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INDUCTION OF POLYPLOIDY IN ORCHIDS  
BY THE USE OF COLCHICINE.

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ARTIFICIAL INDUCTION OF POLYPLOIDY IN  
ORCHIDS BY THE USE OF COLCHICINE

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By  
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ARTIFICIAL INDUCTION OF POLYPLOIDY  
IN ORCHIDS BY THE USE OF COLCHICINE

INTRODUCTION

The study of chromosome numbers of plants has shown the occurrence of a large number of natural polyploids. In related species of plants entire series of polyploids have been revealed. Muntzing (1) indicates that at least 50 per cent and probably more of the angiosperms are polyploids and that they have played an important role in the evolution of plant species. Presumably, polyploids were able to invade new habitats because of unusual vigor, growth, and general adaptability. Plant breeders and geneticists have become aware of the increased fertility of the naturally occurring amphidiploids over their ancestral hybrids.

Cytological investigations have demonstrated that many plants selected from the wild for domestication are polyploids. These were selected because of specific, superior values even though the ploidy of these plants were unknown at that time. With increased knowledge of the nature of polyploids and the realization of their importance in plant improvement programs and in genetic studies, breeders have specifically bred for polyploid types. They have concluded that in many instances polyploids are superior to their diploid counterparts in vigor, size, quality, texture, and other characteristics.

In the orchid industry, polyploidy has come to assume a prominent role in the production of superior types. Kamemoto (32) has shown that the award winning Cattleya hybrids such as C. Balmar, C. Bow Bells, and C. Joyce Hannington were triploids. Storey (57, 58) and Kamemoto (33,

34) showed that superior selections of hybrids in the vandaceous group were triploids, tetraploids, and pentaploids. Kosaki (36) working with Dendrobiums, showed that a number of superior plants were triploids and tetraploids. Polyploid plants developed in the early years of orchid breeding originated through a number of avenues. Many occurred through spontaneous doubling in the somatic tissues and non-reduction of gametes, while others were the result of polyspermy and polyploidization of gametes. Many present day advance generation hybrids are the results of hybridization of these polyploids.

In the course of an extensive breeding program in orchids, relatively high sterility among intergeneric hybrids and to a lesser degree among interspecific hybrids was encountered. The cytological basis for such sterility in orchids is largely that of non-homology of the chromosomes (32, 57, 58, 33, 34). Because of the highly sterile nature of many diploid hybrids as well as triploid strains, it was found desirable to overcome this sterility barrier in order to advance the breeding program. Previous workers have demonstrated with plants other than orchids that this sterility barrier could be removed by doubling the chromosome numbers of the sterile types, and that certain chemicals such as colchicine are effective in inducing such changes. Thus far, attempts that have been made both here and in other areas indicate considerable difficulty in inducing polyploidy artificially in orchids. Furthermore, no studies have been conducted on the behavior of such induced polyploids in orchids, and very little work has been done relative to comparison of morphological differences between diploids and induced polyploids.

Following upon the above considerations, the thesis as presented

here involved three major objectives. The first objective was to devise practical methods for inducing polyploids in selected orchid groups by the use of colchicine. The second objective entailed the study of the characteristics of the induced polyploids, with these types being compared with their diploid counterparts in respect to morphological differences. The third objective involved an attempt to analyze and explain the induction of cytochimeras found among most of the induced polyploids.

#### REVIEW OF LITERATURE

With the realization of the tremendous importance of natural polyploids in fundamental studies in evolution, genetics, cytology, and morphology, and the vast potentialities in practical application, it was a natural reaction for plant breeders and geneticists to attempt the duplication of these polyploidal conditions artificially.

The first artificially induced polyploid in plants is credited to Girassimov, who treated Spirogyra by means of temperature shock (19). This treatment consisted of subjecting the specimen to cold temperature of 5-10° C. for a specific duration, and alternated by exposure to heat treatment of approximately 35° C. Randolph (48) used high temperature for Maize embryos and produced some polyploids. Dermen in 1938 (9) reported the induction of polyploidy by similar treatment. In both cases positive results were few.

Radiations such as X-rays and ultra-violet rays have also been utilized in the past in attempts to induce doubling of chromosomes (19). However, these radiation methods were found to be relatively inefficient in doubling. Muller (41) and Randolph (49) have reported the frequent occurrence of aneuploids and other chromosomal aberrations when plant

parts were subjected to radiations but no doubling was obtained.

Before the advent of colchicine, other chemicals such as mustard gas and sodium cacodylate were used in the study of mitotic activities (19). However, these chemicals were used more as mutagenic agents and were relatively ineffective in doubling the chromosome numbers.

Studies in cellular activities and nuclear divisions both in Europe and in America through the use of colchicine led to the discovery of the ease with which chromosomes could be doubled. The announcement of colchicine polyploidy by the Brussels workers and independently elsewhere by Gavauden, Blakeslee and Eigsti (28) in the late thirties initiated a "fad" in colchicine polyploidy. The highly efficient nature of colchicine in doubling the chromosome numbers has been unquestionably demonstrated by many investigators in fruit crops such as peaches, grapes, strawberries (12, 13, 14, 15); in floricultural crops such as zinnias, petunias, Easter lilies, cosmos (7, 8, 21, 22, 44); in vegetable crops such as tomatoes, muskmelons, lettuce (3, 45, 54, 59); and in large number of field crops (6, 61, 18, 25, 43). Many of these induced polyploids were found to be superior to their diploid counterparts and have been incorporated in commercial production.

In orchids several attempts at inducing polyploidy with colchicine have been reported, but the results have not been too encouraging or as successful as with other plants. The first report of induced polyploidy in orchids is by MacLeod (39) in 1947. He reported delayed flowering and two sizes of embryos in selfed seeds of treated flowers. In Laelia anceps he reported that the treated flowers were twice the normal size and had intensified color. Moore (40) in the same year reported on his work involving Cattleya Trianaei var. alba. Leaves of some colchicine-

treated plants were tough and wrinkled and the plant seemed to have increased in its blooming capacity. Rotor (51) has reported considerable success in the treatment of a number of genera of orchids. However, in all of these cases achievement of polyploidy was assumed on morphological evidences and not on cytological studies. Although certain gigas conditions are highly correlated with higher ploidy, as evidenced in other plants, cytological determination should be the ultimate determinative in such studies.

The use of colchicine in the immediate production of improved varieties of plants overshadowed the more subtle but fundamental uses in the study of cellular activities. It was found that colchicine in very minute quantities could cause metaphasic arrests much more efficiently than all previously used methods. Furthermore, the ease of application of colchicine made it a powerful tool for the investigation of fundamental studies in cytology.

Levan (37), Hawkes (29), and others have reported the lack of any effect upon the interphase nucleus, even up to around the middle of prophase. From late prophase to metaphase the effects of colchicine were found to be rather pronounced. Chromosomes began to contract and the relational coiling gradually disappeared. Chromosomes became scattered throughout the cell, resembling normal diakenesis (29), (46). According to Levan (37), as the relational spirals uncoiled, the chromosomes formed loops between undivided centromeres. This process resembled the terminalization of chiasmata in diakenesis bivalents. Because of the delay in the division of the centromere, when the chromatids finally separated from the loop formation, a cross-shaped configuration was produced.

Since this is typical of colchicine effect, Levan termed it c-mitosis. The delay of centromere division and the scattered distribution of the chromosomes were attributed to the inactivation of the spindle mechanism.

Another characteristic of c-mitosis was the prolonged period of metaphase with consequent accumulation of metaphasic configurations (37, 29). It was also reported by the above investigators that anaphase and telophase were by-passed and restitution nuclei followed metaphasic stage. A considerable amount of work has been directed towards the study of spindle fibers and cell wall formation (60, 27, 30). According to these workers, cell plate formation is the direct function of the spindle mechanism. The spindle fibers were reported to be responsible for the production of a phragmoplast, which formed the basis for the cell plate.

In meiosis the general aspects of colchicine effects were very similar to those found in mitosis. Levan (38) found that there was an unusual number of univalents and a very low chiasmata frequency in treated material. c-mitosis configurations and the lack of congression at the metaphase plate resembled the mitotic counterparts.

Colchicine was also found to influence the production of aneuploids due to the breakdown of bivalent associations between chromosomes (10, 20, 56). Emsweller (20) found tri, octo, hexa- and some pentavalents in higher ploidy forms. These odd associations led to unequal distribution of chromosomes, resulting in the production of aneuploids.

The use of colchicine as a mutagenic agent was reported by Franzke and Ross (25), (26) and by Ross, et al. (50). Colchicine treated plants of a true breeding sorghum variety gave rise to variants possessing a

number of ancestral characteristics, some of which bred true immediately. In subsequent investigations they induced without change in the chromosome number a lineal series of mutants which were true-breeding (26). Some of these mutants were different from any types known to be in the pedigree. Their earlier hypothesis attributed this change to unmasking of gene blocks through reductional grouping of somatic chromosomes. Recently, they proposed the theory that gene mutations both prior and subsequent to a somatic reductional division followed by chromosomal doubling would result in homozygous mutants (26). If colchicine can induce mutations with the constancy indicated in these experiments, a new line of approach to the use of colchicine would be opened.

Another interesting use of colchicine is in the study of histogenic regions in the root and shoot apex and the ontogenetic relationship of organs to these histogenic areas. Colchicine-induced sectorial chimeras in Datura by Satina, et al. (53), and Satina and Blakeslee (52); in Solanum tuberosum by Baker (1); in Cranberry by Dermen (16) and many others have been used to demonstrate the detailed nature of the germ layers in the apical meristems and to explain the ploidy of organs by means of ontogenetic relationships of these organs and tissues to the origin in the germ layers.

These and many other contributions to science are recorded in the literature relative to the use of colchicine.

#### MATERIALS AND METHODS

##### MATERIALS

Colchicine--Colchicine is an extremely poisonous alkaloid extracted from the seeds and corms of Colchicum autumnale which belongs to the

family Liliaceae. Chemically, its nucleus is composed partially by hydrogenated phanthrene ring and is considered to be highly complex. Its empirical formula is  $C_{22}H_{25}O_6N$  (51). Colchicum autumnale is found in abundance around the eastern part of the Black Sea.

Plant materials--In Table I are listed the identities of the orchid materials used in the treatments. In the case of vandaceous plants, the leaf type distinguishing the three groups of Vanda are indicated. Parts of the plant used in the treatments and the normal diploid chromosome numbers of these plants are also given whenever possible.

Nutrient agar--The nutrient agar used in the germination of treated and untreated seeds is known as Martin's Formula, after Mr. J. P. Martin of the Experiment Station of the Hawaiian Sugar Planters' Association, who first formulated and used it for orchid seed germination. The formula and method of preparation are given under the section on methods.

#### METHODS OF PREPARING COLCHICINE IN VARIOUS CARRIERS

##### Solution method

- a. aqueous solution--Colchicine was weighed out in required amounts and dissolved in tap water.
- b. glycerine-colchicine solution--Required amounts of colchicine were dissolved in the following mixture, modified after Dermen (13): glycerine - 16.5 mls., water - 5.5 mls., and 10 per cent solution of Triton B-1956 - 3 mls. The last ingredient is a sticker and spreading compound. According to Dermen, glycerine provides a non-volatile medium which keeps the treated area moist and holds the colchicine in place to be absorbed gradually.

Lanolin paste method--Measured amounts of lanolin were melted (ca.

TABLE I. LIST OF PLANT MATERIALS USED IN THE EXPERIMENTS.

Name	Cross	Leaf Type	Parts of Plant Used	Chromosome Number (2n)
Vanda Miss Joaquim	V. teres x V. hookeriana	terete	tip cuttings young shoots flower spike seeds	38
V. Michael Beaumont	Manila x Roeblingiana	strap-leaf	mature plant	38
V. Princess Elizabeth	V. hookeriana x V. sanderiana	semi-terete	tip cuttings	38
V. Fair Queen	V. Clara Fisher x V. Rothschildiana	strap-leaf	seeds	--
	V. Ellen Noa x V. Bill Sutton	strap-leaf	seeds	--
Dendrobium Molokai	D. Colin Potter x D. gouldii		young shoots	38
D. undulatum	species		seeds	38
D. undulatum var. Bromfieldii	species		seeds	38
	D. Dr. Carter x D. schulleri		seeds	--

55° C.) and the required amounts of colchicine were added to the melted lanolin, stirred until completely dissolved and left to solidify. This material was applied to plant parts with a toothpick.

Colchicine-nutrient agar--Nutrient agar (Martin's Formula) was prepared according to the following formula:

Ca(NO <sub>3</sub> ) <sub>2</sub> ·H <sub>2</sub> O . . . . .	1.00 gm.
KH <sub>2</sub> PO <sub>4</sub> . . . . .	1.00 "
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0.50 "
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> . . . . .	0.25 "
NH <sub>4</sub> NO <sub>3</sub> . . . . .	0.25 "
Tap water . . . . .	.1000.00 mls.
Peptone . . . . .	5.00 gms.
Sucrose . . . . .	20.00 "
Agar . . . . .	12.00 "
FeSO <sub>4</sub> ·(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ·6H <sub>2</sub> O--0.7 gm. per 100 ml. of water as stock solution. 2 mls. of this solution is added to a liter of nutrient agar to satisfy the iron requirement.	

The inorganic salts were first dissolved in one liter of tap water. To this was added 2 mls. of ferrous ammonium sulfate from the stock solution and the entire contents heated to boiling. Peptone, sugar, and agar were then added with constant stirring. After the latter three ingredients were completely dissolved, pH was adjusted to approximately 5.2 and the solution poured into 250 ml. erlenmeyer flasks in approximately 100 ml. proportions. To these flasks, required amounts of colchicine were added and stirred until dissolved. Each flask was plugged with cotton stopper and placed in an autoclave for sterilization at 15 pounds pressure for 15 minutes. After sterilization, the flasks were left in room temperature to cool and solidify.

