COLCHICINE-INDUCED POLYPLOIDY IN THE AZALEA CULTIVAR CORAL BELLS

by

Jonathan Richard Weiss

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APPROVED:

R. S. Lindstrom, Chairman

T. M. Starling

G. C. Whiting

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Introduction

Prior to colchicine, various methods were used to double the chromosome number, including a heat treatment and chemicals. Colchicine largely replaced the other methods because it gave a higher percentage of polyploid tissues. One of the reasons for this higher percentage is that the concentration and length of treatment can be easily regulated.

Doubling of the chromosome number can increase the size and improve the texture of flowers, increase fruit size, alter the size and shape of leaves, as well as change other morphological characteristics. Clayberg (4) reported that sterile diploids can be made fertile by this doubling if they are sterile from a lack of chromosome pairing. Fertile tetraploids have been reported to breed true from seed.

Azaleas and rhododendrons are members of the Ericacaeae family, and were grouped by George Don in 1834 under the genus Rhododendron. Azaleas were placed in the series Azalea (subgenus Anthodendron) which is divided into six subseries or sections (18). The somatic (2n) number for the Azalea series is 26. Rhododendron calendulaceum and Rhododendron canadense are the only two tetraploid species (2n=52). Azaleas are divided into two classes, deciduous and evergreen. Coral Bells is evergreen and a member of the Obtusum subseries (section Tsutsutsi) which is Asiatic in origin. Two kinds of leaves (dimorphism) occur on plants of this subseries. There are the so-called "winter" or evergreen leaves, which are usually small and narrow and the broader "summer" leaves which are shed after cold weather begins (3).

The cultivar Coral Bells belongs to the Kurume group which

originated in Kurume, Japan (early 1800's) and was brought to the United States between 1915-1928. This group is believed to have been derived from natural hybrids among the following species: kaempferi, kiusianum, obtusum, and possibly komiyamae, sataense, and obtusum f. amoenum. Further intercrossings and selections were made in Japan from these natural hybrids (3,18) and Coral Bells is often used as a potted plant for the greenhouse trade.

Coral Bells is a low spreading plant with flowers about 1 1/8 inches in diameter. It flowers in early midseason, and its coral pink flowers are hose-and-hose and tubular in shape (18). Hose-and-hose flowered azaleas will not set seed because of the morphological change which allows the calyx to become the back hose. This change disturbs the normal development of the ovary (21).

There is a difference of opinion among researchers as to the best procedure to be followed with colchicine in order to induce polyploidy. The method used depends to a certain extent upon the type of plant material. The concentration of colchicine is often different among published reports, although .5% is most commonly used with woody material. There is greater variability among researchers concerning the number and length of treatments. Therefore, this study was designed to evaluate the best treatment for inducing polyploidy in azaleas. Three concentrations (.25%, .5%, .75%) of colchicine were applied on the second, third, fourth, fifth and sixth day after the terminal growth was removed and in another series of tests for 2, 3, and 4 consecutive days, to determine the optimum concentration of colchicine and/or length of treatment to induce polyploidy. Changes in certain

morphological characteristics would be desirable as a result of induced polyploidy. As an example, polyploid pollen could be used as a new source of germplasm for future breeding. Published techniques for evaluating polyploid tissue were modified in certain cases to permit better evaluation of treated material.

Review of Literature

Colchicine has been known since 1550 B.C. This drug is derived from the autumn crocus which belongs to the genus Colchicum. Colchicine is an alkaloid or a neutral substance with a homocyclic ring structure and its chemical formula is $C_{22}H_{25}O_6N$ (13).

There are several characteristics of colchicine that account for its effectiveness in inducing polyploidy in plants. It is soluble in water and alcohol, is not toxic to plant cells even in strong dosages, is effective in concentrations ranging from .01-1%, and the change brought about during treatment is wholly reversible (13).

Two important conclusions emerge from the numerous studies dealing with polyploidy and evolution. Polyploid species are abundant in nature (50% of all flowering plants are polyploid), and there are valuable horticultural as well as economic crop species that are polyploid. The two principle types of polyploids are autoploids, which have more than two genomes coming from the same species, and allopolyploids, which have more than two genomes coming from different species (13). Polyploidy results in a numerical change in the chromosomes, involving the addition of at least one genome or set of chromosomes (6).

In 1937, Gavaudan published the first paper dealing with polyploidy induced by colchicine (13). Since 1937, many herbaceous and woody plants have been treated with colchicine. In working with semi-woody and woody material, polyploidy was easily induced by colchicine treatments in pears, cranberries, and grapes. Although with more difficulty, colchicine has also been used to induce polyploidy in plums, black-

berries, cherries, peaches, and apples (12).

Arisumi (2) reported that as far as polyploidy is concerned, the important difference between monocots and dicots is the histogenic layer in which the gametes are produced. In monocot genera such as Hemerocallis and Lilium, gametes are produced partly from the L-I and partly from the L-II layers, whereas gametes arise from the L-II layer in dicots. Dermen believed that all herbaceous cells reached by colchicine were affected because cells in the nuclear phase advanced practically unhindered to metaphase. This accounted for the complete polyploidy in herbaceous plants. In semi-woody and woody plants, the occurrence of polyploidy is rare, and is usually confined to sectorial and periclinal cytochimeras (7).

Dermen (7) devised a system to determine ploidy by means of the histogenic layers in the vegetative shoot. He showed that the tip to the vegetative shoot resembles a dome and there are two principle directions of cell division in the dome. Anticlinal division takes place when cells multiply and grow in a spiraling manner to form a uniseriate layer. In periclinal division, cells are added one over another resulting in growth in depth. The rate of periclinal division increases from the outermost layer of cells to the inner region of the dome. The first or outermost layer of cells (L-I) divides anticlinally. Dermen (8) said that cells in the L-I layer may rarely divide periclinally, a condition occurring more frequently in other layers. Hunter (16) believed that a tetraploid epidermis did not necessarily signify internal tetraploid layers. Polyploidy may be determined by examining the leaves and stomata of the plant (7).

The second layer (L-II) is composed of central cells which usually divide anticlinally (7,16). Ackermen & Dermen (1) concluded that reproductive tissue arises from the L-II layer and 2-4-4 (L-I,L-II,L-III) cytochimeral branches should function as tetraploids in forming seed. Semeniuk & Arisumi (23) found that flower size and substance were good indicators of polyploidy in the L-II layer. Polyploidy may be determined by the pollen grain size (7,16).

