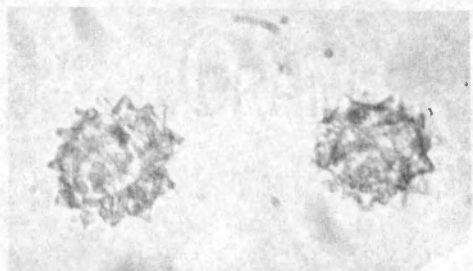
Transverse section of lower epidermis of a ligule (x100).



Cross section of a leaf (x35).

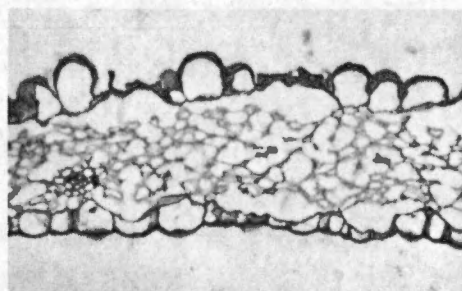
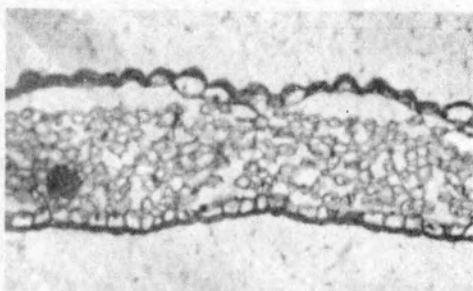


Transverse section of the lower epidermis of leaf (x100).

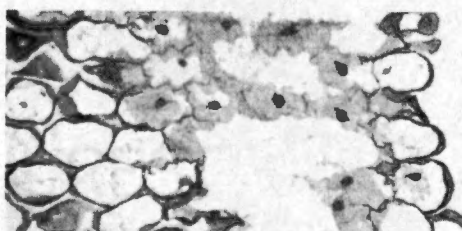
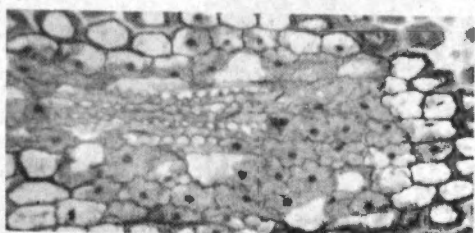


Pollen (x430)

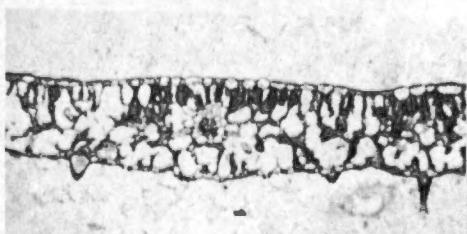
Figure 7. Histological differences in various organs between yellow (left) and red (right) flowering Chrysanthemum morifolium cv. "Mandalay."



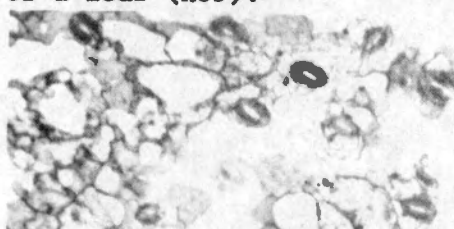
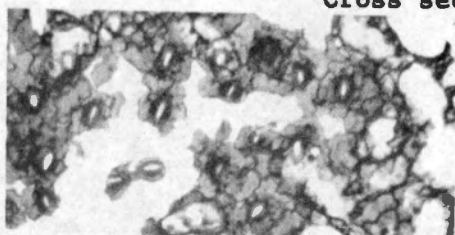
Cross section of a ligule (x100).



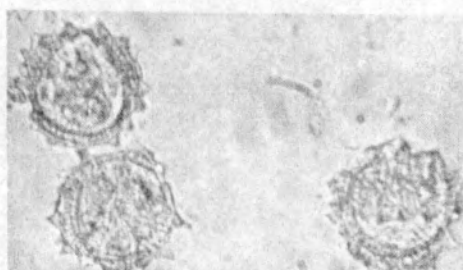
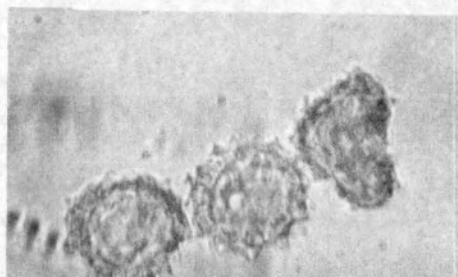
Transverse section of lower epidermis of a ligule (x100).



Cross section of a leaf (x35).



Transverse section of lower epidermis of leaf (x100).



Pollen (x430).

Figure 8. Histological differences in various organs between diploid (left) and polyploid (right) flowering Chrysanthemum morifolium cv. "Mandalay."

Only 10 percent of the apical meristems of EMS treated apple buds were effectively in contact with the chemical, 33 percent of the apical meristems were in contact when colchicine was used, and 17 percent when chlorflurenol plus colchicine was used.

In contrast to the response of chrysanthemum, apple buds treated with EMS had the longest period of growth stoppage among the three treatments. The other two mutagenic chemicals did not have a strong effect on growth. It was observed microscopically that there was no physical damage to the apical meristem as a result of mutagenic chemical treatment except one bud treated with EMS which showed damage to L-I. Since no severe damage to apices was evident, it is difficult to determine if recovery or replacement of tunica layers took place. In so far as observations permitted, the pattern of recovery appeared to be similar to that of chrysanthemums.