## METHODS OF COLCHICINE TREATMENT

Treatment of seeds

a. Treatment of seeds before sowing--Seeds of Vanda and Dendrobium plants were first soaked in five concentrations of aqueous solutions of colchicine ranging from .05 to 1.0 per cent and for durations of one to ten days. Control lots were soaked in water. As the colchicine treatments ended, the seeds were washed in water and disinfected in diluted "Clorox"<sup>1</sup> solution (1:50) for 15 to 20 minutes. Seeds were then pipetted into erlenmeyer flasks containing nutrient agar under aseptic conditions. Flasks were left on shelves in the transfer room for germination and growth. Because of relatively high incidence of contamination, several series of the same treatment were repeated to ensure availability of all series of treatments. In all series each treatment was replicated four times.

When seedlings were approximately 1.5 to 2 centimeters high with sufficient roots and leaves, they were removed from the flasks and transplanted in flats containing peatmoss as the planting medium. Peatmoss was drenched with a dilute (2 tablespoons/gal.) solution of Tersan to prevent damping off of seedlings. These flats were placed in a shaded glasshouse for further growth and observation. Flats were watered daily and a complete fertilizer was

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<sup>1</sup> "Clorox" - Commercial disinfectant containing 5.25% dry weight of sodium hypochlorite as active ingredient.

applied bi-monthly. When plants were approximately 10 months old, with good root system, chromosome counts were made from root-tip squashes.

b. Seeds sowed in colchicine agar--Seeds of Vanda and Dendrobium were sterilized in "Clorox" and sown in colchicine incorporated nutrient agar following the procedure outlined in the above section. Colchicine concentrations ranged from 0.01 to 1.5 per cent. Again, several series with each treatment replicated 3 to 4 times were run to allow for contaminations. Seedlings surviving the colchicine treatments were transplanted in flats containing peatmoss when sufficient roots were established. Control lots contained no colchicine in the agar.

c. Treatment of protocorms--On the premise that the actively growing condition is most conducive to treatment effects, seeds of Vanda Miss Joaquim, Dendrobium undulatum, and D. undulatum var. Bromfieldii were first sown in nutrient agar without any pre-treatment with colchicine. When seeds germinated to form green protocorms, approximately 0.6 cc. of sterilized aqueous colchicine solution was pipetted into each flask under aseptic condition. The colchicine solution was swirled around in the flask each day to wet the protocorms. Concentrations ranged from 0.01 to 1.5 per cent and each treatment was replicated four times. Again, several series were run to allow for contaminations.

#### Treatment of seedlings

D. undulatum seedlings, approximately 1 to 3 centimeters tall, with good root system were soaked in aqueous colchicine solutions

ranging in concentrations from 0.01 to 2.00 per cent under two methods. The first method was designated as controls wherein seedlings were placed in vials containing the colchicine solution and left immersed for three hours. At the end of three hours, the seedlings were washed in tap water and planted in flats containing peatmoss as the planting medium. The second method, designated as infiltration method after Braak and Zeilinga (4), consisted of immersing the seedlings in vials containing colchicine solution and placing the vials in an exsiccator in which a vacuum was created by means of water vacuum pump. In seven minutes the solution began to bubble. At that point, the pump was stopped and the plants were allowed to remain in vacuum for 10 minutes. This procedure is supposed to evacuate the air from the plants and allow the solution to penetrate the tissues more readily than by mere soaking without the vacuum. After 10 minutes, air was admitted gradually into the exsiccator and the plants were removed, washed, and planted in peatmoss flats for later chromosome counts.

Treatment of inflorescence, cuttings, young shoots, and apical meristems of mature plants

a. Treatment of inflorescence--Flower spikes approximately 2.5 to 20 centimeters long were selected and the apical region of the spikes was covered with absorbent cotton saturated with an aqueous solution of colchicine ranging in concentration from 0.1 to 2.0 per cent. The tips were then covered with polyethylene bags held in place with rubber bands to prevent drying of cotton. Duration of treatments extended from 8 hours to 5 days. For the

treatments of longer durations, the cotton wrappers around the tips of the spikes were wetted twice. At the end of the treatment, the polyethylene covering and cotton were removed and the spikes were left to develop. Whenever possible bud materials from these treated spikes were used to determine ploidy.

b. Treatment of tip cuttings of mature plants--Tip cuttings of V. Miss Joaquim and V. Princess Elizabeth, approximately 8 to 10 inches long, were made and the basal ends were immersed in vials containing aqueous solutions of colchicine. The concentrations ranged from 0.1 to 2 per cent and the duration of treatments ranged from one to twenty days. Cuttings were removed after the prescribed time and planted in cutting boxes containing wood shavings as a growing medium. When growth resumed and roots emerged from sections above the original terminal positions, root-tip smears were made for microscopic confirmation of ploidy. In a few cases, chromosome counts were made from bud materials. In some instances, apical growth after treatment was arrested for prolonged periods and axillary shoots emerged from the first or second node below the treated apex. Chromosome counts were made from root tips of these shoots.

c. Treatment of tip cuttings with young flower spikes--Tip cuttings of V. Miss Joaquim with young developing flower spikes were treated by immersing the freshly cut basal ends of the stem into vials of aqueous-colchicine solution. This experiment is identical to the one above except for the emerging flower spike. This was based on the theory that colchicine would be absorbed and

translocated to the plant apex as well as the flower spike apex emerging in the axillary position. All other procedures are identical to that of experiment b, above.

d. Treatment of young shoots--Mature plants of V. Miss Joaquim and V. Princess Elizabeth were cut back to force lateral shoot development. Approximately three months after cutting back, young shoots were 3 to 4 inches high. These young shoots were excised at the base of the shoot and treated as described in the above experiments. Concentration ranged from 0.1 to 1.0 per cent and duration of treatment was from two to six days. After treatment, these shoots were planted in 5-inch clay pots with a 12-inch tree fern (Cibotium chamissoi) pole in the center of the pot upon which these cuttings were secured for further growth. Chromosome counts were made from roots which appeared on nodes of new growth.

In another experiment of a similar nature, young shoots of V. Miss Joaquim were removed with a portion of the old stem with one node still attached to them. This was done on the basis that the old stem would be less apt to injury when immersed in colchicine solution and would root more rapidly than the young stems of the shoots.

In the case of V. Princess Elizabeth, cuttings approximately 6 to 8 inches long, each with a young lateral shoot near the terminal end, were used. Treatment method was the same as above wherein the basal end was immersed in aqueous-colchicine solution for varying lengths of time. These cuttings were also attached to tree fern poles and planted in 5-inch clay pots for further growth and observations.

Shoots of D. Molokai, 3 to 8 inches tall with roots 2 to 5 mm. long, were excised from the parent plants and the basal end immersed in aqueous-colchicine solutions. Concentration ranged from 0.1 to 1.5 per cent with durations from one to four days. After treatment, plants were transplanted into 5-inch clay pots with tree fern fibers as the medium for growth.

e. Treatment of vigorously growing plants--Plants of V. Miss Joaquim and V. Princess Elizabeth were attached to tree fern poles and planted in 5-inch clay pots several months before treatment time. When these plants were well established with sufficient roots attached to the poles, the lower part of the stem approximately 4 inches above the base of the plant was cut off and the newly cut base was immersed in aqueous-colchicine solution. The untreated bottom section of the plant produced shoots which were used as the controls. This experiment was based on the theory that plants in vigorous and uninterrupted state of growth would have a greater absorbing capacity than cuttings removed from plants and interrupted in their growth processes. Concentrations of colchicine solution ranged from 0.5 to 2.0 per cent with durations from 2 days to 6 days at 2-day intervals. After treatment, these plants were left in a shaded glasshouse for further growth and observation.

f. Treatment by incision of apical area--In this experiment the apical region of V. Miss Joaquim and V. Michael Beaumont was incised longitudinally to expose the apical meristem and colchicine-lanolin paste ranging in concentration from 0.1 to 5.0 per cent was

administered into the meristem region with a toothpick (see Figures 6, 11, and 12). They were then left to make further growth for observations and chromosome counts. Controls were treated with lanolin paste without colchicine.

In a similar experiment involving V. Miss Joaquim and V. Princess Elizabeth, instead of using colchicine-lanolin paste, glycerine-colchicine solution was applied into the incisions with a camel's hairbrush. For the controls, only glycerine solution without colchicine was used. Concentrations ranged from 0.1 to 1.0 per cent. To account for the possible effects of time of exposure to the colchicine and the possible drying of the solution, colchicine-glycerine solution was applied 2, 4, and 6 times, each application being given at two-day intervals. These plants were grown in planting boxes on the nursery benches in full sunlight during and after the treatments. Chromosome counts were made from roots developing on new growth beyond the treated area.

g. Treatment by removing terminal leaves--In this experiment all the leaves at the apical portion of V. Michael Beaumont, a spatulate-leaf type, were cut off as close as possible to the apex, thus permitting the application of colchicine-lanolin paste into the region of the apical meristem, between the folds of the leaf sheaths. Concentrations ranged from 1.0 to 5.0 per cent, with each treatment consisting of five plants. After treatment, plants were grown in the lath house for further growth and observation. Chromosome counts were made from roots emerging from subsequent new growth.

h. Treatment by injection of colchicine--In separate experiments involving an aqueous-colchicine solution and glycerine-colchicine solution, the apical regions of V. Michael Beaumont (spatulate type) and young shoots (3 to 15 inches long) of D. Molokai were treated by injecting the colchicine solutions with capillary pipettes. These pipettes were made by stretching glass tubing to a thin point. In the case of V. Michael Beaumont, excess terminal leaves were cut off to facilitate the penetration of the pipette into the meristematic region between the folds of the leaf sheaths. There was no need to remove the terminal leaves in treating the Dendrobium plants.

In one of the above experiments, the capillary pipettes were inserted and left in the plant for gradual release of the solution towards the growing point. Water or glycerine was used in the controls. In the second experiment, three to six drops of colchicine solution were injected and the pipette was removed immediately. Injections were given 1, 3, and 6 times at approximately three-day intervals. Colchicine concentrations ranged from 0.1 to 2.0 per cent. Plants were grown in the lath house for further observations.

#### METHOD OF TAKING DATA

a. Visual effects on plants--All plants were examined periodically to observe changes in appearance of the plant parts treated. Any enlargements, shrivelling, discoloration, and mortality were recorded.

b. Techniques in chromosome counts--For root smears, root tips from aerial roots were cut and pretreated in 0.002 M 8-oxy-

quinoline for two to four hours and fixed in a modified Carnoy's fluid (3 parts of 95% ethyl alcohol, 2 parts of chloroform and 1 part of glacial acetic acid) after Kamemoto (51) for ten or more hours. Following fixation, roots were hydrolyzed in a 1:1 mixture of concentrated hydrochloric acid and 95 per cent alcohol for 10 minutes and washed in water. Roots were then placed on a slide in a drop of 1 per cent aceto-orcein in 45% acetic acid and squashed. The coverslip was placed over the preparation and the slide was slightly heated to intensify staining. The coverslip was then pressed to remove excess stain and finally sealed with a paraffin-gum mastic mixture for microscopic examination.

When bud material was available from treated plants or treated inflorescences, it was killed and fixed in modified Carnoy's solution for 10 hours or longer and the pollinia were removed, hydrolyzed and mounted on glass slides using the same schedule as described for root tips.

c. Paraffin sections for comparison of cell size between polyploid and diploid tissues--Root tips, shoot apex and nodal regions were killed and fixed in FAA (formalin - 5 cc., acetic acid - 5 cc., 50 or 70% ethyl alcohol - 90 cc.) for 18 hours or longer. Materials were then dehydrated in tertiary butyl alcohol series, followed by infiltration with parowax and tissue mat according to the standard paraffin method outlined by Johansen (54). After materials were embedded, sections were microtomed at 13 microns and stained in safranin-fast green for microscopic examination and photomicrographing. Sectioning of nodal sections in

the older stem tissues was extremely difficult due to the fibrous nature of monocotyledonous stem. Tissues became hard and brittle and made it necessary to soak them in softening solution such as hydrofluoric acid (95% ethyl alcohol - 80 cc., glycerol - 10 cc., and hydrofluoric acid - 10 cc.). Even this solution did not help in some instances.

d. Number and size of stomata--Pieces of epidermal tissue were peeled from the leaves of diploid and polyploid plants and placed on the slide in several drops of water. After adding a coverslip, they were examined under the microscope for stomatal count and measurement of size of guard cells. Leaf tissues were placed in water to maintain maximum turgidity of guard cells and measurements were taken on the lengths of those cells. Each leaf was divided into four sections, e.g., tip, median, base, and sheath. For each section, 10 microscopic fields constituted one replicate, and this was replicated 4 times. Variance analysis as outlined by Snedecor (55) was employed in determining significance of difference between means of size and between means of number of stomata on polyploid and diploid leaves. Where variance analysis showed significance between several means, Duncan's (17) multiple range and multiple F test was employed and the means were presented in ranked order.

### EXPERIMENTAL RESULTS

#### Treatment of seeds

a. Treatment of seeds before sowing--Seeds have been a popular material for treatment because of the ease of handling

large numbers. An effective method reported by others (2, 9, 59) is the pre-treatment of seeds in aqueous solutions of colchicine for several hours to a few days before planting. Apparently, the soaking period in colchicine solution can initiate the germination process. To determine whether orchid seeds could be effectively treated by a similar method, seeds of several species and hybrids of Vanda and Dendrobium were soaked in aqueous-colchicine solutions ranging in concentration from 0.1 to 1.0 per cent for durations of 1 to 10 days. After the pre-treatment period was over, the seeds were sown in nutrient agar under aseptic conditions.