Dermen (7) found that the third layer (L-III) of the dome divides both anticlinally and periclinally. Semeniuk & Arisumi (23) believed that because roots are derived from the L-III layer, thickening of the roots may indicate polyploidy in this layer. Dermen (8) stated that when the third layer became polyploid the inner portion of the dome was of the same ploidy. Root mitosis is used to check for polyploidy (7).

The polyploid condition of the central cell or cells determines the extent of polyploidy in each histogenic layer. Each histogenic layer is independent of the other layers. Dermen (7) also decided that it was a matter of chance as to whether one, two, or three cells held the central position of each histogenic layer. Consequently, if one cell was the most polyploidized, the layer in which that cell was found and the tissue to which it gave rise would be wholly polyploid. If two cells were centrally located and one was polyploidized and the other normal, half of the layer and tissue derived from that layer would be polyploid, and the other half would be normal. When three cells were centrally located, either one-third or two-thirds of the layer and the derived tissue could be polyploidized, and the rest normal. Sectorial polyploidy results when there is a partial effect in one layer (only

certain cells affected). Periclinal polyploidy results when one entire layer is affected but one or both of the other layers are not.

Apical polyploidy results when one, two, or all three of the central cells in the apical dome are polyploid. Polyploidy of apical origin would be detected in the part of the branch directly derived from the central cells of the apical dome following colchicine treatments. Polyploidy occurring in the meristematic cells of the apical dome other than centrally located cells is designated axial polyploidy. The closer the cells are to the apical center, the greater is the number of derivatives from them, and the greater will be the dimensions of the resulting polyploid tissue. If polyploidy does not occur in the center of the dome, the new tissue produced by these cells will be unaltered. The polyploidy in the portion of the branch derived from tissues in the treated bud would be of the axial type when colchicine is applied to a bud of a woody plant. Apical polyploidy would be found either in the new terminal bud, the new lateral buds, or in both types of buds. New shoots must form from these buds before the presence of apical polyploidy can be detected (7).

Mergen (19) noted that polyploid cells were larger than normal cells and irregular in pattern. Dermen (5) stated that a close correlation existed between the chromosome number and cell size. He later found that chromosome duplication could result in one of three types of tetraploids. In the first group, tetraploids could cause an appreciable increase in the size of each vegetative cell. However, the total number of cells making up the plant remained relatively the same as in the diploid form. Secondly, there might have been an increase in cell

volume following a doubling of the chromosomes but there was a decrease in the total number of cells making up a tetraploid plant. Finally, a doubling of the chromosomes may not have had any effect on the size of the cell but a change in cell size resulted in changes in the protoplasm. Dermen found that the ratio of cell volume to cell surface became altered and occurred because the surface of the cell body did not increase at the same rate that the cell volume increased. This volume and surface change affected the depth of the cells. A change in this relationship would occur between nuclear surface, and between these and the cytoplasmic volume and chromosome surface, nuclear volume and nuclear surface, and between these and the cytoplasmic volume and cell surface (6). Polyploidy seems to bring about an enlargement of cells in the polyploidized internal tissue without increasing the cell number in the internal tissue (11).

Dermen (8) used the term mixoploidy to describe a truly mixed polyploid condition found in stems, leaves, and roots a short time after colchicine treatment. Hunter (16) found that the colchicine treatment of stem tips on woody plants usually resulted in the formation of cytochimeras. The difficulty lies in the identification of the tetraploid sectors and their propagation. Dermen (8) determined that the chimeral polyploid conditions, found in shoot-tips of colchicine treated material after a growth period of several months to several years, should be termed cytochimera or periclinal cytochimera. These terms also apply to naturally occurring cytochimeral sports. Conditions presumably of genetic origin may be known merely as chimeras or periclinal chimeras when polyploidy is not involved in a chimeral plant. The term mixo-

chimeras indicates chimeras which occur in a haphazard fashion. Dermen (7) found a preponderance of sectorial and periclinal polyploidy in cranberry. This indicated that not all of the cells of the meristematic tissue in a treated area became colchicine affected. Affected cells might have included cells at the prophase stage as well as metaphase cells.

Dermen (6) found a sectorial polyploid-chimera to occur when one side of a growing bud became polyploid, while the other side remained normal. Workers (2,23) have observed that sectorial cytochimeras are unstable. A polyploid sector could become periclinal, or it could be eliminated depending on the central location of the diploid and polyploid cells of the shoot apex (23). Other workers (11,22,23) found that stable forms of polyploids were formed from these sectors by pruning the affected shoots at appropriate nodes to force the lateral bud located within a sector. Semeniuk & Arisumi (23) determined that when the bud was partly in the sector, the lateral was a sectorial chimera. Arisumi (2) found the chances of recovering tetraploid tissue from plants depended upon the size of the affected sector or sectors, and the probability of a new growing point developing in this sector or sectors.

Dermen (6) has shown periclinal ploid-chimeras to be epidermal layers and inner layers of cells which had different chromosomal constitutions. An example of this would be a plant whose stomata size indicated a polyploid condition while its pollen grains remained normal. Mergen (19) found that meristems of polyploids had a tendency to revert to a diploid condition and the entire meristem would go back to

a diploid condition or the meristem would produce side branches with diploid cell complements. Dermen (10) observed that in 3-6-6 and 6-3-3 chimeras in apples and pears, shoots could naturally convert to a normal 3x condition.

Pryor & Frazier (22) found that the growth of treated buds was delayed after a colchicine treatment. They forced buds to grow by carefully removing untreated buds, but retaining the leaves. Kehr (17) showed that rhododendron seedlings treated with colchicine grew more slowly than normal seedlings. Arisumi (2) found growth in daylilies to be retarded by six to eight weeks after treating them with colchicine. Dermen (9) stated, when working with grapes, that the first noticeable change from a colchicine treatment was the retardation of shoot growth in treated buds. Plants treated only three times with colchicine showed less retardation than plants treated five times during a similar time interval. Dermen (10) found no noticeable difference in the rate of growth between 2x and 4x tissue in chimeral apples, chestnuts, grapes, pears, peaches, and cranberries. However, he did find a difference in the rate of growth between 8x and 4x tissue in leaves of an 8-4-4 chimeral cranberry. Hull & Britton (15) noted that the early season retardation of shoot elongation in 2x-4x colchiploids was mild. In 4x-8x forms, retardation was severe, whether the latter originated from the doubling of a 4x seedling or from the twice-doubling of a 2x seedling. Dermen & Bain (11) explained that when internally and totally tetraploid branches were removed and grown on their own roots, they no longer showed the shortened internode type of growth. This indicated that the peculiarity of growth when a polyploid stem was

growing on a diploid root system was perhaps due to an unfavorable relationship which existed between the two cytologically different components of the same plant.