The first four to six leaves which emerged after treatment were usually small. Small, thick and misshapen leaves were observed on shoots treated with colchicine and colchicine plus chlorflurenol. On EMS treated apple trees, eleven completely or partially variegated leaves, eight on adventitious shoots and three on normal shoots, were obtained. There were no significant differences in leaf thickness and size of stomata among mutagenic chemical treatments or between adventitious shoots and normal shoots. There were, however, significant differences in leaf shape index (Table 12). Leaves of normal shoots without mutagenic chemical treatment were significantly longer and

TABLE 12

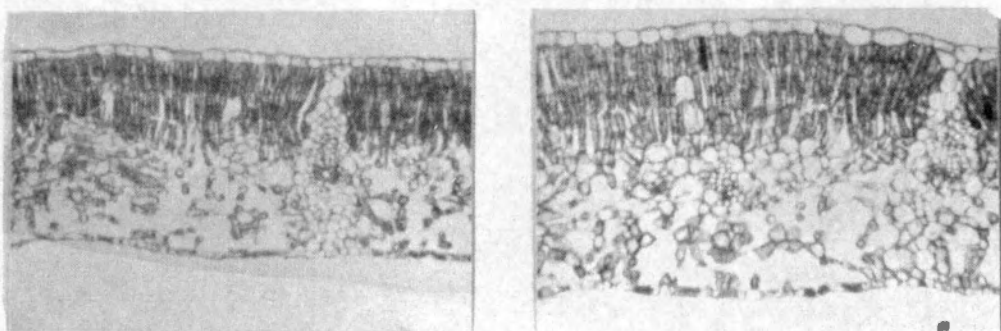
LEAF THICKNESS, LEAF SHAPE INDEX, AND STOMATAL LENGTH OF LEAVES OF
 NORMAL AND ADVENTITIOUS SHOOTS OF PYRUS MALUS cv. "TRIPLE RED
 DELICIOUS" WHICH DEVELOPED FROM BUDS TREATED
 WITH MUTAGENIC CHEMICALS

Treatment	Leaf Thickness (1/100 mm)	Leaf Shape Index (length/width)	Stomatal Length (μ)
Adventitious bud, no mutagenic chemical	24.54 $\pm$ 3.61*	1.56 $\pm$ 0.30*	28.09 $\pm$ 3.64*
Adventitious bud with EMS treatment	24.41 $\pm$ 3.98	1.57 $\pm$ 0.22	26.44 $\pm$ 3.36
Adventitious bud with colchicine treatment	24.94 $\pm$ 5.18	1.51 $\pm$ 0.20	27.96 $\pm$ 2.96
Adventitious bud with chlorflurenol treatment	22.31 $\pm$ 3.20	1.45 $\pm$ 0.16	27.98 $\pm$ 4.41
Normal bud with no mutagenic chemical	24.26 $\pm$ 3.90	1.88 $\pm$ 0.13	26.44 $\pm$ 4.68
Normal bud with EMS treatment	23.17 $\pm$ 2.82	1.67 $\pm$ 0.24	27.27 $\pm$ 3.10
Normal bud with colchicine treatment	24.03 $\pm$ 3.96	1.62 $\pm$ 0.24	28.33 $\pm$ 2.90
Normal bud with chlorflurenol treatment	24.17 $\pm$ 3.08	1.59 $\pm$ 0.18	28.55 $\pm$ 5.78

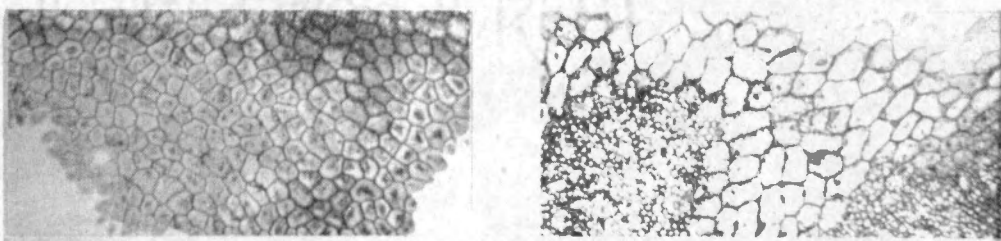
* Mean $\pm$ 2 σ .

narrower than other leaves. Adventitious shoots treated with colchicine plus chlorflurenol produced the broadest leaves.

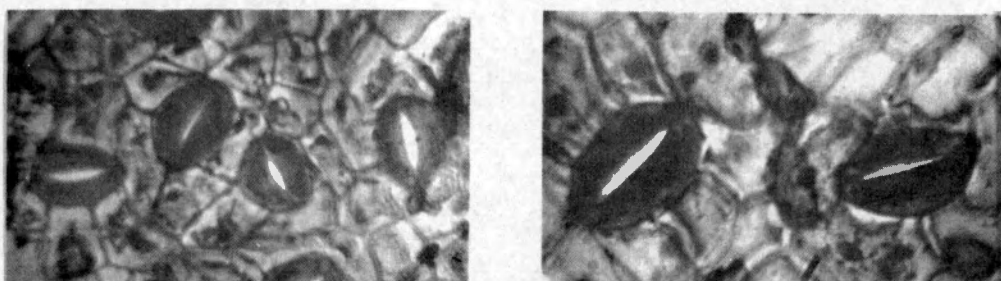
Three apple trees produced shoots with large, thick, doubly serrated leaves with large epidermal and stomatal cells (Figure 9).



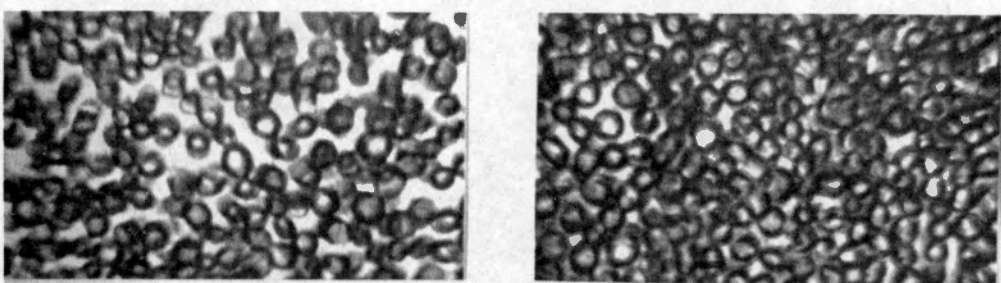
Cross section of a leaf (x100).



Transverse section of upper epidermis of a leaf (x100).



Stomata (x430).



Palisade parenchyma (x430).

Figure 9. Histological differences in various organs between triploid (left) and polyploid (right) Pyrus Malus cv. "Triple Red Delicious."