It was found that at all concentrations and for durations up to four days, germination was good. However, beyond four days even concentrations as low as 0.1 per cent affected germination and post germination mortality. At concentrations beyond 0.5 per cent for durations longer than five days, there were increasing effects on germination rate and post mortality. Survivors in most instances were stunted and made little or no growth. Many succumbed through latent contaminations or died after transplanting into seedling flats. It was also interesting to note that seeds treated over four days at all of the concentrations developed highly proliferated protocorms. At 1.0 per cent and for durations of 8 to 10 days, protocorms enlarged and differentiation was affected. Surviving seedlings planted in flats included only control plants (seeds soaked in water), 0.1 and 0.5 per cent treated plants for durations up to five days. Chromosome counts

from root-tip smears showed that these treated survivors were all diploids with  $2n = 38$  chromosomes.

b. Treatment by sowing seeds in colchicine agar--In another experiment Vanda and Dendrobium seeds were sterilized as described under methods and were sown in colchicine incorporated nutrient agar. Concentration of colchicine in the agar ranged from .05 to 1.0 per cent. Seeds germinated well in low concentrations, but above 0.5 per cent, germination was low and post germination mortality was high. Excessive proliferation of protocorms was noted in concentrations above 0.5 per cent. Most of the surviving plants were from concentrations below 0.3 per cent and all were determined by root-tip smears to be diploid.

c. Treatment of protocorms--Since colchicine is reported to be most effective upon plant tissues in highly active state of growth, the third series of experiments involved the germination of seeds without pre-treatment in Martin's nutrient agar without colchicine. When the seeds were germinated and protocorms were differentiating leaves, various concentrations of sterilized aqueous-colchicine solutions were pipetted into the flasks of protocorms. Sterile water was pipetted into the control flasks.

In the low concentrations (0.01 to 0.05 per cent) protocorms continued their development and differentiation as in the control lots. From 0.1 per cent and up, protocorm mortality increased. Many of the surviving protocorms became enlarged and further differentiation was arrested. Only plants from the checks, 0.01, 0.05 and few of the 0.1 per cent treatments survived transplanting.

These were all determined to be diploids.

#### Treatment of seedlings

Dendrobium seedlings approximately 1.5 to 2.0 centimeters tall, soaked in aqueous-colchicine without vacuum, were designated as controls for the vacuum-treated lot of seedlings. In Table II are given the pertinent information and results for treatment of seedlings. It is quite apparent from the data in this table that seedlings are very sensitive, especially to concentrations beyond 0.10 per cent even without vacuum. When vacuum treated, the mortality increased. The few remaining plants were transplanted into seedling flats. Chromosome counts from root-tip smears showed all of them to be diploids. No doubling of chromosomes were realized.

#### Treatment of young developing flower spikes

A useful method, if successful, is the treatment of young flower spikes to induce the production of  $2n$  pollen grains. These  $2n$  pollen grains could be utilized in pollination of appropriate types to produce  $4n$  or  $3n$  progenies.

With this in mind, attempts were made to treat young spikes 2.5 to 20 centimeters long by covering the spike apex with absorbent cotton saturated with aqueous solution of colchicine. The tips were then covered with polyethylene sacks held in place with rubber bands to prevent drying of the colchicine solution. In Table III are summarized the results of four separate trials. At concentrations of 0.1 and 0.5 per cent and for durations up to two days, the effects were mild. Some swelling occurred and the first

TABLE II. RESULTS OF DENDROBIUM SEEDLINGS SOAKED IN  
AQUEOUS-COLCHICINE SOLUTION. 20 SEEDLINGS PER TREATMENT.

Treatment	Concentration Per Cent	Soaking Time	Number of Plants Dead	Ploidy of Survivors
no vacuum	0.01	3 hrs.	0	diploid (2n=38)
	0.05	"	4	"
	0.10	"	11	"
	0.50	"	9	"
	1.00	"	8	"
	1.50	"	20	no count
	2.00	"	20	" "
vacuum treated	0.01	17 min.	9	diploid (2n=38)
	0.05	"	14	"
	0.10	"	16	"
	0.50	"	13	"
	1.00	"	19	"
	1.50	"	18	"
	2.00	"	20	no count

TABLE III. RESULTS OF TREATING FLOWER SPIKES OF  
VANDA MISS JOAQUIM WITH AQUEOUS-COLCHICINE SOLUTION.

Concentration Per Cent	Treatment Time	Number of Spikes	Immediate Effects on Spikes	Effects on Buds	Floidy*
0.1	8 hrs.	16	normal	normal	diploid (2n=38)
	12 hrs.	15	"	"	"
	1 day	15	slight swelling	first buds yellow	"
	2 days	16	" "	first buds yellow	"
	3 days	16	" "	bud drop	no count
	4 days	16	distorted	" "	diploid (2n=38)
	5 days	16	"	" "	"
0.5	8 hrs.	16	normal	normal	"
	12 hrs.	16	slight swelling	"	"
	1 day	16	" "	distorted	"
	2 days	16	distorted	"	no count
	3 days	16	"	bud drop	" "
	4 days	16	"	" "	" "
	5 days	16	"	" "	" "
1.0	8 hrs.	16	normal	normal	diploid (2n=38)
	12 hrs.	16	slight swelling	distorted	"
	1 day	16	distorted	"	"
	2 days	16	"	bud drop	"
	3 days	16	dead	dead	no count
	4 days	16	"	"	" "
	5 days	16	"	"	" "

\* Chromosome counts from bud materials.

few maturing buds turned yellow and abscised. Later buds were available for chromosome counts. As duration of exposure or concentration was increased, the spikes became distorted and buds turned yellow and abscised at an early stage. Even at 0.1 per cent for durations beyond three days, spikes and buds were distorted and further development was arrested. At 1.0 per cent and for durations up to two days, early buds abscised but the later ones were available for chromosome counts. Considerable distortion was noted. In all cases no doubling of chromosomes was observed. No counts were made in the long duration or high concentrations due to lack of bud material.

#### Treatment of mature plants and shoots

a. Treatment of tip cuttings--Tip cuttings of V. Miss Joaquim were treated as described under methods. A summarized tabulation of data from several similar trials is presented in Table IV. Five different concentrations ranging from 0.5 to 5.0 per cent were used in these experiments. Duration of exposure extended to 20 days. The data presented here show that apex swelled and growth was temporarily arrested but new growth usually resumed (Figures 1, 2, and 3). At concentrations above 3.0 per cent even exposure for one day was sufficient to cause rather severe damage to the cuttings. Plants shrivelled and remained dormant for as long as two years and in most instances did not recover. This is illustrated in Figures 4 and 5. Low concentrations for long durations had the same detrimental effect. Occasionally, a plant recovered and grew when treated with 0.5

TABLE IV. RESULTS OF TREATING VANDA MISS JOAQUIM TIP CUTTINGS  
WITH BASAL ENDS IMMERSSED IN COLCHICINE SOLUTION.  
(SUMMARY OF SEVERAL TREATMENTS.)

Concentration Per Cent	Duration Days	No. of Plants	Symptoms After Treatment*	No. Dead**	Ploidy of*** Survivors
0.5	1	5	no visual effect	0	diploid (2n=38)
1.0	1	5	apex, roots swollen	4	"
1.5	1	5	apex, roots swollen	2	"
2.0	1	5	stems, leaves shrivelled	4	no growth
3.0	1	5	shrivelled	3	" "
5.0	1	5	"	5	no count
0.5	2	5	apex swollen	0	1-tetraploid (4n=76)
1.0	2	5	" "	3	2-tetraploids
1.5	2	5	" " , leaves shrivelled	2	diploid
2.0	2	5	shrivelled	3	1-no growth 1-diploid
3.0	2	5	shrivelled	2	no growth
5.0	2	5	"	5	no count
1.0	3	4	apex swollen, leaves shrivelled	0	diploid
3.0	3	4	apex swollen, leaves shrivelled	1	2-diploids 1-no growth
5.0	3	4	apex swollen, leaves shrivelled	1	3-no growth
0.5	4	6	apex swollen	0	5-diploids 1-tetraploid
1.0	4	6	" "	0	2-no growth 4-diploids
1.5	4	6	" " , leaves shrivelled	1	diploid
2.0	4	6	apex swollen, leaves shrivelled	1	no growth

TABLE IV CONTINUED

Concentration Per Cent	Duration Days	No. of Plants	Symptoms After Treatment*	No. Dead**	Ploidy of*** Survivors
0.5	5	5	apex swollen, leaves shrivelled	1	diploid
1.0	5	5	apex swollen, leaves shrivelled	2	diploid
0.5	6	8	apex swollen, leaves shrivelled	0	diploid
1.0	6	8	apex swollen, leaves shrivelled	2	4-no growth 2-diploids
1.5	6	8	plants shrivelled	4	1-diploid 3-no growth
2.0	6	8	plants shrivelled	3	5-no growth
0.5	10	6	plants shrivelled	4	no growth
1.0	10	6	plants shrivelled	3	diploid
1.5	10	6	plants shrivelled	4	no growth
2.0	10	6	plants shrivelled	5	no growth
3.0	10	6	plants shrivelled	4	no growth
0.5	13	3	plants shrivelled	0	1-diploid 2-no growth
0.5	15	3	plants shrivelled	1	1-diploid 1-no growth
1.0	15	3	plants shrivelled	2	diploid
0.5	20	3	plants shrivelled	1	no growth

\* observed two to three months after treatment.

\*\* data taken six months to two years after treatment.

\*\*\* In some cases where no growth resulted from the apex, ploidy given are from new shoots arising immediately below apical region.

to 1.0 per cent for as long as 15 days. Figure 8 shows a plant which recovered after a long period of inactivity.

Whenever new growth commenced from the treated tips and root-tip material became available, chromosome counts were made from root smears. Tetraploid counts were observed in one plant treated with 0.5 per cent for two days and two plants treated with 1.0 per cent for two days. Treatment with 0.5 per cent for four days resulted in another tetraploid. In all, there were four tetraploid plants. It is interesting to note that doubling occurred at concentrations of 0.5 to 1.0 per cent for durations of two to four days. Low concentrations for long durations and concentrations higher than 1.0 per cent were ineffective in doubling the chromosomes.

b. Treatment of tip cuttings with young flower spikes--In a similar experiment, tip cuttings of Vanda Miss Joaquim possessing young developing flower spikes were selected. The purpose of selecting cuttings with young spikes was to determine whether colchicine could be translocated to the inflorescence apex to effect doubling in the pollen mother cells as well as in the terminal shoot apex.

Results of this trial are presented in Table V. The colchicine effect on the developing spikes was similar to that observed in the direct treatment of the spike apex with colchicine. At concentrations of 0.5 and 1.0 per cent for treatment time of two and four days, spike apices were deformed and swollen. Figure 7 shows a set of three plants with affected spikes. The plant to

the extreme right is the control with normal spike. The first plant to the extreme left was treated with 0.5 per cent aqueous-colchicine for four days and shows definite curvature of the spike. The two plants in the middle show spikes which are beginning to swell. This photo was taken 13 days after treatment. In all treatments the first formed buds turned yellow and abscised. Later buds developed normally and PMC materials were examined where possible. No diploid spores were noted.

The effects on the shoot apex were similar to the previous experiment. Out of a total of 45 plants, two tetraploids were found, one receiving 1.0 per cent colchicine for four days and the other receiving 0.5 per cent colchicine for six days. The effective range of concentrations and duration was similar to the first experiment with tip cuttings.

#### Treatment of Young Shoots

a. Treatment of young shoots excised from mature plants--On the assumption that young, rapidly growing shoots would be more responsive to colchicine treatment, young shoots of V. Miss Joaquim, V. Princess Elizabeth, and Dendrobium Molokai were excised from the parent plants and treated by immersing the basal ends into aqueous-colchicine solutions. Data for young shoots of V. Miss Joaquim are tabulated and presented in Table VI. Young shoots appeared to be more sensitive to colchicine than the tip cuttings of mature plants. Even 0.1 per cent for two days effected considerable swelling at the apex with a long delay in recovery. Most of the shoots treated for six days made no further

TABLE V. RESULTS OF TREATING VANDA MISS JOAQUIM CUTTINGS  
POSSESSING FLOWER SPIKES BY IMMERSING THE BASAL ENDS OF  
STEMS IN AQUEOUS-COLCHICINE SOLUTION.

Concentration Per Cent	Duration Days	No. of Plants	Effects on Spike	No. of Plants Dead	Buds	Floidy Plant
0.5	2	3	swollen, bud drop	0	diploid	diploid
1.0	2	3	swollen, bud drop	0	no count	diploid
1.5	2	3	spikes dead	0	no count	diploid
0.5	4	3	swollen, bud drop	0	diploid	diploid
1.0	4	3	swollen, bud drop	0	no count	1-tetra. 2-dipl.
1.5	4	3	swollen, bud drop	2	no count	1-tetra.
0.5	6	3	bud drop	0	no count	1-tetra. 2-dipl.
1.0	6	3	dead	3	no count	no count
1.5	6	3	dead	0	no count	2-dipl. 1-no growth
0.5	8	3	dead	1	no count	1-damaged 1-diploid
1.0	8	3	dead	0	no count	1-no growth 2-dipl.
1.5	8	3	dead	2	no count	1-no growth
0.5	10	3	dead	1	no count	2-no growth
1.0	10	3	dead	3	no count	no count
1.5	10	3	dead	3	no count	no count

PLATE I

FIGURE 1: AQUEOUS-COLCHICINE TREATED V. MISS JOAQUIM CUTTINGS SHOWING SWELLING OF APEX. LEFT - 4.0%-1 DAY; CENTER - 3.0%-1 DAY; RIGHT - 0.5%-20 DAYS.