Ackerman & Dermen (1) and Moore et al. (20) stated that the abnormalities of basal leaves were good indicators that the colchicine solution had penetrated to the apical meristems. Dermen & Bain (11) established five categories of polyploid types of leaves and stems. These five categories were 1) total diploid, 2) epidermal tetraploid, 3) internal tetraploid, 4) total tetraploid, and 5) epidermal octoploid. Moore et al. (20) found distorted leaves near the base of the affected shoot while sectorially or totally polyploidized leaves were found further away from the base. Colchicine-induced changes within treated plants as reported in the literature are given in Table 1.

Although there is a wide range of flower shapes and colors in azaleas, as yet there are few tetraploid varieties. It is possible that tetraploid azaleas could add a new dimension in plant improvement and in breeding work especially with Rhododendron calendulaceum and Rhododendron canadense (22).

Table 1. Reported effects of polyploidy due to colchicine treatment.

Plant part	Anatomical changes due to polyploidy	Reference
Leaves	Distortion, usually large and/or thickened. Exceptionally dark green leaves. Leaves with halves of unequal widths. Contrasting light and dark green coloration. Deeper serrations and marginal teeth more pronounced on polyploid sectors.	1
	Leaf margins show tendency toward double serration on treated plants. Single serrated leaves on control plants. Stipules of laterals were longer and broader than those of control. First treated leaves to emerge were abnormally thickened and twisted, indicative of typical colchicine injury.	2
	Mosaic pattern in polyploid leaves (dark and light green). Darker leaf portions indicate tetraploidy. U-shape character in leaf at blade/petiole attachment point shows tetraploidy.	9
	Hexaploidy in epidermis of triploid, influenced pubescence, serration and veination of leaves.	10
	Abnormal in shape and patches of epidermis with enlarged stomata.	11
	Wide angle of juncture between the primary veins and midribs. In prominent veins, darkdened interveinal coloration. Primary veins tend to curve irregularly throughout most of their length. Basal portion of midribs of internally colchiploid leaves appear broader.	15
	Treated leaves are coarse, more prominent veins and larger interveinal islets of tissue. Sectorially polyploidized leaves appeared as having half of blade with an increase in width over other half of blade.	20
	Thicker veins and difference in color and texture of blade.	22
Stomata	As number of chromosomes increases, number of stomata decreases. Size of leaf has no influence on number of stomata.	14

Table 1, cont.

Plant part	Anatomical changes due to polyploidy	Reference
	Stomatal cells of tetraploids 1 1/4 times larger in diameter than those of diploids.	17
Flower	Larger, heavier petals	1,9,17,20,22
	Larger anthers, thicker styles and thicker flower buds. Tetraploid flowers have stiffer petals and deeper coloration than diploid.	2
	Flower size difference not as remarkable as difference in leaf size.	11
	Flowers of tetraploid rhododendron were reflexed backwards so they were reverse or saucer-shaped in contrast to tubular shape of diploids.	17
Pollen	No evidence of pollen abortion due to tetraploidy.	2
	Polyploidy increases pollen size and percent of imperfect pollen quartets.	11
	Tetraploid has larger pollen.	17
Fruit	Increase in fruit size due to internal tetraploidy.	9
	Difference in size of fruit of tetraploid plants is greater than difference in flower size. Tetraploidy in epidermis increased seed size but apparently did not influence fruit size.	11
	Size varied directly with ploidy of third histogenic layer.	15

Materials and Methods

Plants of the azalea cultivar Coral Bells were obtained from Blackwell Nurseries, Semmes, Alabama. They were grown in a greenhouse prior to being treated with colchicine. Four days before the treatments began, the plants were put into controlled environmental growth chambers. Plants were treated in the growth chambers in order to maintain optimum growing conditions. Growth chamber conditions included: day temperature 75°F, night temperature 70°F, relative humidity 60%, and artificial light for 12 hours. Following colchicine treatments, the plants were grown in the chambers for several weeks before being removed to a greenhouse. The plants used were liners and were placed in 4 inch azalea pots containing Canadian peat moss with the pH of the peat adjusted by $CaCO_3$ to 5.0-5.5. Plants were fertilized with either $(NH_4)_2SO_4$ or KNO_3 at 2 ounces per gallon based on soil test results.

Colchicine concentrations of .25% (6.25×10^{-3}M), .5% (1.25×10^{-2}M), and .75% (1.875×10^{-2}M) in a 10% glycerine solution were used for each single day treatment and for consecutive day treatments. The single day treatments were applied to plants whose terminal growth was removed at the same time and treated for only one day on the second, third, fourth, fifth, and sixth day after the removal of the terminal growth. Plants used for the control were treated on the sixth day with a 10% glycerine solution. Plants in another series of tests were treated for either 2, 3, or 4 consecutive days beginning on the second day after the terminal growth was removed. Control plants were treated with a 10% glycerine solution for 4 consecutive days.

All plants in this study were prepared and cared for after treatment in the same manner. Plants were pruned to 2 stems per plant prior to treating. Individual treatments were applied to 5 plants. Using random numbers, the plants were randomized in the growth chambers before and during treatments. The plants were kept in a random design after they were removed from the chambers.

The colchicine was dissolved in a few drops of 80% alcohol and then a 10% glycerine solution added. The solution was kept frozen when not being used. The colchicine solution was applied to lateral nodal regions after the terminal growth was removed because colchicine is most effective when the vegetative growth is very active. Using a micro-syringe, colchicine was placed on the lateral buds of the top 4 nodal regions of each of the 2 stems giving a total of 8 nodal regions per plant. Each nodal region was treated once in the morning and once in the afternoon using 2.5ul (microliters) of the colchicine solution per application. During the treatment period, care was exercised to make sure water was not splashed on the foliage while watering to prevent dilution of the solution. The untreated lateral buds were removed in order to promote the growth of the treated lateral buds. Shoots with leaves arising from treated regions were tagged and numbered for identification.