CHAPTER VI

DISCUSSION

Application of growth regulating substances to several plant species in an attempt to stimulate adventitious bud development showed that the response of species to these chemicals differs.

As mentioned by Nybom and Koch (54), the main problem with mutagenic chemical treatment seemed to be that of infiltration. This was particularly true with Triple Red Delicious apple buds which are well protected by heavily pubescent young leaves during the growing season.

I. INDUCTION OF ADVENTITIOUS BUDS

Kinetin in combination with IAA used on two plant species in these experiments increased adventitious bud production and reduced the number of days required to produce such buds (Tables 2, 5, 6, and Figure 1, pages 35, 41, 43 and 36, respectively).

It was found that development of adventitious buds on these two plant species differed. A clear example of adventitious bud development from a prosphaeroblast formed on the internodes was observed on Pyrus Malus cv. "Triple Red Delicious," while Chrysanthemum morifolium cv. "Mandalay" produced callus type adventitious buds on the disbudding wound tissue. Not all of the prosphaeroblasts on Pyrus or adventitious

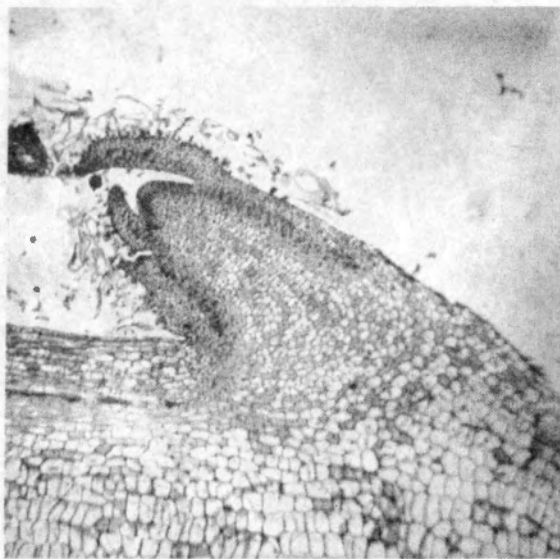
callus on Chrysanthemum produced adventitious shoots. Part of the adventitious buds produced shoots and some of them failed to initiate adventitious shoots.

Chrysanthemum morifolium cv. "Mandalay"

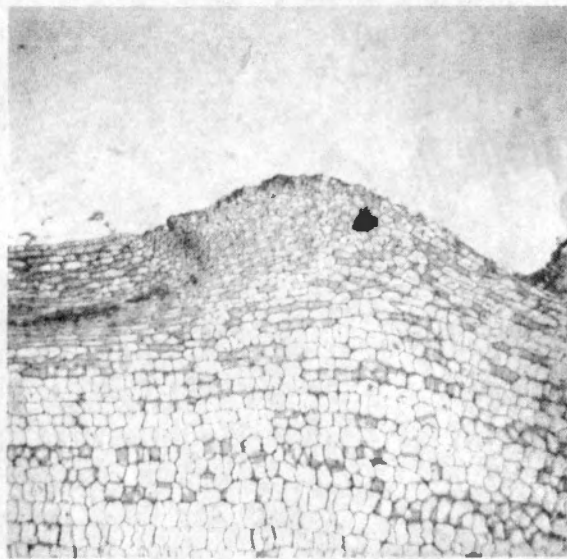
It was found that upon removal of all axillary and apical buds of Chrysanthemum morifolium cv. "Mandalay" 25 percent of the axillary wound calluses formed adventitious buds (Table 2, page 35). Application of kinetin plus IAA to the disbudded shoots increased the formation of adventitious buds up to 154 percent when chemicals were applied in aqueous solution, and the formation was 26 percent increased when the chemicals were applied in lanolin paste. It is assumed that the difference in adventitious bud formation, as related to the method of application, was due to the amount of growth regulating substances absorbed by the plant tissue. The amount of actual growth regulating substances in direct contact with the plant materials upon application with the aqueous solution was presumably greater than upon application in lanolin paste. This might be the reason that the number of adventitious buds formed was affected by the method of application, i.e., with lanolin paste translocation within the paste would be necessary for full contact of the chemical with the tissue. It was apparent that application of plain lanolin did not reduce the number of adventitious buds formed (Table 2). Although no research is reported on induction of adventitious buds on chrysanthemum by treatment with kinetin plus IAA, the results with Chrysanthemum morifolium cv. "Mandalay" seems closely correlated with

the results obtained with tissue cultures in vitro of other species (57, 73, and 80).

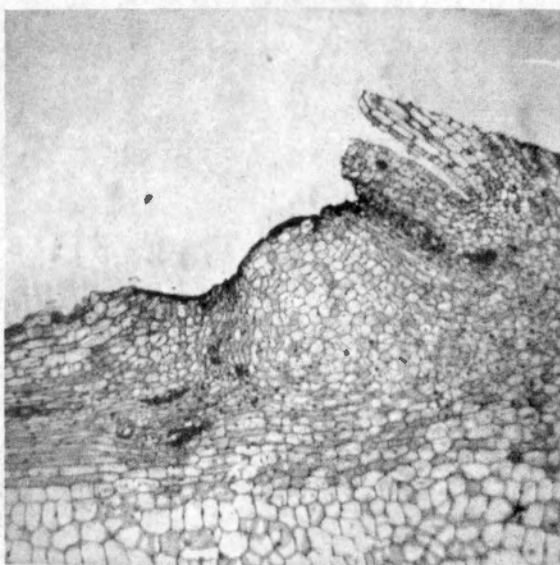
It was observed microscopically that there were two types of histogenesis of adventitious buds in Chrysanthemum morifolium cv. "Mandalay." The first type of histogenesis is presented in Figure 10A through F. Figure 10A shows a normal bud before it was removed. Figure 10B shows that 24 hours after the bud was removed the wound surface was covered with a lignified membrane. Figure 10C pictures a sample collected two days after sample B was collected (3 days after disbudding) and shows callus cell proliferation. Figure 10D was taken from a sample harvested 5 days after disbudding and shows the formation of callus about 0.4 mm high topped with dense lignified cells. Figure E was taken 7 days after disbudding and by this time callus had grown about 0.8 mm in height with well organized cells at the top of the callus. Figure 10F shows the formation of an apical meristem at the tip of the callus. This tissue was collected nine days after disbudding. This bud initial shows several cavities formed during periods of rapid cell proliferation. The cavities were surrounded by lignified tissue. This type of tissue was never found in normal buds. The callus buds seemed to have originated directly from the parenchymatous cells of the wound tissue. It was evident that the adventitious callus buds did not originate from existing redifferentiating tissues, such as endodermis or pericycle. Consequently, it may be possible to obtain an histological arrangement different from that of normal buds. As there is a strong possibility that the tunica layers in the adventitious buds originated from several parenchymatous cells, each