FIGURE 2: SPLITTING OF LEAF-SHEATH CAUSED BY SWELLING OF APEX.

FIGURE 3: SWELLING OF ROOTS ONE MONTH AFTER TREATMENT.

FIGURE 4: SEVERE EFFECTS BY HIGH CONCENTRATION. DEFOLIATION AND LONG DORMANCY.

FIGURE 5: SEVERE EFFECTS BY LONG DURATION OF TREATMENT.

FIGURE 6: INCISION METHOD USING LANOLIN-COLCHICINE PASTE.

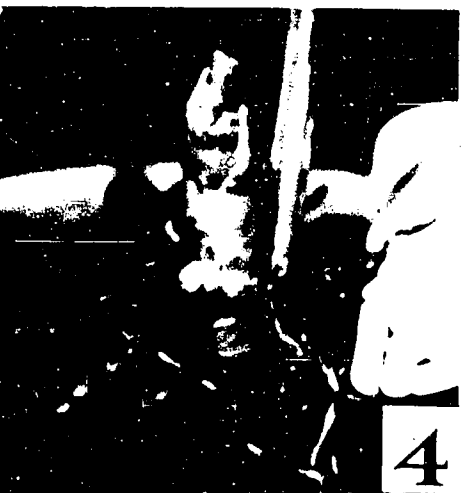


TABLE VI. RESULTS OF AQUEOUS-COLCHICINE SOLUTION TREATMENT FROM BASAL ENDS OF YOUNG SHOOTS OF VANDA MISS JOAQUIM EXCISED FROM PARENT PLANT.

Concentration Per Cent	Duration Days	No. of Plants	Symptoms After Treatment	No. Dead	Ploidy of Survivors
0.1	2	6	apex swollen, growth delayed	0	diploid (2n=38)
0.5	2	6	apex swollen, growth delayed, shrivelled leaves	2	"
1.0	2	6	leaves, stems shrivelled	4	"
0.1	4	6	apex swollen	0	"
0.5	4	6	apex swollen, leaves shrivelled	2	3-diploids 1-tetraploid
1.0	4	6	plant shrivelled	2	diploid
0.1	6	6	apex swollen	0	2-no growth 4-diploids
0.5	6	6	apex swollen, leaves shrivelled	1	3-no growth 2-diploids
1.0	6	6	apex swollen, leaves shrivelled	2	2-no growth 2-diploids

growth from the affected apices. Among those that recovered and grew, chromosome counts were made from root-tip smears, and one tetraploid was found in treatment 0.5 per cent for four days. This particular plant, upon recovery, produced two new shoots from the swollen area and one shoot from below the swollen area. Chromosome counts of the three shoots showed that the one emerging from below the swelling was a tetraploid. This plant is shown in Figure 10.

In the case of the young shoots of V. Princess Elizabeth, only two plants survived the treatments, one of which was 0.1 per cent for four days and the other was 0.1 per cent for six days. Although they survived, no growth was made to date and chromosome numbers have not been determined. V. Princess Elizabeth is a semi-terete Vanda and under normal conditions, the young shoots do not produce adventitious roots as readily as terete Vanda. This may be a partial explanation of the high mortality rate encountered in this experiment.

Shoots of Dendrobium Molokai exhibited exceptional sensitivity to aqueous-colchicine treatments. Table VII gives the results of this experiment. Young shoots three to eight inches tall with very short roots at the base were treated by immersing the base in colchicine solution. Ten to 15 days after treatment, plants receiving concentrations of 1.0 per cent and higher and for durations as short as one day showed yellowing of leaves and defoliation. For longer durations, plants treated with concentrations as low as 0.1 per cent showed yellowing of leaves.

TABLE VII. RESULTS OF AQUEOUS-COLCHICINE SOLUTION TREATMENT FROM BASAL ENDS OF YOUNG DENDROBIUM MOLOKAI SHOOTS SEPARATED FROM PARENT PLANTS.

Concentration Per Cent	Duration Days	No. of Plants	Effects After Treatment*	No. Dead**	Floidy of Survivors***	
0.1	1	4	none	2	no count (2n=38)	
0.5	1	4	none	3	"	"
1.0	1	4	leaves yellow defoliating	4	"	"
1.5	1	4	leaves yellow defoliating	4	"	"
0.1	2	4	none	2	"	"
0.5	2	4	none	2	"	"
1.0	2	4	leaves yellow defoliating	3	"	"
1.5	2	4	leaves yellow defoliating	4	"	"
0.1	3	4	leaves yellow	1	"	"
0.5	3	4	leaves yellow	3	"	"
1.0	3	4	leaves yellow	4	"	"
1.5	3	4	defoliation	4	"	"
0.1	4	4	leaves yellow	4	"	"
0.5	4	4	leaves yellow	4	"	"
1.0	4	4	leaves yellow	4	"	"
1.5	4	4	leaves yellow	4	"	"

\* observed 10 to 15 days after treatment.

\*\* observed 2 to 3 months after treatment.

\*\*\* no count due to lack of flowering of survivors.

Mortality counts made two to three months later showed that all plants in the four-day duration treatment involving all concentrations were dead. Most of the plants receiving 1.0 per cent and over were dead regardless of the exposure time. No chromosome determinations were made of the few survivors since none of them flowered. No aerial roots are produced in Dendrobium.

Diploid chromosome counts presented in Table VII were derived from root tips of untreated plants.

b. Treatment of young shoots attached to mature stem sections--On the assumption that immature tissues at the base of young shoots of Vanda and Dendrobium were too sensitive to colchicine, shoots of V. Miss Joaquim and V. Princess Elizabeth were removed with sections of mature stem of the parent plant still attached. The bases of the mature stems were immersed in colchicine solution. In Table VIII are presented the results of one such experiment. The symptoms after treatment were similar to that of other experiments using identical methods. However, the mortality rates of the shoots in this case were much lower than those observed for young shoots immersed directly into the colchicine solution. Terminal growth was arrested in a number of cases for a long time. Ploidy determinations among the survivors which recovered and grew showed that doubling was effected in one shoot treated with 0.5 per cent for three days. Again, the ranges of effective concentration and duration are in agreement with the results obtained in other experiments already discussed.

Shoots of V. Princess Elizabeth again showed greater sensi-

TABLE VIII. RESULTS OF AQUEOUS-COLCHICINE SOLUTION TREATMENT  
OF NEW SHOOTS OF VANDA MISS JOAQUIM ATTACHED TO OLD STEMS.

Concentration Per Cent	Duration Days	No. of Plants	Symptoms After Treatment	No. Dead	Ploidy of Survivors
0.5	2	3	apex swollen	2	diploids
1.0	2	3	apex swollen	0	diploids
1.5	2	3	apex swollen	0	2-diploids 1-no growth
2.0	2	3	apex swollen, leaves shrivelled	1	1-diploid 1-no growth
0.5	3	3	apex swollen	0	2-diploids 1-tetraploid
1.0	3	3	apex swollen	0	2-no growth 1-diploid
1.5	3	3	apex swollen, leaves shrivelled	1	1-no growth 1-diploid
2.0	3	3	plant shrivelled	2	1-diploid
0.5	4	3	apex swollen	0	diploid
1.0	4	3	apex swollen	0	diploid
1.5	4	3	apex swollen, leaves shrivelled	1	1-no growth 1-diploid
2.0	4	3	apex swollen, leaves shrivelled	1	2-no growth

## PLATE II

- FIGURE 7: VANDA MISS JOAQUIM CUTTINGS WITH FLOWER SPIKES AFFECTED BY COLCHICINE. CUTTING ON THE EXTREME RIGHT IS THE CONTROL. (13 DAYS AFTER TREATMENT.)
- FIGURE 8: NEW SHOOT DEVELOPMENT FROM SEVERELY AFFECTED V. MISS JOAQUIM APEX.
- FIGURE 9: COLCHICINE EFFECT ON A YOUNG SHOOT JUST EMERGING WHEN THE CUTTING WAS TREATED.
- FIGURE 10: TWO NEW SHOOTS FROM THE SWELLING ON THE LEFT AND ONE SHOOT BELOW THE SWELLING OF V. MISS JOAQUIM CUTTING.
- FIGURE 11: TWO SHOOTS EMERGING FROM INCISED APEX OF V. MICHAEL BEAUMONT TREATED WITH LANOLIN-COLCHICINE PASTE.
- FIGURE 12: SHOOT DEVELOPMENT FROM WITHIN THE INCISED AREA OF V. MICHAEL BEAUMONT.



tivity to colchicine in spite of their attachment to older stem. Shoots were arrested in growth in most instances, and new shoots appeared below the treated shoots. In a few cases, growth of the affected shoots resumed and chromosome determinations were made. In one plant treated with 1.5 per cent for four days, initial chromosome count from the first root showed tetraploid cells but second and third counts from the same root which regenerated new tips and counts from other root tips gave diploid counts. This seems to be a case of mixoploid condition which will be discussed in greater detail in another section.

c. Treatment of plants well established and in vigorous state of growth--To conduct this experiment, V. Miss Joaquim cuttings were attached to tree fern poles as described in the section on methods and were grown for several months until sufficient aerial roots were established and attached to the tree fern poles. At that point the lower part of the stem, approximately two nodes above the base, was cut in each case and the freshly cut base was immersed in colchicine solution. This method allowed treatment of plants with least disturbance in their growth processes. Results of this experiment are presented in Table IX. External symptoms of the apex were similar to other treatments already discussed. Apices of plants were swollen and growth was temporarily arrested. In some cases growth of the treated apices was more or less permanently arrested and new shoots emerged from nodes below the swelling.

TABLE IX. RESULTS OF AQUEOUS-COLCHICINE SOLUTION TREATMENT OF VANDA MISS JOAQUIM CUTTINGS WELL ESTABLISHED ON TREE FERN POLES.

Concentration Per Cent	Duration Days	No. of Plants	Symptoms After Treatment	No. Dead	Ploidy of Survivors
0.1	2	3	none	0	diploid
0.5	2	3	apex swollen slightly	0	diploid (2n=38)
1.0	2	3	apex swollen slightly	0	diploid
2.0	2	3	apex swollen, leaves shrivelled	0	2-diploid 1-no growth
0.1	4	3	slight swelling	0	diploid
0.5	4	3	apex swollen	0	diploid
1.0	4	3	apex swollen	0	2-no growth 1-tetraploid
2.0	4	3	apex swollen, leaves shrivelled	0	2-no growth 1-diploid
0.1	6	3	apex swollen	0	diploid
0.5	6	3	apex swollen	0	diploid
1.0	6	3	apex swollen	0	diploid
2.0	6	3	apex swollen	1	2-no growth

Chromosome determinations from root-tip smears of survivors showed doubling in only one plant treated with 1.0 per cent colchicine for four days. Here again the effective concentration was between 0.5 and 1.0 per cent and the duration of treatment fell between two and six days. Chromosome counts of new shoot growth from nodes below the apex of plants whose terminal growth was arrested were all diploids.

d. Treatment of plants by incision method using colchicine-lanolin paste--Apical regions of V. Michael Beaumont and V. Miss Joaquim were incised to allow application of colchicine-lanolin paste into the meristem regions. Eight concentrations ranging from 0.5 to 5.0 per cent were utilized. Data for treatments on V. Michael Beaumont and V. Miss Joaquim are presented in Tables X and XI, respectively. In the case of V. Michael Beaumont, growth was retarded by 1.5 per cent colchicine while V. Miss Joaquim was retarded by concentration as low as 0.5 per cent. In most instances growth was arrested temporarily and chromosome counts were made from subsequent growth either from the treated apices or from new shoots emerging from the incised area. Figure 6 shows the incision on V. Miss Joaquim, while Figures 11 and 12 show Vanda Michael Beaumont with shoots emerging from the incised area. These new shoots were all determined to be diploids. Results of these two trials were completely negative.

e. Treatment with colchicine-lanolin paste by removal of apical leaves--In another experiment involving V. Michael Beaumont (strap-leaf type), instead of the drastic treatment of incision,

TABLE X. RESULTS OF COLCHICINE-LANOLIN PASTE TREATMENT ON INCISED APEX OF VANDA MICHAEL BEAUMONT (STRAP-LEAFED TYPE).

Concentration Per Cent	No. of Plants	Effects on Apex	No. Dead	Ploidy of Survivors
Check*	4	none	0	diploid (2n=38)
0.5	4	none	0	"
1.0	4	none	0	"
1.5	4	growth retarded	0	"
2.0	4	growth retarded	0	"
2.5	4	growth retarded	0	"
3.0	4	growth retarded	0	"
4.0	4	growth retarded	0	"
5.0	4	growth retarded	0	"

\* Lanolin paste without colchicine

TABLE XI. RESULTS OF COLCHICINE-LANOLIN PASTE TREATMENT ON INCISED APEX OF VANDA MISS JOAQUIM (TERETE-LEAFED TYPE).  
SUMMARY OF THREE SIMILAR TRIALS.