Methods of Determining Polyploidy

Leaves. Five leaves were sampled from each shoot to determine the effects of the treatments relative to induction of polyploidy within the individual leaves or for the entire shoot. Five stomata, including guard cells, from each leaf were measured for a total of 25 stomata per

shoot. The sampled leaves were determined by taking the total number of leaves on each shoot and dividing by 5. These numbers were used to determine how many leaves to skip between samples.

A 5mm disc was removed from the center, usually the widest point, of each sampled leaf. The sampling was kept constant to minimize the variability since polyploidy may change within a leaf. All discs were placed in separate vials and were later placed into a 1:1 solution of hydrogen peroxide and acetic acid, and boiled for 8-13 minutes in a 300° F water bath. The leaf tissues separated from each other after several rinsings in distilled water. The disc was divided so that a stoma from each quarter and one from the middle could be measured. The stomata were examined under 250 power and a mean size in square microns for the 5 stomata of each leaf was determined by multiplying the length x width x pi.

Mean stoma size was compared to the control mean by the use of confidence intervals at the 5% level. This test was used for the individual leaves and for the entire shoot. That is, significance was judged according to overlapping and non-overlapping of these confidence intervals. This type of statistical analysis was necessary because of the unequal variances of both leaf and shoot measurements.

For each concentration, equality of proportions of diploid leaves for different treatments was tested by a contingency table chi-square procedure. For example, with a .25% concentration for the consecutive day treatments, the contingency table and chi-square statistics are:

Consecutive days	Polyploid leaves	Non-polyploid leaves	Total leaves
2	3	102	105
3	6	99	105
4	1	69	70
	10	270	280

$\chi^2 = 2.48$ (5% critical point was 5.99)

A similar table was set up for each concentration, treatment day(s) and additional combinations of data.

Flowers. Early azalea cultivars are usually pre-cooled for 6 weeks prior to being forced into flowering in a greenhouse. However, irregular flowering will occur without this cool period. Treated plants did not receive a cooling period but were allowed to flower naturally. The central flower was removed from the shoot and several stamens squashed in a drop of water on a glass slide to get a mixture of the pollen tetrads. Fifteen pollen tetrads were randomly selected and the diameter at the widest point measured under 250 power.

When a shoot failed to flower, the central flower bud was removed and used for sampling pollen tetrads. The immature flowers selected were generally at the same stage of development. There is a difference in size between pollen tetrads found in a bud and those in an open flower. Due to this difference, pollen tetrads in the treated flowers were compared with pollen tetrads from buds of the control plants. Pollen tetrads from open flowers were compared to pollen tetrads of open flowers from the control plants.

A mean pollen tetrad size, in microns, was calculated for each

shoot. Statistical analyses based on confidence intervals and chi-square were used and are explained under the paragraph entitled leaves.

Roots. Treated shoots were removed from their plants and placed in a propagation bench containing perlite as soon as the pollen samples were taken. A heating cable provided bottom heat (70-72^{o}F). Each shoot was dipped into a rooting hormone (Hormodin 1) to induce quicker rooting. After the roots were removed, the shoot was again placed in the bench.

Roots were rinsed in distilled water and then placed for 2-4 hours in a killing solution made up of 1 part acetic acid, 3 parts 95% alcohol, and 1/2 part chloroform. The roots were then rinsed with distilled water and placed in a softening solution of 1 part hydrochloric acid and 9 parts distilled water for 2 minutes. Roots were immediately removed from the softening solution and after the root-tips were cut from the main root, they were placed in a few drops of aceto carmine stain, squashed with a flat needle, and placed under a cover slip. The root-tips under the cover slip were squashed again, heated slightly, and sealed with Krylon acrylic spray.

Root-tips were examined to determine their chromosome numbers. While actual counts were not possible, if a cell appeared to have the diploid number of chromosomes, then the L-III layer was labelled as being diploid.

Results

The number of polyploid leaves, based on significant deviations in stoma size from the control, for each concentration and day of treatment is presented in Table 2. A significant difference in the number of induced polyploid leaves was observed among the 3 concentrations of colchicine applied on the fifth day after the removal of the terminal growth. Even though the .5% solution induced a larger number of polyploid leaves, there was no significant difference among the 3 concentrations summed over the day of treatment or among these concentrations within any day of treatment other than the fifth. The combined totals (for all 3 concentrations) for each treatment day showed no significant effect relative to the day on which the treatments were applied.

The relationship of leaf position to the frequency of polyploid leaves at the various days after pinching is reported in Table 3. Treated shoots were found to have anywhere from 5 to 45 leaves (nodal regions) per shoot. Polyploid leaves were found to be located no higher than the twenty-first position even though leaves as high as 45 were sampled. The first leaf position represents the first leaf on a shoot starting from the base and progressively working toward the apex. Out of 71 polyploid leaves, 68 were located between the first and fourteenth leaf position on a stem.

A significant difference existed among the 5 sampled leaves for the total number of polyploid leaves for each individual concentration and the combined overall total (Table 4). A significant difference in the number of polyploid leaves existed among the 3 concentrations for

the second sample. No polyploid leaves were found in the fifth sample.

The number of shoots which exhibited a significantly larger mean stoma size than the control is presented in Table 5. There was no significant difference among the treatments but there was a noticeable reduction in the number of treated shoots which survived as the strength of colchicine increased. Consequently, there were fewer shoots having a mean stoma size significantly larger than the control among the 3 concentrations but this difference would not be true if percentages were used. When the number of shoots having significantly larger stoma than the control are combined for all 3 concentrations, 61% (173 out of 272) of the shoots had significantly larger stoma than the control.

The single day treatments induced a significantly larger number of polyploid leaves (71 out of 1360) than the consecutive day treatments (14 out of 500) based on chi-square analysis (Tables 2 and 6). The failure of the .75% concentration to induce polyploid leaves is reported in Table 6. A noticeable, although not statistically significant, difference existed among the single and consecutive day treatments in relation to the position of polyploid leaves on a shoot (Tables 3 and 7). In the consecutive day treatments (Table 7) polyploid leaves were not found above the ninth position, whereas in the single day treatments (Table 3) polyploid leaves were found up to the twenty-first position. The majority of polyploid leaves from the consecutive day treatments were found from the first through fifth position since these were the first leaves to come in contact with the colchicine (Table 7). A significant difference was found among leaves sampled within the .25% concentration and the overall total (Table 8).