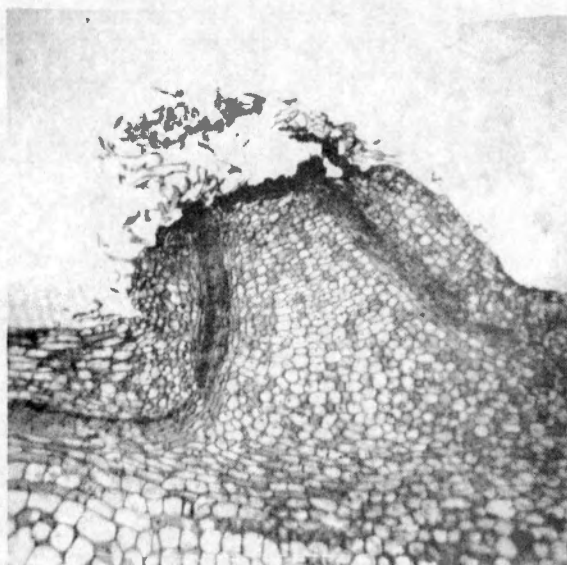
A. Normal bud before disbudding.



B. Twenty-four hours after disbudding. A lignified layer is formed on the surface of the wound.

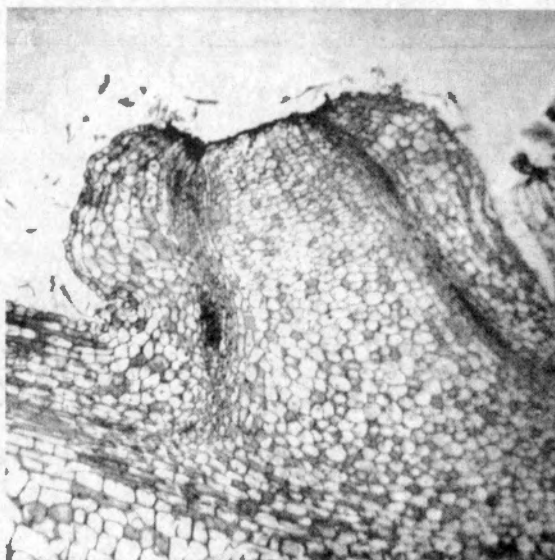


C. Three days after disbudding. Wound starts to proliferate.

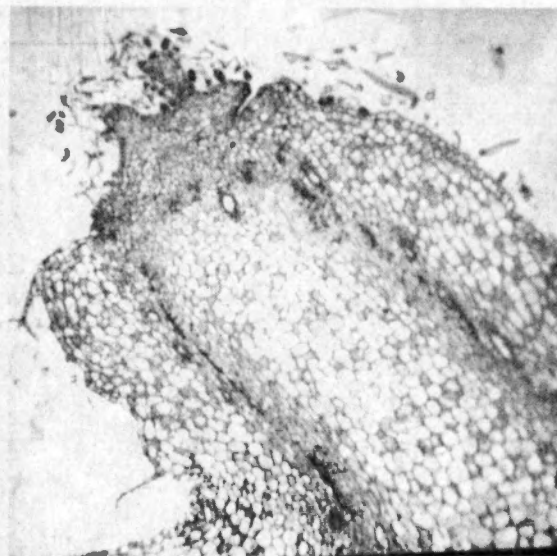


D. Wound tissue with considerable growth and heavy lignified layers.

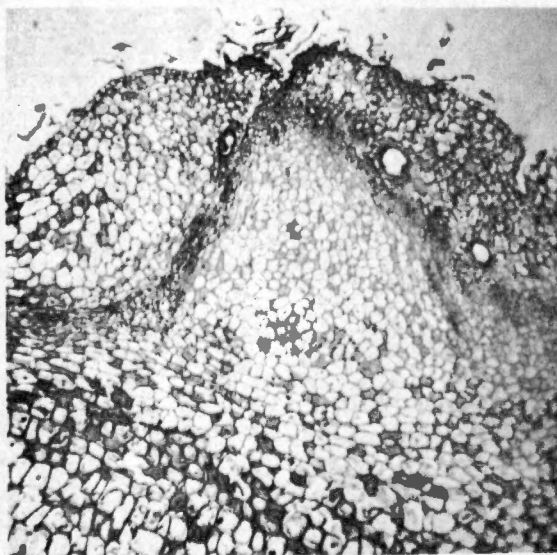
Figure 10. Development of adventitious callus buds from disbudded Chrysanthemum morifolium cv. "Mandalay" (x35).