Concentration Per Cent	No. of Plants	Effects on Apex	No. Dead	Ploidy of Survivors
Check*	20	none	0	diploid (2n=38)
0.1	20	none	0	"
0.5	20	growth retarded	0	"
1.0	20	growth retarded	0	"
1.5	20	growth retarded	0	"
2.0	20	growth retarded	0	"
3.0	20	growth retarded	0	"
4.0	20	growth retarded	0	no growth
5.0	20	growth retarded	0	" "

\* Lanolin paste without colchicine

all the terminal leaves were cut off as close as possible to the apical region and colchicine-lanolin paste was applied into the area between the folds of the leaf sheaths. The very young and unexpanded leaves in the interior made it somewhat difficult to apply the paste. In Table XII are shown the results of this experiment. In all concentrations ranging from 1.0 to 5.0 per cent, results were negative. There were no visible effects common to colchicine treatment on apices. It is quite apparent that the colchicine applied did not reach the meristematic area of the apex and that this method is a poor one to be employed.

f. Treatment of plants by incision of the apical area using glycerine-colchicine solution--Apices of V. Miss Joaquim and V. Princess Elizabeth were incised longitudinally in the same manner as in the experiments previously discussed and glycerine-colchicine solution after Dermen (13) was applied into the incised area with a camel's hairbrush. Concentrations were limited to 0.1, 0.5, and 1.0 per cent.

Results of the trials for V. Miss Joaquim are presented in Table XIII. Results for V. Princess Elizabeth were very similar to the one presented here. Since the plants were grown on nursery benches in direct sunlight, glycerine-colchicine was applied 2, 4, and 6 times to allow for possible drying. In all cases, some swelling occurred at the incised areas but cytological studies from root tips of subsequent growth showed no doubling of chromosomes. In some cases the incised terminals failed to grow, but secondary shoots emerged from nodes immediately below the incised

TABLE XII. RESULTS OF COLCHICINE-LANOLIN PASTE APPLIED INTO APICAL AREA OF V. MICHAEL BEAUMONT BY CUTTING OFF THE LEAVES AT THE APEX.

Concentration Per Cent	Number of Plants	Effects on Apex	Ploidy*
Check**	5	none	diploid (2n=38)
1.0	5	none	"
1.5	5	none	"
2.0	5	none	"
2.5	5	none	"
3.0	5	none	"
4.0	5	none	"
5.0	5	none	"

\* root tip and bud smears.

\*\* lanolin paste without colchicine.

TABLE XIII. RESULTS OF GLYCERINE-COLCHICINE SOLUTION APPLIED TO INCISED APICES OF VANDA MISS JOAQUIM PLANTS.

Concentration Per Cent	Number of Colchicine Application	No. of Plants	Ploidy*
Check**	2	10	diploid (2n=38)
0.1	2	10	"
0.5	2	10	"
1.0	2	10	"
Check	4	10	diploid (2n=38)
0.1	4	10	"
0.5	4	10	"
1.0	4	10	"
Check	6	10	diploid (2n=38)
0.1	6	10	"
0.5	6	10	"
1.0	6	10	"

\* In most instances apical growth was arrested. Chromosome counts were made from roots of new shoots emerging from below incised area.

\*\* Glycerine without colchicine.

area and chromosome counts were made for these shoots.

g. Treatment by injection of colchicine solution--Aqueous and glycerine-colchicine solutions were injected into the apical meristem by means of fine capillary pipettes. The results of aqueous-colchicine solution injected into D. Molokai and V. Michael Beaumont are given in Tables XIV and XV. The results of glycerine-colchicine treatment on these plants were similar to that reported here for the aqueous solution.

In the case of Dendrobium, the apices of all treated plants died, indicating that treatment was too severe.

The results determined for V. Michael Beaumont are quite variable as far as effects on growth are concerned. Even the control plants with water injected into the apex failed to grow. This may indicate injury to the apical bud by the capillary tubing injected into that area rather than by direct effects of the substance injected into it. For the plants that made new growth after treatment, chromosome counts showed no doubling. In several cases, pollen mother cells were examined and all were found to be diploid,  $n = 19$  pairs.

#### EVALUATION OF METHODS AND THE NATURE OF THE INDUCED POLYPLOIDS

A summary of all induced polyploids and the methods by which they were obtained are presented in Table XVI. It is obvious from this table that the greatest number of polyploids were obtained from treatment of tip cuttings of V. Miss Joaquim. Young shoots and vigorously growing plants of V. Miss Joaquim were also responsive but not to any

TABLE XIV. RESULTS OF ONE ATTEMPT IN INJECTING AQUEOUS-COLCHICINE SOLUTION INTO APEX OF YOUNG SHOOTS OF DENDROBIUM MOLOKAI.

Concentration	No. of Plants	Symptoms	Ploidy
Check (water)	6	shoots normal and growing	diploid (2n=38)
1.0% colchicine	6	apex dead	no count

TABLE XV. RESULTS OF AQUEOUS-COLCHICINE SOLUTION INJECTION INTO APEX OF VANDA MICHAEL BEAUMONT (STRAP-LEAF TYPE). THREE TO FIVE DROPS OF SOLUTION INJECTED AT ONE APPLICATION.

Concentration Per Cent	No. of Applications	No. of Plants	No. of Plants Dead	Ploidy of Survivors
Water	1	3	2	1-no growth*
0.1	1	3	1	1-no growth 1-diploid
0.5	1	3	1	1-no growth 1-diploid
1.0	1	3	2	1-diploid
2.0	1	3	2	1-no growth
Water	3	3	0	2-no growth 1-diploid
0.1	3	3	1	1-diploid 1-no growth
0.5	3	3	1	2-no growth
1.0	3	3	2	1-no growth
2.0	3	3	2	1-no growth
Water	6	3	1	1-no growth 1-injured
0.1	6	3	2	1-diploid
0.5	6	3	3	no count
1.0	6	3	2	1-no growth
2.0	6	3	0	3-no growth

\* treated apex has not made any growth.

TABLE XVI. SUMMARY AND EVALUATION OF INDUCED TETRAPLOIDS.

Treated Material	Treatment Method	Nature of Ploidy*
tip cuttings	0.5%-2 days	temporary
tip cuttings	0.5%-4 days	temporary
tip cuttings	0.5%-6 days	temporary
tip cuttings	1.0%-4 days	temporary
tip cuttings	1.0%-2 days	temporary
tip cuttings	1.0%-6 days	continuing
tip cuttings	1.5%-4 days	continuing
young shoot	0.5%-4 days	pure, cont.
young shoot on old stem cutting established on	0.5%-3 days	temporary
totem pole	1.0%-4 days	temporary

\* Temporary indicates that tetraploidy was noted in the first few observations but not found in subsequent counts.

Continuing indicates that tetraploidy is still present.

Pure indicates that counts at all nodes have been tetraploid.

TABLE XVII. CHROMOSOME COUNTS OF ROOT TIPS AT SUCCESSIVE NODES OF SIX INDUCED POLYPLOIDS.

Treatment	Root	N O D E S																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1.0%-6 days	A	4n	4n	2n	2n	2n	2n	2n	2n	2n	2n	4n				4n-2n		2n mixo	2n	4n-2n	4n		2n-4n	4n	4n-2n	
	B								4n	4n									4n				2n-4n	2n	4n	
1.0%-2 days	A				2n	2n	2n						2n	2n	2n		2n	2n		2n	2n	2n				
	B	4n				4n															2n					
1.5%-4 days	A	4n	2n	4n	4n	4n		2n	4n			4n	4n				4n	4n-2n	4n		4n					
	B											4n							4n							
1.0%-4 days	A	4n	4n	2n	2n		2n																			
	B	2n	2n	2n	2n																					
No. 1	A							2n		2n	2n	4n-2n	2n	2n	2n	2n										
0.5%-4 days	B											2n	2n	2n		2n			4n-2n							
0.5%-4 days, No. 14a	A	2n																								
Shoot No. 1	B																									
Same (14a)	A	2n				2n	2n																			
Shoot No. 2	B	2n					2n																			
(14a)	A		4n	4n	4n		4n	4n																		
Shoot No. 3	B		4n		4n																					
1.0%-6 days	A						2n-4n	4n-2n	4n																	
Shoot at Node 10	B						4n	4n-2n	2n																	
1.0%-6 days	A																									
Shoot at Node 3	B					2n																				
1.0%-6 days	A		4n	4n-2n																						
Shoot at Node -1	B			4n-2n																						
1.5%-4 days	A			4n	4n																					
Shoot at Node 2	B																									
1.5%-4 days	A	2n	2n	2n																						
Shoot at Node 5	B																									
1.5%-4 days	A																									
Shoot at Node 8	B			4n																						

great extent. The method of colchicine application in all of these cases is by immersing the basal ends into colchicine solution. None of the other methods employed was effective.

The concentration and duration of exposure to colchicine are presented in the second column of the table. It is quite evident that concentrations between 0.5 and 1.0 per cent are most effective in doubling the chromosome number. Only in one case 1.5 per cent was effective.

The duration of treatment was also confined to a relatively narrow range between two and six days. Durations less than two days or longer than six days have not been effective at any of the concentrations used.

The nature of the polyploids obtained is very interesting. In seven out of the ten polyploids obtained, continued chromosome counts from successive roots showed that doubling was temporary. After the first few counts showing tetraploidy, complete reversion to diploidy occurred.

In one case, doubling appeared to be complete since no diploid cells were found in the examination of root tips at several nodes. In two cases, tetraploidy has been maintained although diploid cells have been observed at various nodes. Attempts were made to determine the possible cause of this phenomenon and the results are presented in the following section.

#### PRODUCTION OF CYTOCHIMERAS AND MIXOPLOIDS

When the temporary nature of the induced polyploids was indicated by variable chromosome counts at different nodes, six induced tetraploids were isolated and chromosome counts were taken at each root .

node thereafter. In a few cases, plants were cut into sections to induce root formation, but some nodes failed to produce any roots. At each node of V. Miss Joaquim, up to three or even more roots may form but usually two roots were found at each node. The first root at each node was designated as A and the second as B. If a third root emerged, it was designated as C.

The largest number of determinations were made on plant treatments 1.0%-6 days, 1.0%-2 days, and 1.5%-4 days. Hereafter, for clarity and simplicity, plants will be designated as 1.0/6, 1.0/2, 1.5/4, etc., the top figure indicating colchicine concentrations in percentage and the bottom figure indicating days of treatment. In plant 1.0/6, the first two nodes were tetraploids and nodes 3 to 10 were diploids. Root B at node 5 gave tetraploid counts, indicating differences at the same node. The same situation was found at node 19. In Figures 13 to 17 are photomicrographs of chromosomes at the various nodes of this plant. Figures 13 and 14 show diploidy in root A, node 3 and at root A, node 17, respectively. Figure 14 shows a tetraploid cell in root A, node 19. Figures 16 and 17 show diploid and tetraploid cells in root A, node 20. This condition is also found in root B, node 10; root A, node 15; roots A and B at node 23; and in root A, node 25. A similar situation is found in the other induced polyploids. It should be pointed out that at some nodes such as 10, 15, and 20, the condition of ploidy is indicated as  $4n-2n$  and in other nodes such as node 23 of the first plant, ploidy is indicated as  $2n-4n$ . These designations were based on the preponderance of one ploidy type in a root tip. If  $4n$  cells were found in greater quantity than  $2n$  cells, then it was designated as  $4n-2n$ .

Table XVIII shows the frequency of 2n and 4n cells for three plants taken at specific nodes and roots.

TABLE XVIII. FREQUENCY OF 2n AND 4n CELLS IN A SINGLE ROOT-TIP SMEAR.

Treatment	Node	Root	Ploidy*	Observations**	Number of	
					2n Cell	4n Cell
0.5%-4 days	17	B	2n-4n	8	31	9
1.0%-6 days	10	B	4n-2n	9	2	15
1.0%-6 days	25	A	4n-2n	8	7	27

\* In a single root, if more than 4n cells are observed, ploidy for that root is indicated as 2n-4n. The reverse order is indicated as 4n-2n.

\*\* Each microscopic field at 250X is considered as one observation. The figures in this column represent the number of microscopic fields examined in each root-tip smear.

Another condition is the difference in ploidy between two roots of the same node. Figure 18 represents a 2n condition in root A at node 5, and Figure 19 represents a 4n condition in root B of the same node. This condition also occurred in other plants.

Figures 20 and 21 show tetraploid cells in different roots of the same node for plant 0.5/4, shoot 3. This shoot was given 4n counts at all nodes examined and appears to be a complete tetraploid. Figures 22 to 24 show tetraploidy and diploidy at different nodes of plant 1.5/4.

Since root designation, A and B, was placed on the first and second emergence basis, it does not show any relationship of ploidy to the roots in the same positions on succeeding nodes above. In order to ascertain the possible relationship of ploidy from one node to another in the same relative position, an arbitrary scheme was devised. Figure 26 shows a schematic division of a stem into eight parts with numbers

TABLE XIX. RELATIONSHIP OF PLOIDY BETWEEN ROOTS AT THE SAME NODE AND BETWEEN ROOTS OF DIFFERENT NODES AT THE SAME EMERGENCE POSITION. (REFER TO FIGURE XXVI FOR SCHEMATIC DRAWING OF POSITIONS ON THE NODE.)

Plant	Node Number*	Root	Position on Node	Ploidy of Roots
1.5%-4 days	1	A	5	4n
		B	--	--
	2	A	2-3	2n
		B	--	--
	3	A	4-5	4n
		B	--	--
	4	A	3	4n
		B	--	--
	5	A	6	4n
		B	2	--
	6	A	--	--
		B	2-3	4n
	7	A	3	2n
		B	--	--
	8	A	1	4n
		B	--	--
	11	A	6-7	4n
		B	4	4n
	12	A	8-1	4n
		B	--	--
13	A	8	--	
	B	--	--	
16	A	4-5	4n	
	B	--	--	
17	A	1	4n·2n	
	B	--	--	
18	A	4	4n	
	B	7	4n	
19	A	8-1	--	
	B	--	--	
20	A	8-1	4n	
	A	--	--	

\* Nodes 9, 10, 14, 15 no readings.