There was significant difference among the 3 concentrations when applied for both 2 and 3 consecutive days as shown in Table 9. There was significant difference among the 3 concentrations of colchicine for the total number of shoots having significantly larger stoma than the control. Forty-one percent of the total number of examined shoots (41 out of 99) for the consecutive day treatments had significantly larger stoma than the control. Based on the number of shoots which had an overall stoma mean significantly larger than the control, the single day treatments induced a significantly greater number of shoots (173 out of 272) than the consecutive day treatments (41 out of 99) based on chi-square analysis.

Of 46 shoots treated for single days, only 5 shoots exhibited polyploid pollen (Table 10). Although polyploid pollen was induced there was no difference as a result of colchicine concentrations or the time of application. There was a significant difference among the 3 concentrations only when applied on the fifth day, for the number of shoots having polyploid pollen (Table 11). Five out of 39 shoots exhibited polyploid pollen when treated for consecutive days as shown in Table 12. The .25% concentration was the only concentration to induce polyploid pollen in the consecutive day treatments.

Based on chi-square analysis, the single day treatments did not significantly induce a higher number of shoots which contained polyploid pollen (45 out of 214), compared to the consecutive day treatments (5 out of 39). Fewer total shoots were sampled for the pollen tetrads (272) as compared to the number sampled for stomata measurements (371). This resulted because only certain shoots developed flower buds and

could not be examined for polyploidy in the L-II layer.

Table 2. Number of leaves out of total examined which exhibited symptoms of polyploidy, based on stoma size, when treated with 3 concentrations of colchicine for single days on various days following pinching of the terminal growth.

.25% colchicine concentration

Days following pinching	Polyploid leaves	Total leaves
2	7	100
3	4	110
4	5	105
5	5[z]	130
6	5	115
Total	26	560

.5% colchicine concentration

Days following pinching	Polyploid leaves	Total leaves
2	5	80
3	5	80
4	5	90
5	10[z]	95
6	5	105
Total	30	450

.75% colchicine concentration

Days following pinching	Polyploid leaves	Total leaves
2	1	55
3	2	65
4	4	55
5	2[z]	80
6	6	95
Total	15	350

Total for 3 colchicine concentrations

Days following pinching	Polyploid leaves	Total leaves
2	13	235
3	11	255
4	14	250
5	17	305
6	16	315
Total	71	1360

[z]Significant difference at the 5% level among the 3 concentrations for the number of polyploid leaves, applied on the fifth day after pinching.

Table 3. Relationship of leaf position to frequency of polyploid leaves, based on stoma size, from single day treatments.

Leaf position[z]	Number of polyploid leaves on indicated days following removal of the terminal growth.[y] 2	3	4	5	6	Total
1	0	0	1	2	0	3
2	3	3	2	4	1	13
3	1	3	0	1	1	6
4	2	1	2	4	3	12
5	1	0	2	0	3	6
6	1	0	3	1	1	6
7	0	0	0	1	1	2
8	2	2	1	1	4	10
9	1	1	0	0	0	2
10	0	0	1	0	0	1
11	0	0	0	0	0	0
12	0	1	2	2	1	6
13	0	0	0	0	0	0
14	1	0	0	0	0	0
15	0	0	0	0	0	0
16	0	0	0	0	0	0

[z]Position of leaf on treated shoot starting at the base and counting progressively toward the apex. Leaves were sampled above the 21 position but were not found to be polyploid.

[y]The number of leaves is the total from the 3 colchicine concentrations for each indicated day.

Table 3, cont.

Leaf position[z]	Number of polyploid leaves on indicated days following removal of the terminal growth.[y] 2	3	4	5	6	Total
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	1	1	2
21	1	0	0	0	0	1
Total	13	11	14	17	16	71

[z]Position of leaf on treated shoot starting at the base and counting progressively toward the apex. Leaves were sampled above the 21 position but were not found to be polyploid.

[y]The number of leaves is the total from the 3 colchicine concentrations for each indicated day.

Table 4. Number of polyploid leaves, based on stoma size, from the single day treatments in relation to the 5 sampled leaves taken per shoot for the indicated colchicine concentrations.

.25% colchicine concentration

Leaf sampled[z]	Days following pinching 2	3	4	5	6	Total
1	2	3	4	4	3	16[y]
2	3	0	1	0	0	4[u]
3	2	0	0	1	1	4
4	0	1	0	0	1	2
5	0	0	0	0	0	0

.50% colchicine concentration

Leaf sampled[z]	Days following pinching 2	3	4	5	6	Total
1	3	2	2	5	2	14[x]
2	2	1	2	3	3	11[u]
3	0	1	1	1	0	4
4	0	1	0	0	0	1
5	0	0	0	0	0	0

.75% colchicine concentration

Leaf sampled[z]	Days following pinching 2	3	4	5	6	Total
1	1	1	3	1	3	9[w]
2	0	0	1	0	2	3[u]
3	0	1	0	0	0	1
4	0	0	0	1	1	2
5	0	0	0	0	0	0

Total for 3 colchicine concentrations

Leaf sampled[z]	Days following pinching 2	3	4	5	6	Total
1	6	6	9	10	8	39[v]
2	5	1	4	3	5	18
3	2	2	1	3	1	9
4	0	2	0	1	2	5
5	0	0	0	0	0	0

[z]Five leaves sampled from each shoot with the first sample being taken near the base of the shoot and then sampling progressively toward the apex.

[y,x,w,v]Significant difference at the 5% level among concentrations and overall total.

[u]Significant difference at the 5% level among the 3 concentrations for the second leaf sample.

Table 5. Number of shoots out of total examined which exhibited significantly larger mean stoma size than the control when treated with 3 concentrations of colchicine for single days on indicated days following pinching of the terminal growth.