E. Seven days after disbudding.

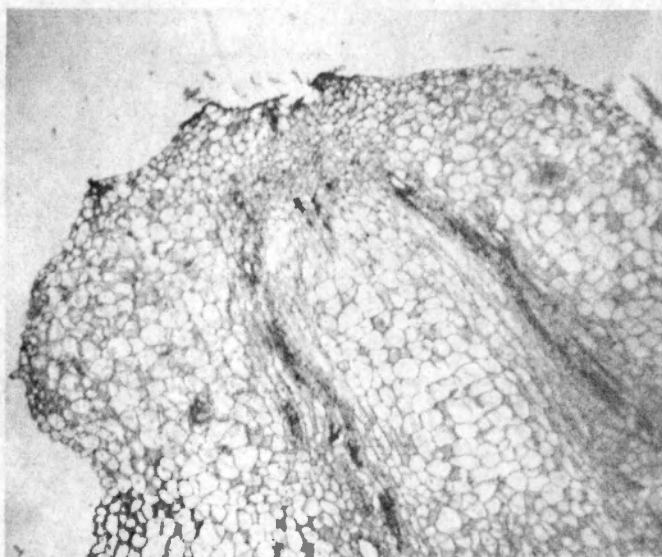


F. Nine days after disbudding.

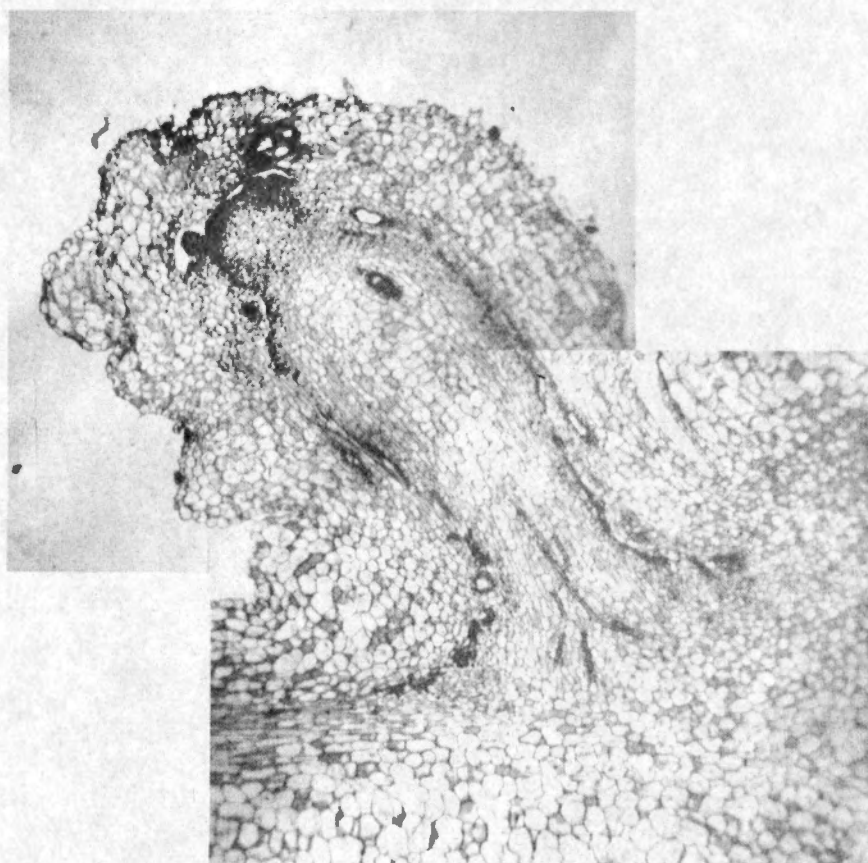


G. Five days after disbudding.
Initiation of an adventitious
bud in the endodermis-pericycle
region.

Figure 10 (Continued)

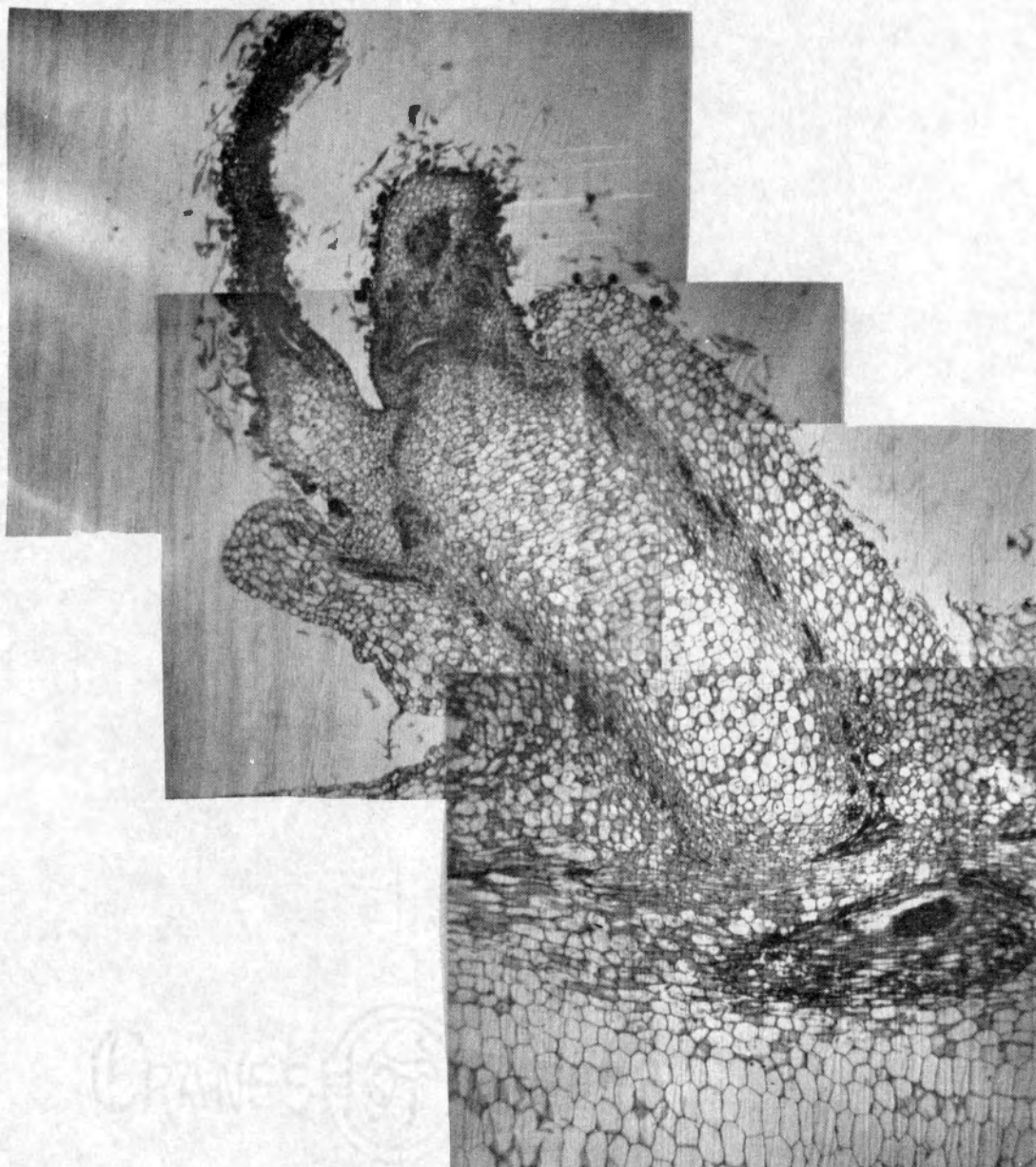


H. Seven days after disbudding.



I. Nine days after disbudding.

Figure 10 (Continued)