TABLE XIX CONTINUED

Plant	Node Number*	Root	Position on Node	Ploidy of Roots
1 $\frac{1}{2}$ -6 days	1	A	4	4n
		B	--	--
	2	A	8	4n
		B	--	--
	3	A	2-3	2n
		B	--	--
	4	A	2	2n
		B	--	--
	5	A	5	2n
		B	1	4n
	6	A	6-7	2n
		B	--	--
		C	5-6	4n
	7	A	6	2n
		B	1	--
	8	A	6	2n
		B	2-3	--
	9	A	7	2n
		B	2-3	4n
	10	A	4	2n
		B	6-7	4n-2n
	11	A	2-3	4n
		B	--	--
	12	A	6	--
		B	--	--
	13	A	6-7	--
		B	2	--
	15	A	2	4n-2n
		B	--	--
	17	A	2	2n
B		--	--	
18	A	6	Mix	
	B	3	--	
19	A	6-7	2n	
	B	2	4n	
20	A	2-3	4n-2n	
	B	--	--	
21	A	2	4n	
	B	--	--	
22	A	2-3	--	
	B	--	--	
23	A	2-3	2n-4n	
	B	6-7	2n-4n	
25	A	3	4n-2n	
	B	6	4n	

\* Nodes 14, 16, and 24 no readings.

PLATE III

(Chromosome Figures Magnified 2700x)

FIGURE 13: DIPLOIDY IN ROOT A, NODE 3 OF PLANT 1.0/6.

FIGURE 14: DIPLOIDY IN ROOT A, NODE 17 OF THE SAME PLANT.

FIGURE 15: TETRAPLOIDY IN ROOT A, NODE 19 OF THE SAME PLANT.

FIGURE 16: DIPLOIDY IN ROOT A, NODE 20 OF THE SAME PLANT.

FIGURE 17: TETRAPLOIDY IN THE SAME ROOT AND NODE OF FIGURE 16.

FIGURE 18: DIPLOIDY IN ROOT A, NODE 5 OF THE SAME PLANT.

FIGURE 19: TETRAPLOIDY IN ROOT B, NODE 5 OF THE SAME PLANT.

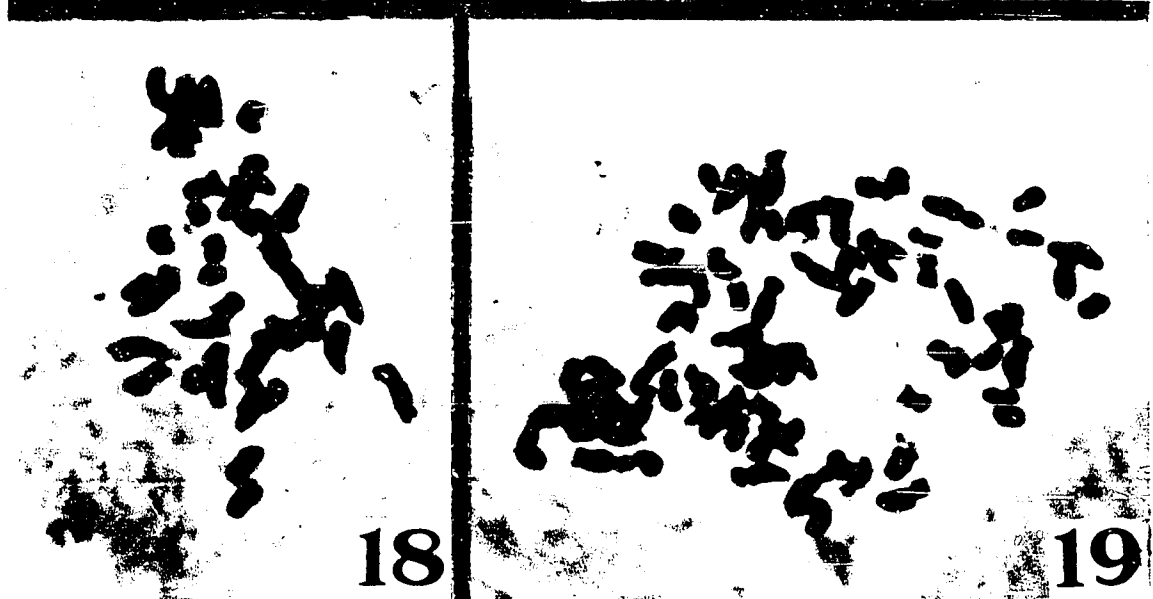
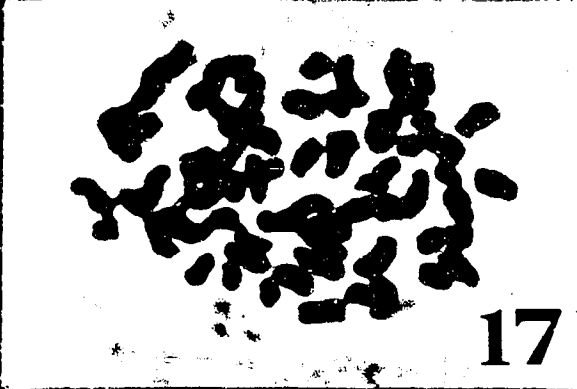
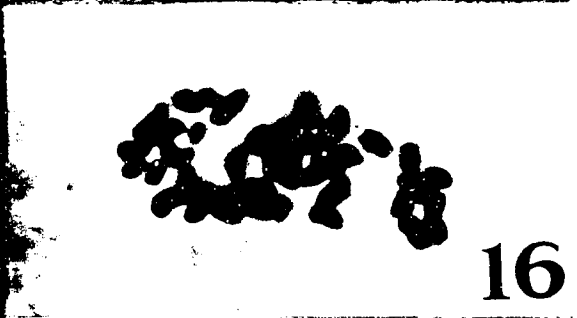
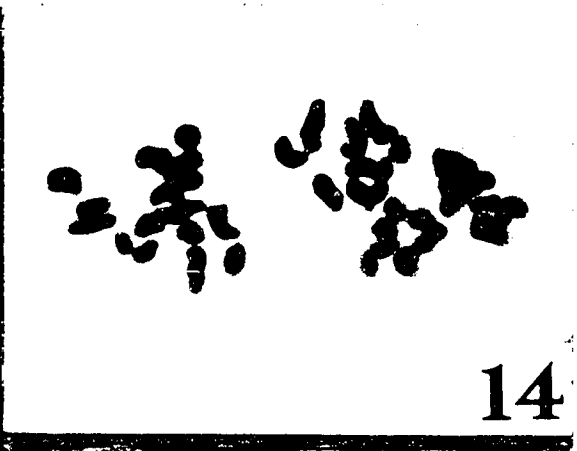


PLATE IV

(Chromosome Figures Magnified 2700x)

FIGURE 20:  $4n$  CELL IN ROOT A OF NODE 2, SHOOT NO. 3 OF PLANT  
0.5/4.

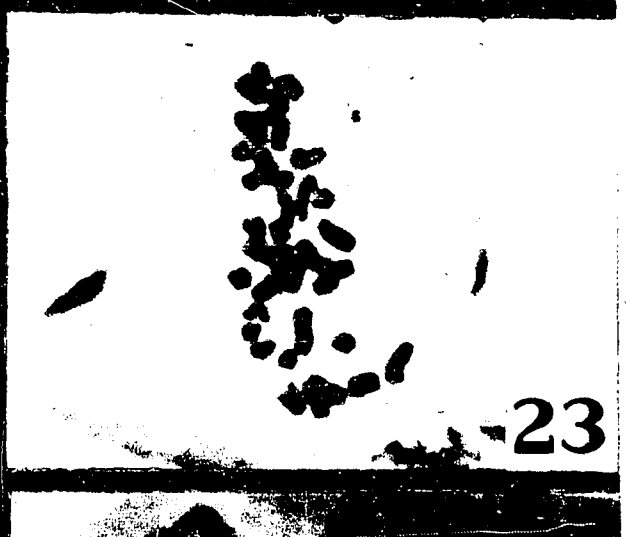
FIGURE 21:  $4n$  CELL IN ROOT B OF NODE 2, SHOOT NO. 3 OF THE SAME  
PLANT.

FIGURE 22: TETRAPLOIDY AT ROOT B, NODE 6 OF PLANT 1.5/4.

FIGURE 23: DIPLOIDY AT ROOT A, NODE 7 OF THE SAME PLANT.

FIGURE 24: TETRAPLOIDY AT ROOT A, NODE 8 OF THE SAME PLANT.

FIGURE 25: TETRAPLOIDY AT ROOT B, NODE 11 OF THE SAME PLANT.



one to eight running clockwise. A Vanda Miss Joaquim plant is placed in front of the person and all roots emerging directly towards him is given the numerical designation 4-5 or in between position 4 and 5. If the root is slightly to the right, it would be in position 4. Thus, two plants were examined and all available roots at each node were given position numbers. The data are tabulated in Table XIX giving the position on the stem for each available root and the ploidy of the roots. Unfortunately, many nodes did not possess roots and in some cases, although roots were present, they did not possess good root tips for chromosome counts. A disadvantage of this method of designating relative position of roots is that there is no way of accounting for possible twisting of stems in the process of elongation. However, for the lack of a better method, this system may suffice to determine possible relationships.

Examination of the position numbers for plant 1.0/6 shows that there were roots at seven nodes in position 2-3. Two of the nodes do not have ploidy determinations. Root A, node 3, was found to be  $2n$ , but root B at node 9, and root A at node 11 were  $4n$ . Root A, node 20, and root A, node 23 were  $4n-2n$  and  $2n-4n$ , respectively. Position 2 at the various nodes was also represented by  $2n$ ,  $4n$ , and  $4n-2n$  conditions. There were two nodes with roots in position 3, one of which was determined to be  $4n-2n$ . Thus, from position 1 to 4 which encompasses one-half the stem on this plant, there is no consistent association of ploidy with nodal position.

In section 4 and 5 of the same plant, ploidy determinations for all roots emerging in these two sections were  $4n$ . The nodes involved were 1, 3, 11, 16, and 18. The sectors made up of numbers 4 and 5

seemed to be  $4n$  all the way. Sectors 6 and 7 also were tetraploid for nodes 5, 11, and 18. Thus, in plant 1.5/4, all chromosome counts made for sections 5, 6, 7, and 8 were completely tetraploid at all nodes tested from the first to the 20th node. However, the entire picture is still not clear since counts on all nodes were not available. It is tentatively designated that the left half of the stem, as indicated by the scheme presented here, is tetraploid and that it could be considered a vertically extended chimera of a large sector in the stem.

In the case of plant 1.0/6, diploidy and tetraploidy were represented in both halves of the stem and no single ploidy was found consistent from node to node along any one sector.

An interesting aspect of ploidy relationship of sectors within a node and between sectors on different nodes was the ploidy evaluation of axillary shoots which emerged in several nodes. The axillary shoots for two plants, 1.5/4 and 1.0/6, were analyzed and the data presented in Table XX. In the case of plant 1.5/4, the first shoot appeared on node 2 at position 6-7 and the ploidy of this shoot was  $4n$  on two nodes examined. Root A at node 2 is in position 2-3, directly opposite the shoot and was determined to be  $2n$ . The second shoot is at node 5, position 3 and the ploidy, although determined only once, was  $2n$ . Root A on this same node but in position 6 was  $4n$ . At node 8, the shoot is in position 2-3 and its ploidy was determined to be  $4n$ . Root A at this node is in position 1 just adjacent to this shoot and the ploidy was also  $4n$ . In the case of plant 1.0/6, both shoot and roots at two nodes had the same ploidy, although situated in different positions. Information of this sort would be valuable in isolating

TABLE XX. RELATIONSHIP OF AXILLARY SHOOT PLOIDY TO PLOIDY OF ROOTS AT THESE NODES.