.25% colchicine concentration

Days following pinching	Shoots with larger stoma	Total shoots
2	13	20
3	17	22
4	15	21
5	14	26
6	14	23
Total	73	112

.50% colchicine concentration

Days following pinching	Shoots with larger stoma	Total shoots
2	12	16
3	8	16
4	11	18
5	15	19
6	12	21
Total	58	90

.75% colchicine concentration

Days following pinching	Shoots with larger stoma	Total shoots
2	7	11
3	11	13
4	5	11
5	8	16
6	11	19
Total	42	70

Total for 3 colchicine concentrations

Days following pinching	Shoots with larger stoma	Total shoots
2	32	47
3	36	51
4	31	50
5	37	61
6	37	63
Total	173	272

Table 6. Number of leaves out of total examined which exhibited symptoms of polyploidy, based on stoma size, when treated with 3 concentrations of colchicine for consecutive days following the pinching of the terminal growth on the second day.

.25% colchicine concentration

Number of consecutive days	Polyploid leaves	Total leaves
2	3	105
3	6	105
4	1	70
Total	10	280

.50% colchicine concentration

Number of consecutive days	Polyploid leaves	Total leaves
2	1	95
3	2	45
4	1	25
Total	4	165

.75% colchicine concentration

Number of consecutive days	Polyploid leaves	Total leaves
2	0	40
3	0	5
4	0	10
Total	0	55

Total for 3 colchicine concentrations

Number of consecutive days	Polyploid leaves	Total leaves
2	4	200
3	8	150
4	2	95
Total	14	500

Table 7. Relationship of leaf position to frequency of polyploid leaves, based on stoma size, from consecutive day treatments.

Leaf position[z]	Number of polyploid leaves from consecutive day treatments following pinching of terminal growth[y]			
	2	3	4	Total
1	1	3	0	4
2	0	2	0	2
3	0	0	0	0
4	2	1	2	5
5	0	2	0	2
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	1	0	0	1
Total	4	8	2	14

[z]Position of leaf on shoot starting at the base of the shoot and counting progressively toward the apex. Leaves were sampled above the ninth position but were not polyploid.

[y]The number of leaves is the total from the 3 colchicine concentrations for each consecutive day treatment.

Table 8. Number of polyploid leaves, based on stoma size, from the consecutive day treatments in relation to the 5 sampled leaves taken per shoot for the indicated colchicine concentrations.

.25% colchicine concentration

Leaf sampled[z]	Number of consecutive days 2	3	4	Total
1	1	6	0	7[y]
2	1	0	1	2
3	1	0	0	1
4	0	0	0	0
5	0	0	0	0

.50% colchicine concentration

Leaf sampled[z]	Number of consecutive days 2	3	4	Total
1	1	1	1	3
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	1	0	1

.75% colchicine concentration

Leaf sampled[z]	Number of consecutive days 2	3	4	Total
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0

Total for 3 colchicine concentrations

Leaf sampled[z]	Number of consecutive days 2	3	4	Total
1	2	7	1	10[x]
2	1	0	1	2
3	1	0	0	1
4	0	0	0	0
5	0	1	0	1

[z]Five leaves sampled from each shoot with the first sample being taken near the base of the shoot and then sampling progressively toward the apex.

[y,x]Significant difference at the 5% level among the 5 sampled leaves within individual concentration and overall total.

Table 9. Number of shoots out of total examined which exhibited significantly larger mean stoma size than the control, when treated with 3 concentrations of colchicine for consecutive days following the pinching of the terminal growth on the second day.

.25% colchicine concentration

Number of consecutive days	Shoots with larger stoma	Total shoots
2	13[z]	21
3	12[y]	21
4	5	14
Total	30[x]	56

.50% colchicine concentration

Number of consecutive days	Shoots with larger stoma	Total shoots
2	3[z]	19
3	2[y]	9
4	3	5
Total	8[x]	33

.75% colchicine concentration

Number of consecutive days	Shoots with larger stoma	Total shoots
2	2[z]	8
3	0[y]	0
4	1	2
Total	3[x]	10

Total for 3 colchicine concentrations

Number of consecutive days	Shoots with larger stoma	Total shoots
2	18	48
3	14	30
4	9	21
Total	41	99

[z]Significant difference among colchicine concentrations at the 5% level.

[y]Significant difference among colchicine concentrations at the 5% level.

[x]Significant difference among colchicine concentrations at the 5% level.

Table 10. Number of shoots out of the total examined which exhibited polyploid pollen tetrads, based on pollen tetrad size from open flowers, when treated with 3 concentrations of colchicine for single days on indicated days following pinching of the terminal growth.

.25% colchicine concentration

Days following pinching	Shoots with polyploid pollen	Total shoots
2	0	1
3	1	2
4	1	8
5	0	3
6	0	2
Total	2	16

.50% colchicine concentration

Days following pinching	Shoots with polyploid pollen	Total shoots
2	0	2
3	1	6
4	0	5
5	1	2
6	0	4
Total	2	19

.75% colchicine concentration

Days following pinching	Shoots with polyploid pollen	Total shoots
2	1	4
3	0	3
4	0	0
5	0	1
6	0	3
Total	1	11

Total for 3 colchicine concentrations

Days following pinching	Shoots with polyploid pollen	Total shoots
2	1	7
3	2	11
4	1	13
5	1	6
6	0	9
Total	5	46

Table 11. Number of shoots out of the total examined which exhibited polyploid pollen tetrads, based on pollen tetrad size from flower buds, when treated with 3 concentrations of colchicine for single days on indicated days following pinching of the terminal growth.

.25% colchicine concentration

Days following pinching	Shoots with polyploid pollen	Total shoots
2	3	14
3	4	11
4	2	9
5	3[z]	17
6	3	14
Total	15	65

.50% colchicine concentration

Days following pinching	Shoots with polyploid pollen	Total shoots
2	3	9
3	2	8
4	2	7
5	7[z]	12
6	2	14
Total	16	50

.75% colchicine concentration

Days following pinching	Shoots with polyploid pollen	Total shoots
2	2	3
3	1	9
4	1	12
5	1[z]	14
6	4	15
Total	9	53

Total for 3 colchicine concentrations

Days following pinching	Shoots with polyploid pollen	Total shoots
2	8	26
3	7	28
4	5	28
5	11	43
6	9	43
Total	40	168

[z]Significant difference at the 5% level among the 3 concentrations applied on the fifth day.