J. Eleven days after disbudding.

Figure 10 (Continued)

tunica layer or even cells within a layer could have different genetic constituents. At this stage of development this bud had a primary vascular system which was located in the area between the callus and the bud initial. The second type of adventitious bud histogenesis in this species is presented in Figure 10A, B, G, H, and I. Pictures A and B have been described earlier and picture G shows a complete healing of the wound with an exception of the center portion where a dense layer of lignified cells is formed. This adventitious bud initiated from the internal tissue, from either endodermis or pericycle. This picture was taken five days after disbudding. Both the formation of callus tissue and the initiation of the adventitious bud took place rapidly. There were several cavities in the area between the callus tissue and the adventitious callus bud. The largest cavity was about 100 μ in diameter. Also many of the parenchymatous cells in the callus tissue were compressed by the growing bud.

Figure 10H, which was taken 7 days after disbudding, shows a strong mitotic activity at the tip of the callus tissue where a large number of cells appear to be compressed. Figure 10I was taken 9 days after disbudding and it shows the development of the apical meristem inside the callus tissue with a large number of cavities. One was found inside an adventitious bud. The outer surface of the callus tissue is a wrinkled, highly proliferated tissue, a typical feature of callus. This adventitious bud shows vascular development along with the primary vascular system between callus and pith of the adventitious callus bud. Picture J was taken 11 days after disbudding. It shows the emergence of a new adventitious bud through a large opening in the callus tissue.

This is the last stage of development studied for both types of adventitious bud development.

It may be assumed that the tunica layers of the second type of adventitious bud originated from a single cell or a few cells as is usually the case with adventitious buds (4).

Pyrus Malus cv. "Triple Red Delicious"

It is known that disbudded trees of some Pyrus cultivars produce adventitious buds quite readily (5, 19, and 22). Although the total number of prosphaeroblasts and the number of adventitious shoots on disbudded apple trees was increased through application of growth regulating substances (Table 5, page 41), it is not clear whether these chemicals directly stimulated the formation of adventitious buds or that the number of adventitious buds was increased as a result of the increased life span of the treated apple trees. It is evident that application of kinetin plus IAA decreased the rate of senescence in disbudded apple trees (Tables 6 and 8, pages 43 and 46). The relation between application of these two growth regulating substances and the number of adventitious buds formed on disbudded apple trees is presented in Table 8. The number of dead trees at the end of the experiment was less and also the rate of senescence was delayed upon chemical treatment. The combination of growth regulating substances applied to disbudded apple trees hastened the formation of prosphaeroblasts and initiation of adventitious buds. It also extended the period of adventitious bud production (Table 5). The average number of prosphaeroblast and adventitious buds produced per surviving treated tree were not

different from that produced by the controls (Table 6, page 43).

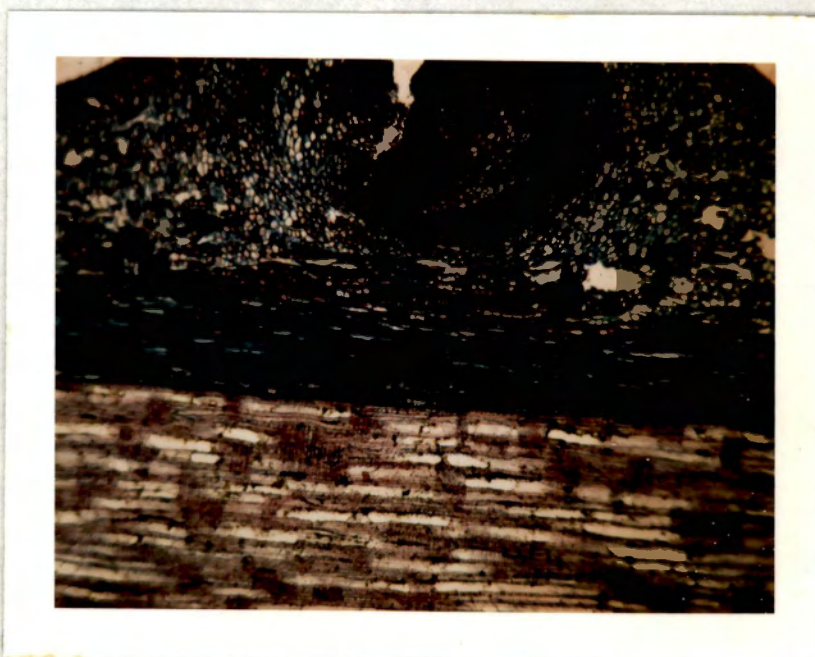
Treatment with these chemicals produce neither a larger number of prosphaeroblasts per tree nor a larger number of adventitious shoots per prosphaeroblast (Table 7, page 45). This appears to indicate that the combination of kinetin and IAA did not contribute to either initiation or differentiation in this study. The chemical treatment decreased both the number of dead trees and the rate of senescence. The number of prosphaeroblasts produced on treated trees that died later was much greater than that on nontreated trees (2.47 per treated tree vs. 1.67 per control) (Table 8, page 46).

There seem to be two different sites of origin of adventitious buds on apple trees, from deep tissue origin and from shallow tissue. It could be assumed that application of kinetin plus IAA on disbudded apple trees stimulated bud formation in shallow tissue (possibly cortex). In this case an adventitious bud generally has poor vascular connections and has less chance to survive. Treatment of disbudded Triple Red Delicious apple trees with these growth regulating substances increased both the total number of adventitious buds formed and the total number of adventitious buds on living trees but the average number of surviving adventitious buds per living tree was not affected by the chemical treatment.