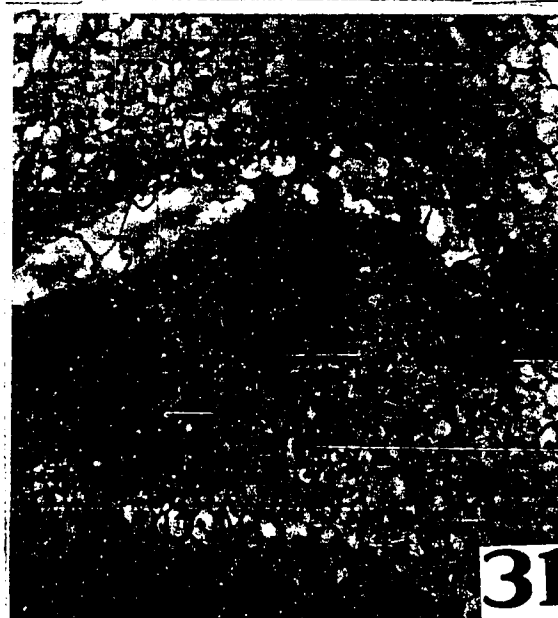
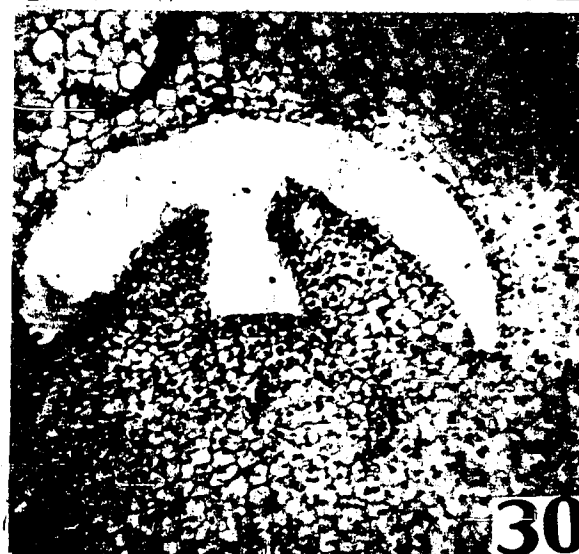
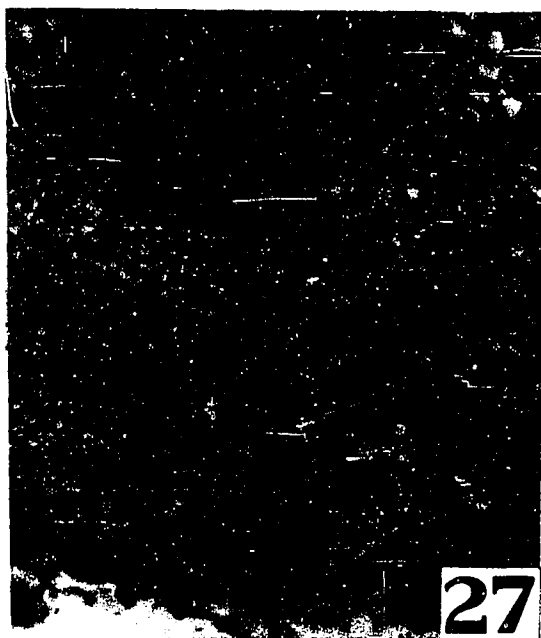
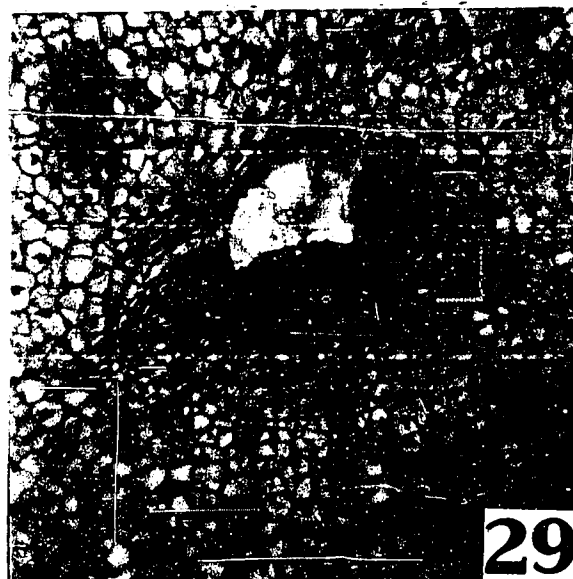
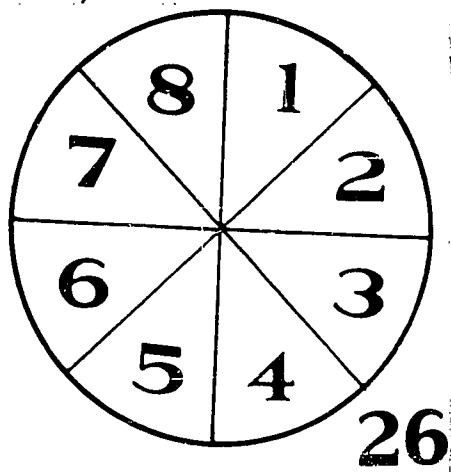
Plant Identity by Treatment	Node Number	Ploidy and Position of Roots				Shoot Position on Node	Ploidy of Shoot**
		Root A	Position*	Root B	Position		
1.5%-4 days	2	2n	2-3	----	----	6-7	4n
" "	5	4n	6	----	----	3	2n
" "	8	4n	1	----	---	2-3	4n
1.0%-6 days	3	2n	2-3	----	---	4-5	2n
" "	10	2n	4	4n-2n	6-7	4-5	4n-2n

\* Position refers to Figure 26, showing schematic division of cross-section of node from which roots and shoots can emerge. The number of the section indicates the position of the root or shoot at a node.

\*\* Since axillary shoots were young, ploidy indicated in this column represents only two to three nodes.

## PLATE V

- FIGURE 26: SCHEMATIC DIVISION OF STEM FOR CLASSIFYING THE POSITION OF ROOTS OR SHOOTS ON THE NODES.
- FIGURE 27: ROOT APEX OF  $4n$  PLANT (0.5%-4 DAYS) SHOWING MERISTEMATIC AREAS. MAGNIFICATION AT 170x.
- FIGURE 28: ROOT APEX OF DIPLOID PLANT. MAGNIFICATION AT 170x.
- FIGURE 29: SHOOT APEX OF  $4n$  PLANT (0.5%-4 DAYS) SHOWING TWO LAYERS OF TUNICA AND A CORPUS LAYER. MAGNIFICATION AT 170x.
- FIGURE 30: SHOOT APEX OF  $2n$  PLANT. MAGNIFICATION AT 170x.
- FIGURE 31: SHOOT APEX FOR PLANT 1.0%-6 DAYS (NODE 36) SHOWING DIFFERENCES IN CELL SIZE BETWEEN LEFT AND RIGHT SIDE OF THE TWO TUNICA LAYERS. MAGNIFICATION AT 170x.



shoots of different ploidies for the study of chimeral types. Isolation and culture of pure tetraploid shoots from nodes of known ploidy is entirely possible.

#### ONTOGENETIC AND CYTOHISTOLOGICAL CONSIDERATIONS OF THE INDUCED CHIMERAL TYPES

The chimeral nature of the colchicine-induced polyploids in these experiments affords an opportunity to investigate the ontogenetic relationships of the germ layers of the shoot apex and their tissue derivatives in the mature stem relative to the conditions of ploidy.

In the chimeral types induced in Vanda Miss Joaquim, an attempt is made to explain the phenomenon from the histogenic and ontogenetic standpoint. In Figures 29 and 32 are shown the apical dome region of a  $4n$  plant, and in Figure 30 a diploid apical dome. A close examination of Figures 29 and 30 shows that there are two layers of tunica over the corpus region. In Figure 32, however, the tunica is represented rather clearly by three layers. This difference in number of tunica layers within the same plant is not surprising in the light of recent studies by Popham (47), who reported differences in the organization, dimensions and genetics of the apices, even within an individual plant.

Since the outermost layer of the tunica gives rise to the protoderm, the precursor to the epidermis of the older tissues, a change in ploidy in this layer would produce the same changes in the epidermis. Figures 29 and 32 show complete change to  $4n$  in the tunica layers. If there were several initials, only one or two of which changed in ploidy, anticlinal divisions would create a situation in which epidermis on one side would be of one ploidy and the other side would be of another

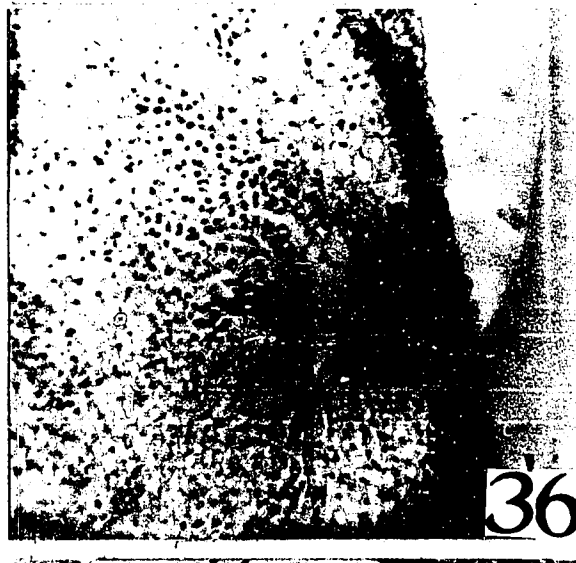
ploidy. This situation appeared to have occurred here in one case shown in Figure 31. The cells in the outermost layer of the tunica on the right side of the dome are definitely larger than cells in similar position on the left of the dome.

The second and third tunica layers usually contribute to tissues of the sub-epidermal layer and the procambium system, although much of the procambium is derived from the group of cells in the corpus region. A complete change from  $2n$  to  $4n$  in the corpus region would give rise to  $4n$  vascular system and all secondary organs resulting from this region. Part of the ground meristem also comes from the corpus cells dividing periclinally as well as anticlinally. Therefore, some cortical tissues and pith cells would also be of  $4n$  nature if the cells in the corpus were changed from diploidy to tetraploidy. Chimeras and mixoploids would result if only partial changes took place in the corpus initials. It is difficult to determine from the slide preparations the number of such initials, but it is obvious that there is a group of these cells rather than one, two, or even three. In the case of the plant 1.5/4, all nodes tested on one-half of the plant were  $4n$  and the other half was composed of  $4n$  and  $2n$  cells. It appears safe to assume that the central cell initials giving rise to tissues on the  $4n$  half of the plant were completely doubled in their chromosome numbers, while on the other half, a few  $2n$  cells were still present, thus giving rise to a mixoploid condition.

In the list of induced tetraploids in Table XVI, seven out of the ten are indicated as temporary tetraploids. These were classified as such because the tetraploid condition appeared to be lost after the

## PLATE VI

- FIGURE 32: A  $4n$  SHOOT APEX OF V. MISS JOAQUIM SHOWING THREE TUNICA LAYERS. MAGNIFICATION AT  $170\times$ .
- FIGURE 33: FLOWER SPIKE PRIMORDIUM AT THE AXIL OF A YOUNG LEAF IN THE APICAL MERISTEM OF V. MISS JOAQUIM. MAGNIFICATION AT  $100\times$ .
- FIGURE 34: FLOWER SPIKE PRIMORDIUM WITH BRACT FORMATION. MAGNIFICATION AT  $170\times$ .
- FIGURE 35:  $2n$  V. MISS JOAQUIM NODE SHOWING ROOT PRIMORDIUM ON THE LEFT AND FLOWER SPIKE PRIMORDIUM ON THE RIGHT. MAGNIFICATION AT  $100\times$ .
- FIGURE 36: FLOWER SPIKE PRIMORDIUM WITH BRACT FORMATION TAKEN AT MAGNIFICATION AT  $170\times$ .
- FIGURE 37: DIPLOID ROOT PRIMORDIUM SHOWN IN FIGURE 35 TAKEN AT A MAGNIFICATION AT  $170\times$ .



initial two or three readings. In several cases, after the first root was determined to be tetraploid, all other subsequent roots were diploids.

Since none of the induced polyploid plants flowered during the course of the study, no ploidy studies were conducted for the flowers. However, from the ontogenetic standpoint, certain predictions may be made. Figure 33 shows an inflorescence primordium developing in the axil of the leaf at the shoot apex. Figure 34 shows the same primordium with bract formation. If the first tunica layer gave rise to the epidermal layer and the second tunica layer and the corpus both participated in the ontogeny of the inner tissues of the flower spike or the vegetative shoot, the ploidy of the flower parts would be similar to that of the cell initials giving rise to these organs. A tetraploid condition of the apical dome cells as shown in Figures 29 and 32 should give rise to tetraploid flower spikes. A partial change in ploidy of the tunica layer as seems to be the case in Figure 31 should give rise to mixoploid condition in the epidermal layer of the flower spike. Figure 35 shows a nodal region with a root primordium on the left and a floral spike primordium on the right. Figures 36 and 37 show these primordia separately at a higher magnification.

The aerial roots of V. Miss Joaquim are adventitious roots and in all probability arise endogenously from one or several procambial meristems in the vascular bundles. This assumption appears to be a valid one in the light of similar occurrences cited by Esau (24). Figures 27 and 28 show the root apices of a  $4n$  and  $2n$  root tip respectively. The organization of the root apex appears to be similar to that of

V. luzonica and V. parishii which Engard (23) classified as being Haberlandt's type VI. In this type tissues are derived from a mass of cells at the root apex but the protoderm takes no part in the formation of the root cap. Consequently, a few diploid cells among tetraploid cells in this group of meristems will give rise to a mixoploid condition.

Morphological differences between tetraploid and diploid cells are quite evident in the photomicrographs presented here. In the case of the root apex, although Figures 27 and 28 are not at exactly the same region, the  $4n$  cells in the meristem region in Figure 27 appear to be much larger than those of comparable region in Figure 28. At the shoot apex, the  $4n$  cells shown in Figure 29 and 32 are obviously larger than the cells in the  $2n$  shoot apex in Figure 30. In the actual slide preparations from which the photomicrographs in Figure 29 and 32 were taken, approximate tetraploid counts of chromosomes were made in two cells, thus further confirming the ploidy of the shoot.

The development of the leaf still has to be considered. The photomicrographs in Figures 29 and 32 show rather clearly that the first tunica layer takes part in the development of the epidermis of the leaf. The second tunica layer and the corpus appear to contribute to inner tissues of the leaf. Since the entire first tunica layer appears to be tetraploid, the epidermis of the leaves of this plant should be tetraploid. Stomatal measurements showed the polyploidal nature of the epidermis.

To determine the differences in number and size of stomata, counts and measurements were made from leaf samples of the diploid shoot no. 2

and the tetraploid shoot no. 3 of plant 0.5/4. Table XXI shows the variance analysis for the mean number of stomata for  $4n$  and  $2n$  leaves. There was no difference in the number of stomata between diploid and tetraploid leaves based on counts per microscopic field at 250X magnification. However, the F values observed for number of stomata on the different positions on the leaf approached the .05 per cent level of significance. When analysis of variance was calculated for the diploid and tetraploid leaves separately to determine differences in number according to position on the leaf, there was no evidence of differences in the number of stomata in the three regions of the tetraploid leaf. However, in the diploid leaf there was a highly significant difference in the number of stomata between positions. Table XXIII gives the mean number of stomata for base, center and tip of leaf in ranked order according to Duncan's (17) method. It is obvious that the big difference is between the basal portion and the tip portion of the leaf. There appears to be more stomates near the tip than at the base of the leaf.