Table 12. Number of shoots out of the total examined which exhibited polyploid pollen tetrads, based on pollen tetrad size from flower buds, when treated with 3 concentrations of colchicine for consecutive days following the pinching of the terminal growth on the second day.[z]

.25% colchicine concentration

Number of consecutive days	Shoots with polyploid pollen	Total shoots
2	4	20
3	0	12
4	1	7
Total	5	39

[z]The .5% and .75% concentrations did not induce polyploid pollen and are not presented.

Discussion

Based on significant differences from the non-overlapping confidence intervals, induced polyploidy in the first histogenic layer, based on stoma size, resulted in 71 polyploid leaves out of 1360 in the single day treatments (Table 2). Forty-five of the 71 leaves appeared singularly while 10 shoots had 2 polyploid leaves out of the 5 sampled. Two shoots had 3 polyploid leaves out of the 5 sampled, but no more than 3 polyploid leaves were recorded for any one shoot.

There is a relationship between the number of polyploid leaves and leaf position on the shoot. The majority of polyploid leaves from single day treatments were located between the first and fourteenth leaf (Table 3). While samples were made of leaves above the twenty-first position, no polyploid leaves were found. This does not imply that polyploid sectors do not exist above the twenty-first leaf, but rather, that the greatest concentration of polyploid leaves is below the fourteenth leaf. It is apparent that the colchicine induced polyploidy in only a portion of the L-I layer, namely those cells which were first to come in contact with the solution.

It was found that most of the polyploid leaves fell into the first and/or second sample leaf positions (Table 4). Based on sampling multiples, the first or second sample leaf would lie within the first to fourteenth leaf range. The sampling procedure is very arbitrary in that only 5 leaves were sampled per shoot. Many of the sampled shoots had but one polyploid leaf above the first leaf position but there should have been additional polyploid leaves beneath this polyploid

leaf. When a polyploid leaf was reported above the first or lowest node, it must be assumed that much of the tissue below this reported leaf was in contact with the colchicine and this tissue should have been polyploid.

One of the side effects of colchicine is the retardation of plant growth. This was most noticeable in the consecutive day study. There was a remarkable reduction in the number of buds which developed as the colchicine concentration or length of treatment increased. The colchicine treatments not only retarded growth, but caused some treated buds to die. Apparently, the length of treatment is crucial in determining the number of buds which will survive. The single day as compared to the consecutive day treatments had a higher percentage of buds which grew even when compared at the .75% concentration.

The emergence of deformed leaves suggests a toxic level of colchicine on leaf primordia in the bud apex. The first few leaves were usually misshapen but leaf deformity was absent as the shoot elongated. Often, these deformed leaves were smaller in size. No effort was made to observe or measure other morphological changes in the leaves.

Many treated buds died within consecutive day treatments and there were fewer shoots and fewer polyploid leaves (Table 6). Fourteen polyploid leaves were discovered out of 500 sampled. Thirteen shoots had a single polyploid leaf while 2 leaves were found on one shoot. The majority of polyploid leaves were found between the first and fifth leaf on a stem (Table 7), and were usually the first sampled leaf (Table 8). A total of 85 polyploid leaves out of 1860 or about 5%

were induced by using colchicine in both the single and consecutive day studies. There was a significant difference between the overall number of polyploid leaves for the single day and consecutive day treatments (Tables 2 and 6). It may be concluded, based on chi-square analysis, that single day treatments induced significantly more polyploid leaves than the consecutive day treatments.

There is an indication based on the location of the polyploid leaves that the chemical acted on the young developing leaves surrounding the bud apex and/or the first few leaf primordia. Incomplete polyploidy of the first histogenic layer suggested the formation of sectorial cytochimeras since none of the shoots had 5 polyploid leaves. Sectorial cytochimeras are not usually stable and shoots displaying them may revert back to the diploid state. Periclinal cytochimeras might also be present but further cytological work would be necessary before a final decision could be reached. Complete or partially polyploid shoots can be forced from the axil of the leaf by pruning the stem to the leaf where the sector of polyploid tissue is found. The growing point of the lateral bud would be another sectorial or periclinal cytochimera or possibly a complete polyploid, depending on the nature of the polyploid sector.

Sixty-four percent of the L-I layers in the single day treatments and 41% in the consecutive day treatments were significantly larger than the control when a mean stoma size was calculated for the entire shoot (Tables 5 and 9). Very few shoots having an overall stoma mean significantly larger than the control had individual polyploid leaves.

The reason is that the sample size for an individual leaf was too small for an adequate measurement of the leaf. To have a shoot without leaves which were polyploid, there must be some polyploid tissue within the shoot. It is possible that the leaf discs were taken from diploid tissue rather than polyploid tissue, or on the fringe of a diploid/polyploid sector. The mean stoma size of the individual leaves was within the upper range of the control and was not significantly different from it because of the small sample size that was used. When all 5 leaves are added together, their accumulated mean becomes significantly greater than that of the control.

A larger sample size is recommended for individual leaves and more than one area of the leaf should be sampled in order to get a better representation of the leaf. Had larger samples been used, it is believed that additional polyploid leaves would have been found. The analysis of an entire shoot may be a better method to evaluate polyploidy in the L-I layer than individual leaves because it uses a larger sample size than the individual leaf, which in turn tends to magnify the differences between treated and nontreated material. Shoots having an overall stoma size significantly larger than the control, are either partially or completely polyploid for the L-I layer. The single day treatments induced a significantly greater number of polyploid L-I layers than the consecutive day treatments based on chi-square analysis (Tables 5 and 9).

Published papers have rarely given any precise methods of measuring polyploidy in the L-I layer and have not used statistical analyses.

This study has shown that statistical analyses can be successfully used to determine the difference between treatments in inducing polyploidy in both the L-I and L-II layers.

Polyploidy was successfully induced in the L-II layer. Five polyploid L-II layers were found from 46 sampled shoots in the single day treatments (Table 10). Flowers having polyploid pollen were not noticeably larger and it is possible that in the case of Coral Bells, polyploidy does not result in an enlargement of the flower. In this study, the majority of shoots were tested by using pollen tetrads from flower buds, and this allowed for earlier evaluation of treated material. It is a faster method for plants requiring a cooling period prior to flowering or for those which require a long time to flower.