It was confirmed microscopically that there are two different origins of adventitious buds on apple trees. Picture A of Figure 11 shows a meristem of deep tissue origin, possibly the cambium or the outer phloem (21). It is difficult to say exactly which tissue initiated the



A. Cross section of adventitious bud of deep tissue origin.



B. Longitudinal section of adventitious bud of shallow tissue origin.

Figure 11. Two different origins of adventitious buds on Pyrus Malus cv. "Triple Red Delicious."

adventitious bud in this case because the tree had increased about 1.2 mm in diameter since initiation of the prosphaeroblast. A strong vascular connection was formed through the vascular cambium and deep into the secondary xylem. In contrast to this type of adventitious bud development, picture B of Figure 11 shows an adventitious bud initiated in the cortex (of shallow origin) where no proper conductive tissue connection into either secondary phloem or xylem was observed. Most adventitious buds which had no vascular connection into the existing conductive system remained inactive for a long time. If this type of adventitious bud remained dormant long enough to develop proper phloem and xylem connections, it produced a healthy adventitious shoot. When a cell prosphaeroblast developed during the last stage of senescence of a particular tree, the adventitious bud of shallow origin had no chance to develop vascular connections, so consequently such bud would die. It was observed that several adventitious shoots, which had emerged from prosphaeroblasts, suddenly died because of lack of proper vascular connections.

It can be concluded that kinetin plus IAA applied to disbudded apple trees decreased the rate of senescence of the treated trees, and therefore each adventitious bud initial had a better chance to develop vascular connections.

It is generally accepted that in vitro the quantitative interaction between kinetin and IAA is very critical (82). Although

this is assumed to be less critical in vivo or in situ than in vitro, determining the optimum ratio of these two chemicals for adventitious bud induction on different plant species offers a future challenge.

II. SOMATIC VARIATIONS

Chrysanthemum morifolium cv. "Mandalay"

As with many other cultivated plant species (20, 23, and 59) colchicine treatment on chrysanthemum did not result in many complete tetraploids. Commonly, periclinal chimeras were produced. Phenotypic appearance of induced periclinal chimeras varied among individual plants. Some showed increased flower color intensity with increased size of cells and others showed increased cell size without change of color. The most valuable progressive mutant obtained in this experiment was a dark red flowering plant (Figure 4, page 53) which might possibly be the result of a combination of an increased gene population governing anthocyanin formation and an alteration of tunica layers (48). Furthermore, the appearance of blooms with a darker or lighter than normal center strongly suggests an effect of the periclinal arrangement of tunica layers on the ligule pigmentation of this composite flower. Another factor indicating the alteration of tunica layers is the different degree of pubescence of leaves of red flowering plants and that of yellow flowering plants. The red flowering plants had more pubescent leaves than yellow flowering plants. This corresponds with the finding of Darrow on the thornless blackberry (19) because both hairs on leaves and thorns on stems are derived from L-I. Both hairs on

the leaf surface and the pigment containing surface cells of the ligules are supposedly derived from L-I. Thus there might be a relationship between leaf pubescence and flower color on chrysanthemum also. Assuming that in a red flowering plant L-I has been replaced by L-II, and this L-II is also affected by colchicine which caused a partial chromosome duplication, then this plant could produce hairy leaves and dark red flowers.

No increase in red pigmentation was observed on EMS treated chrysanthemum plants. Instead, two yellow flowering plants were obtained. In addition to these two, there were several yellow-bronze chimeras in this treatment. This suggests that this mutagenic chemical suppressed the action of the gene(s) that governs formation of anthocyanin in chrysanthemum ligules. This could have happened by either abnormal pairing of purine or minor deletion of particular chromosome segments as suggested by Sato and Gaul (67). The effect of colchicine on chrysanthemum seems to be more complicated than that of EMS since some plants had increased cell size, stomatal size, and pollen size, combined with increased flower color intensity (from bronze to dark bronze and dark red) and others had an increase in cell size without increased flower pigmentation. This is probably due to exchange of L-I and L-II. Again this L-II may have been affected by the mutagenic chemical. When only relocation of the tunica layers occurred, cell size increased without flower color change. This could be vice versa.

Color intensity was expressed by two factors; pigmentation of the enlarged surface cells above the upper epidermis, and the population density of cells with a different degree of pigmentation (Figure 5, page

41). Actually it was observed that on the surface of the ligules there were large cells which were located above the upper epidermis (Figures 5 and 6, pages 43 and 45). These cells were full of cell sap containing various concentrations of crystalline anthocyanin. As was mentioned earlier, the thickness of these enlarged surface cells was greater on ligules of red blooms than of yellow blooms but there was no difference in width of the cells. Four different hues of the ligules were observed, these were dark red, dark bronze, bronze and yellow (Figures 5 and 6). The number of different colored cells per unit area determined the color expression of the ligules of the mutants of this chrysanthemum cultivar. Occurrence of several different color intensities in this cultivar may be due to differences in the number of genes governing these characteristics, as was discussed earlier. This could happen in two ways: the first possibility is based on the nature of this particular species which is an allopolyploid with about 54 chromosomes. There may be a tendency toward aberration during cell division in an allopolyploid plant. Consequently there may be a possibility of translocation or inversion of controller gene(s), resulting in color differences within a single ligule. The second possibility is change in the makeup of duplication of the gene(s) which are associated with color formation during mitotic division (1, 2, 16, 34, 62, and 69). This can be affected by application of either colchicine or EMS.

Although all mutations which were induced in this study may arise spontaneously, over a long period of time, the rate of occurrence of these mutations was unusually high in this study. If this particular

cultivar produced spontaneous mutations at the rate of 10 percent upon each vegetative propagation, this cultivar could no longer be called a stable cultivar. Fifteen percent of the adventitious buds produced some type of somatic aberration. In the case of adventitious buds treated with colchicine, in which group the highest number of somatic mutations was obtained, as many as 25 percent or one out of four buds produced a mutant.

Pyrus Malus cv. "Triple Red Delicious"

It is difficult to say whether any valuable mutation was induced in this species since no fruit has been produced as yet.

There appeared several single variegated leaves on apple trees treated with EMS. As was mentioned in the previous chapter infiltration of the mutagenic chemical, especially of EMS, was a major problem (54) and only a few apical meristems were physically affected by chemical treatment. The foliar variegation obtained in this study was limited to no more than three consecutively produced variegated leaves. None of these trees produced a stable, totally variegated shoot.

Similar results were obtained upon treatment with colchicine or colchicine plus chlorflurenol, but there were some trees which consistently produced large, deeply serrated highly pubescent leaves which are sometimes considered to be polyploids (28). Also, increased stomatal length was found on some of the leaves of the adventitious shoots. This indicates that L-I was affected by this chemical, producing these distinct characteristics (Figure 9, page 64). Also L-II appears to have been

affected as the thickness of the leaves often increased greatly upon treatment. Since there was a limited number of buds which were distinctly affected by the mutagenic chemical treatments, it could not be determined whether exchange, relocation or elimination of tunica layers occurred in this plant species.

CHAPTER VII

SUMMARY

The purpose of this investigation was to obtain somatic mutations of Chrysanthemum morifolium cv. "Mandalay," and Pyrus Malus cv. "Triple Red Delicious" through induction of adventitious buds and treatment with several mutagenic chemicals.

Both plant species produced adventitious buds. The process of adventitious bud development varied from species to species. Chrysanthemum produced a callus type adventitious bud on its disbudding wounds while on Pyrus adventitious shoots were initiated from prosphaeroblasts developed in the internodal region.

It was not expected that adventitious bud development would take place on Chrysanthemum morifolium cv. "Mandalay" under normal conditions. It was found that when this cultivar was disbudded and all primary shoots were topped to eliminate apical dominance, the wound tissue on the auxillary bud sites proliferated and gave rise to adventitious callus buds. The formation of adventitious buds on wound tissue of disbudded chrysanthemum plants was significantly increased by application of kenetin plus IAA. There were two types of adventitious buds in this cultivar; one from inner tissue origin, possibly from either endodermis or pericycle, and another directly from wound tissue callus, apparently parenchymatous cells.

The number of adventitious buds formed on disbudded Triple Red Delicious apple trees was not significantly increased by application of kinetin plus IAA in lanolin paste. The increased number of adventitious buds was mainly due to increased life span of the disbudded trees as affected by the application of kinetin.

There were two different origins of adventitious bud initiation in apple trees. The first type was of deep tissue origin probably from the pericycle, cambium or outer phloem. This type of adventitious bud had a good chance to survive and usually produced a single adventitious shoot shortly after it was formed. The second type was of shallow tissue origin directly beneath the epidermal tissue of the stem, possibly from the cortex. This type of adventitious bud lacked vascular connection to the existing conductive tissue, and had less chance to survive. For those that survived it usually took a longer time to initiate adventitious shoots but then they often produced more than one shoot.

Application of EMS on Chrysanthemum morifolium cv. "Mandalay" produced no progressive mutation. Instead, loss of certain characteristics, such as intensity of pigmentation and plant vigor was common. Both colchicine and chlorflurenol plus colchicine treated chrysanthemums produced a considerable range of somatic mutations, especially in regard to color intensity. Occurrence of increased color intensity in ligules was assumed to be due to both alteration of tunica layers and duplication of gene(s) or an increase in number of those chromosomes which govern color intensity of the flowers of this cultivar. Adventitious buds treated with mutagenic chemicals produced more somatic mutations than nontreated

adventitious buds and treated or nontreated normal buds. Twenty-five percent of colchicine treated adventitious buds produced some type of mutation.

Application of mutagenic chemicals on adventitious buds of this chrysanthemum cultivar increased the number of somatic mutations by 277 percent over that of normal buds treated with mutagenic chemicals.

It was found that infiltration of the mutagenic chemical, especially of EMS, was a major problem in Pyrus Malus cv. "Triple Red Delicious."

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APPENDIXES



APPENDIX I

TABLE 13

LEAF THICKNESS, LEAF SHAPE INDEX, AND STOMATAL LENGTH OF TWO
ADVENTITIOUS SHOOTS PRODUCED FROM A SINGLE
PROSPHAEROBLAST ON DISBUDDED PYRUS
MALUS cv. "TRIPLE RED DELICIOUS"

	Leaf Thickness (1/100 mm)	Leaf Shape Index (length/width)	Stomatal length (μ)
Shoot I	23.21 $\pm$ 3.49*	1.66 $\pm$.24	26.49 $\pm$ 3.37
Shoot II	23.95 $\pm$ 2.74	1.74 $\pm$.28	26.55 $\pm$ 2.99

*Mean $\pm$ 2 δ .

APPENDIX II

TABLE 14

LEAF THICKNESS, LEAF SHAPE INDEX, AND STOMATAL LENGTH OF TWO
ADVENTITIOUS SHOOTS PRODUCED FROM TWO DIFFERENT
PROSPHAEROBLAST ON ONE DISBUDDED PYRUS
MALUS cv. "TRIPLE RED DELICIOUS"

	Leaf Thickness (1/100 mm)	Leaf Shape Index (length/width)	Stomatal length (μ)
Shoot I	25.26 $\pm$ 2.17*	1.53 $\pm$.28	28.58 $\pm$ 2.45
Shoot II	21.36 $\pm$ 2.93	1.73 $\pm$.28	24.53 $\pm$ 1.34

*Mean $\pm$ 2 σ .

VITA

The author, son of Mr. Jin-Soo Choo, and Mrs. Jung-Hei (Kim) Choo, was born in Tokyo, Japan, on April 8, 1941, when his father was a law student at Waseda University in Tokyo. He went back to Korea in 1944 and was raised in Korea. He entered Seoul National University in 1959 and received the Bachelor of Science degree in 1963 with a major in Agricultural Biology. Upon graduation he joined the Korean Army and served as a lieutenant for two and one-half years. In September, 1966, he came to the United States of America and entered the graduate school of The University of Tennessee and received the Master of Science degree in 1968 with a major in Horticulture. In the summer of 1971, he was granted the Doctor of Philosophy degree with a major in Plant and Soil Science. He was employed as a laboratory assistant by the Department of Horticulture while completing the degree requirements.

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