If pollen tetrads are measured from flower buds, they should be compared with those of flower buds from the control and not open flowers. Apparently, as the flower bud develops, the tetrad increases in volume so that it is larger when the flower is open.

Forty polyploid L-II layers were induced out of 168 (24%) in the single day treatments (Table 11) as indicated by the size of pollen tetrads from flower buds. There was also a difference among the 3 concentrations applied on the fifth day. A similar response was obtained as measured by the number of polyploid leaves (Table 2), and this can be related to the effect of concentration for that one day because no other difference was observed among the 3 concentrations for other single day treatments.

Twenty-two out of 45 polyploid L-II layers were polyploid for the L-I and 15 were polyploid for the L-I and had at least one

polyploid leaf (Tables 10 and 11). Eight shoots were found to be polyploid in the L-II layer but did not exhibit polyploidy in the L-I. These particular shoots probably possess internal polyploidy. Internal polyploidy exists when either the L-II and/or L-III are polyploid but not the L-I. One hundred and thirty-six shoots were not polyploid for the L-II but were polyploid for the L-I and 27 of these also had polyploid leaves. A shoot exhibiting polyploidy only in the L-I is an epidermal polyploid.

In the consecutive day treatments, 5 out of 39 shoots displayed polyploid L-II layers (Table 12). Of these, 2 were internal polyploids, 2 were polyploid for the L-I, and 1 was polyploid for the L-I and had a polyploid leaf. These shoots were found only on plants treated with the .25% concentration. Thirty-eight shoots were not polyploid for the L-II but were polyploid for the L-I and of these, 9 had polyploid leaves.

Fifty polyploid L-II layers were induced out of 253 (20%) when the single day and consecutive day treatments were combined. There was no significant difference between the single or consecutive day treatments in the total number of induced polyploid L-II layers, indicating that no one treatment was superior to another in inducing polyploid L-II layers.

Results of the root-tip squashes were very poor. Cuttings were made after the wood had become somewhat hardened and azaleas will not root well if the wood is too hard. Despite a rooting hormone and good rooting conditions, very few of the cuttings rooted. Due to poor

rooting, only a few shoots were analyzed. Though actual chromosome counts were not possible, the cells appeared to have the diploid number of chromosomes which would indicate diploidy in the L-III layer.

Care must be taken to obtain a complete polyploid shoot if asexual propagation is desired. If the plant breeder is interested only in polyploid pollen, a completely polyploid shoot is not needed. There appeared to be no major change in visual morphological characteristics and without these changes, it is doubtful that a polyploid Coral Bells would be beneficial to a greenhouse grower.

This study has shown that colchicine can induce polyploidy in the azalea cultivar Coral Bells. None of the individual methods of treatment or concentrations were decisively different from each other. The single day treatments as a whole, induced a greater number of polyploid L-I layers compared to the consecutive day treatments. As the length of and/or strength of concentration increased, the number of surviving lateral buds decreased. For this reason, consecutive day treatments and concentrations above .5% are not recommended. It is believed that the length of treatment is more crucial than the strength of the concentration and it would be interesting to see what kinds of results would occur using a .25% or .5% concentration for alternate days.

The techniques used to determine polyploidy were basically the same as those employed by other published reports. Two major differences were that statistical analyses were successfully used to determine polyploidy instead of visual morphological changes, and flower buds as well as open flowers were used to obtain pollen tetrads.

Sample size is very important when testing for polyploidy especially in the L-I layer. It is suggested that more than 5 stomata be sampled from each leaf and several samples be taken from the leaf in order to magnify differences between treated and nontreated material.

Induced polyploidy was found in the L-I and L-II layers but not in the L-III layer. No completely polyploid shoots were discovered but the presence of polyploid pollen could add new germplasm for future breeding.

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Appendix

Examples of stoma size for individual polyploid leaves, shoots polyploid for the L-I layer based on overall stoma size, and pollen tetrad size for polyploid L-II layers, compared to their respective control means.

I. Mean stoma size in square microns for individual leaves.

A. Single day treatments.

Control	Polyploid examples
1962 ± 291	2538 ± 282
	2643 ± 345
	3357 ± 307

B. Consecutive day treatments.

Control	Polyploid examples
1969 ± 268	2498 ± 217
	2501 ± 189
	3410 ± 285

II. Mean overall stoma size in square microns for individual shoots.

A. Single day treatments.

Control	Polyploid examples
1962 ± 90	2145 ± 87
	2366 ± 88
	2483 ± 99

B. Consecutive day treatments.

Control	Polyploid examples
1969 ± 82	2153 ± 99
	2322 ± 104
	2400 ± 60

III. Mean pollen tetrad size in microns for individual L-II layers.

A. Single day treatments.

1. Open flower

Control	Polyploid examples
40.91± 1.14	43.32± 1.09
	43.46± 1.07
	44.65± 1.29

2. Flower bud

Control		Polyploid examples	
36.87±	.92	39.04±	1.23
		40.94±	1.02
		44.48±	1.21

B. Consecutive day treatments

Control		Polyploid examples	
37.55±	1.15	40.00±	1.17
		42.47±	1.89
		56.68±	1.43

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COLCHICINE-INDUCED POLYPLOIDY IN THE AZALEA CULTIVAR CORAL BELLS

by

Jonathan Richard Weiss

(ABSTRACT)

Lateral buds of the azalea cultivar Coral Bells were treated twice a day on either the second, third, fourth, fifth, or sixth day following the terminal pinch with one of 3 colchicine concentrations (.25%, .5%, .75%). The remaining plants were pinched the same day and the consecutive day treatments were started 2 days after the pinch. Plants were treated for 2, 3, or 4 consecutive days.

Colchicine-induced polyploidy was found in the first and second histogenic layers but not in the third layer and a completely polyploid shoot was not isolated. Evidence indicates the formation of sectorial and periclinal cytochimeras. Within the single and the consecutive day treatments, none of the 3 colchicine concentrations or length of treatments were decisively different from each other. However, the single day treatments, when combined, induced a significantly higher number of polyploid L-I layers compared to the consecutive day treatments. It was found that as the concentration and/or length of treatment increased, there was a decrease in the number of viable vegetative buds.

Morphological changes were not observed within treated material. Induced polyploidy in the second histogenic layer resulted in the formation of polyploid pollen which could be used as a new source of germplasm.