In the test for size differences in stomata between  $4n$  and  $2n$  leaves, variance analysis was again employed and results are shown in Table XXIV. The difference in mean stomata size between  $4n$  and  $2n$  leaves was highly significant with  $4n$  leaves possessing larger stomata. In the test for differences in size of stomata in the different sections of the leaf, a highly significant F value was observed. Again, by Duncan's multiple range and multiple F test method, the real differences between means of the base, center, tip and sheath were determined. On the bottom of Table XXIV, the means are presented in ranked order. The tip seems to possess the smallest stomata with increasing size towards

TABLE XXI. VARIANCE ANALYSIS FOR NUMBER OF STOMATA ON  
LEAVES OF 4n SHOOT AND 2n SHOOT OF PLANT 0.5/4.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Values Observed	F Values .05	F Values .01
Total	23	30.34				
Between Ploidy	1	0.10	0.10	<1	4.95	8.40
Position on Leaf	2	7.93	3.96	3.04	3.59	6.11
Replication	3	0.11	.04			
Error	17	22.20	1.30			

TABLE XXII. VARIANCE ANALYSIS FOR NUMBER OF STOMATA ON  
LEAVES OF 4n SHOOT OF PLANT 0.5/4.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Values Observed	F Values Calculated .05
Total	11	6.65			
Position on Leaf	2	2.12	1.06	1.84	5.14
Replication	3	0.98	.32	<1	4.76
Error	6	3.55	.59		

the base and sheath. When number and size of stomata are considered together, there seems to be an inverse relationship showing decreasing size of stomata with increasing number relative to position on the leaf. This information becomes extremely important when stomatal size is used to determine ploidy. In all probability, measurements taken from the epidermal layer at the tip of the leaf for one plant and at the base for another plant, will cancel each other and show no real difference in size, in spite of the possible existence of higher ploidy. Measurements taken in the same positions on both leaves may show real difference.

TABLE XXIII. VARIANCE ANALYSIS FOR NUMBER OF STOMATA  
ON LEAVES OF  $2n$  SHOOT OF PLANT 0.5/4.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Values Observed	F Values .05	F Values .01
Total	11	23.59				
Position on Leaf	2	22.55	11.27	161.00**	5.14	10.92
Replication	3	0.61	.20	2.86	4.76	
Error	6	0.43	.07			

Results: Means in ranked order

Position on Leaf	<u>Base</u>	<u>Center</u>	<u>Tip</u>
Mean Number	<u>6.50</u>	<u>6.93</u>	<u>7.88</u>

Note: Any two means not underscored by the same line are significant.

TABLE XXIV. VARIANCE ANALYSIS FOR MEASUREMENT OF SIZE OF STOMATA  
FOR 4n SHOOT AND 2n SHOOT OF PLANT O.5/4.

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Squares	F Values Observed	F Values .05	F Values .01
Total	31	1311.47				
Between Ploidy	1	518.10	518.10	31.90**	4.24	7.77
Position on Leaf	3	346.57	115.52	7.13**	2.99	4.68
Replication	3	40.81	13.60			
Error	25	405.99	16.24			

	4n Plant	2n Plant		
Means of Stomata Size	55.08 Microns	47.03 Microns		
Means of Stomata Size for Four Positions on the Leaf				
	<u>Tip</u>	<u>Center</u>	<u>Base</u>	<u>Sheath</u>
Mean Size in Microns	<u>46.49</u>	<u>49.83</u>	<u>52.49</u>	<u>55.41</u>

## DISCUSSION AND CONCLUSIONS

In the various methods employed to induce polyploidy by the use of colchicine, it was found that immersing the base of cuttings and young shoots in aqueous-colchicine was most effective. Although seed soaking method was found to be an effective method with seeds of other plants, it was not found to be such with orchid seeds under the conditions of the experiments conducted and reported here. One probable reason is that orchid seeds are relatively slow in germination as compared to the germination rates of other seeds. The period of soaking may have been entirely too short and seeds could have been in a dormant stage when removed from the colchicine solution. Another probable reason is the extreme sensitivity to colchicine injury due to lack of substantial protection of the embryo. A third difficulty is the ever-present chances of fungal and bacterial contaminations of the nutrient agar in which these seeds are germinated. A large proportion of the seeds sowed in these experiments were lost by contaminations. Transplanting mortality takes another toll of seedlings due to damping off. Therefore, there may have been seedlings which resulted in doubling but did not survive up to the period of testing their ploidy.

Dermen and Darrow (14) found that there was no effect on strawberry seeds treated with 0.2 per cent colchicine solution for several days but seedlings with identical treatment were highly responsive. Newcomer (44) also found Cosmos seedlings to be highly responsive to colchicine. Braak and Zeilinga (4) found that seedlings soaked in colchicine and placed in a vacuum for a few minutes increased the incidence of chromosome doubling. The method reported above were

tried with Dendrobium seedlings. Seedlings treated by soaking only and by soaking under vacuum were found to be seriously injured as indicated by the high mortality rate in Table II. No tetraploids were obtained by this method. However, Rotor (51) reported highly successful doubling with Cattleya and Phalaenopsis seedlings immersed in aqueous solutions of colchicine, although he gives no cytological evidences. No information relative to concentrations or durations of exposure are given for the reported successes in inducing doubling of chromosomes in Cattleya and Phalaenopsis seedlings.

Because of the relatively slow rate of growth of orchid seedlings and their extreme sensitivity to colchicine when soaked in it, even for as little as three hours, a less drastic treatment over a longer duration may be more effective. A few drops of aqueous or glycerine-colchicine applied to the apex of the seedlings over several days or even weeks might be more effective. This method appears to be suitable for Dendrobium seedlings because they possess natural pockets formed by the leaves at the terminal. However, the actual apical meristem is embedded far below the leaves and is surrounded by layers of leaf sheath that penetration of the colchicine may be a problem.

Treatment of inflorescences also produced negative results. Although Rotor (51) reported success in doubling the chromosome numbers in Cattleya and Phalaenopsis, he found that most of the buds turned yellow and abscised. Concentrations lower than 0.1 per cent over two to three days may be more effective than concentrations or durations used in the experiments reported here. Also, the time element becomes very important since the colchicine must be applied at a time when pollen

mother cells are in a rapid state of division in the production of spores.

The method of immersing the basal ends of cuttings possessing flower spikes does not appear to be a good method of inducing doubling in the flower spikes because cuttings without a good root system can hardly develop and support flower buds to maturity. Even in the controls, only a few buds actually reached maturity and these flowers did not open fully, although the pollinia appeared to be well developed.

Tip cuttings from mature plants and excised shoots immersed in aqueous-colchicine for several days induced some polyploids. Similar treatment for plants first established on tree fern poles appeared to be promising. In both methods although a few polyploids were induced, the low frequency still indicates induction by chance and no definite percentage success can be predicted for these methods. Apparently, there must be some favorable physiological condition besides the cytological condition of rapid division which must exist within the plant material at the time of treatment. Defining these conditions remains an interesting research problem.

The lanolin and glycerine-colchicine applications on incised apices also do not seem to be an effective means of treatment. Incision is too drastic a treatment of the apex, with injury of the apical dome sometimes occurring. In a few cases, new vegetative shoots emerged from both sides of the incision but the meristems producing these shoots were not affected as shown by chromosome counts. These shoots were not developed from the true apical meristem but from axillary apices.

Dendrobium and strap-leaf Vanda, by the nature of their leaf shape

and position of leaves, appeared to afford a good area at the tips of the plant to inject colchicine solutions. However, the apical meristems are embedded deeply and protected by many folds of leaf sheaths. Reaching the apical meristem becomes difficult. Measurements of the distance between the base of the last leaf and the meristem area after cutting the plants longitudinally were not consistent. Other means of determining the exact area of the apical meristem must first be developed before this method can become effective.

In evaluating the methods, concentrations, and durations of exposure, it was found that cuttings and shoots treated by immersing the base in colchicine solution gave the best results. It was found that concentrations between 0.5 and 1.5 per cent and durations of two to six days were most effective in inducing doubling. Even within these concentrations and durations, a large number of cuttings or shoots must be treated because doubling still appears to be by chance. Furthermore, this method is conducive only to orchids with monopodial growth habits like that of Vanda. Even with Vanda, the terete and semi-terete types seem to be more suited to this method than the strap-leaf type because of the slender stems.

The ploidy of the roots at the various nodes when correlated with the position of these roots on the nodes by the method presented in this paper, indicates the presence of sectorial chimeras and mixoploids. These sectorial chimeras and mixoploids could be used to advantage in further studies relative to ontogeny and histogenesis in orchids as has been done with other plants. Satina, et al. (53) and Satina and Blakeslee (52) have reported on this type of study with Datura,

demonstrating the organization of the apical dome and the nature of periclinal chimeras relative to ploidy. Dermen and Bain (16) and Dermen (11) did similar work on periclinal chimeras of cranberries, demonstrating the histogenesis of primary tissues and the ontogeny of the various plant organs.

Among the chimeral types found in this study, one case is shown (Figure 31) in which the tunica layer on one side of the apical dome was much larger than comparable cells on the other side. This supports the chimeral nature of the shoot as suggested by the correlation of ploidy of roots with the various positions on the nodes.

The temporary conditions of polyploidy can be explained on the basis of cell replacements in the initial region. A tetraploid initial may be pushed out of place by a diploid initial or a single cell in the periphery of the initial area may have been doubled but later replaced or pushed out of the initiating area and ceased to take a prominent role in the histogenesis of tissues. These conditions would allow tetraploid numbers initially. The  $4n$  condition could reappear in a random fashion in roots of subsequent nodes.

In the apical meristem of the V. Miss Joaquim, there are at least two and possibly three tunica layers. The corpus seems to be represented by a group of cells composing the central mother cell area. Complete polyploidy can result only when all the cell initials in both the tunica layers and the corpus are affected. The difficulty in achieving complete polyploidy is a good indication that the number of initial cells are many and are not limited to one or two cells. To effect doubling in a whole group of cell initials, there must be an effective concentration around

the apical dome over a period long enough to allow karyokinesis to go on in all of the cells during this period. It is common knowledge that even within a group of meristematic cells such as is found in the shoot apex, all cells in the group do not divide simultaneously.

The origin of the root primordium assumed to be in some meristematic region in the vascular area. If this assumption is correct a large portion or all of the tissues in the root would take the ploidy of the corpus initials giving rise to the procambium system. If a group of mother cell initials giving rise to the vascular system in one sector of the stem were  $4n$ , than the roots emerging from this sector would undoubtedly be of  $4n$  composition. If the origin were mixed, the roots may also exhibit the same mixture, or be of completely  $2n$  or  $4n$  composition depending upon the ploidy of the initiating mother cell and the number of initiating cells. However, here as in the shoot apex, histogenic organization of the initiating cells may shift so that a  $4n$  cell or cells could replace the  $2n$  cells in the initial area in the germ layers.

The size of the cells as shown in the photomicrographs are different between  $4n$  and  $2n$  tissues. This is in agreement with the results presented by numerous workers for plants other than orchids. The gigas conditions exhibited by natural polyploids must be due to increase in cell size.

Although stomata numbers have been found to be the same between  $2n$  and  $4n$  plants, the size has been determined to be significantly different. Tetraploids possess larger stomata than diploid plants. This is in agreement with the increased size of cells in the tunica layer of the

shoot apex.

#### SUMMARY

The thesis involved three major objectives. The first objective was to devise practical means for inducing polyploidy in selected orchid groups by the use of colchicine. The second objective entailed the study of the characteristics of the induced polyploids. The third objective involved an attempt to analyze and explain the induction of cyto-chimeras and mixoploids found among most of the induced polyploids.

To achieve the first objective, seeds, seedlings, young shoots, stem cuttings, and mature plants of several Vanda and Dendrobium species and hybrids were treated in various ways, using different concentrations of colchicine in carriers such as water, glycerine, nutrient agar, and lanolin. Seeds were either soaked in colchicine solution before sowing in nutrient agar or sowed directly in colchicine-incorporated nutrient agar. Seedlings were soaked in various concentrations. One lot of seedlings was soaked in aqueous-colchicine under vacuum. Cuttings and young shoots were treated by immersing the basal ends in aqueous-colchicine for different durations. Lanolin-colchicine and glycerine-colchicine were applied into incised areas in the apical regions on the plants.

When the results were evaluated, it was found that tetraploidy was induced only in cuttings and young shoots of Vanda Miss Joaquim, whose basal ends were immersed in aqueous-colchicine. Effective concentrations were between 0.5 and 1.5 per cent and durations of exposure were between two and six days. Even within these concentrations and durations, successful doubling still remained a matter of chance and

no definite percentage success can be predicted.

Among the ten tetraploids induced, only one gave  $4n$  number consistently at all nodes tested. The others gave variable numbers of  $4n$  and  $2n$  in the same roots or in different roots of the same plant.

A scheme was devised to classify the position of the roots on the nodes to determine the relationship of the ploidy of roots in the same positions on all nodes. By this method, one plant was found to be of tetraploid nature throughout one-half of the stem on all nodes tested. The other longitudinal half showed a mixture of  $4n$  and  $2n$  numbers from node to node. This condition is indicative of a wide sectorial chimera on one side and a mixoploid condition on the other.

Anatomical sections of a shoot apex from a plant known to give  $4n-2n$  numbers showed distinctly that cells in the first tunica layer on one side were visibly larger than those on the opposite side, indicating that doubling was not complete. From these sections, it was found that in Vanda Miss Joaquim there appears to be two and possibly three layers of tunica over the corpus. It also appears that there is a group of cells composing the central cell initials. This would mean that complete tetraploidy could be obtained only if all cell initials were affected. If a few or even one initial remained diploid, a mixoploid condition would arise. If cell initials giving rise to tissues of one specific sector of the stem were affected, sectorial chimeras would result. These two conditions seem to have occurred in the tetraploids induced in these experiments.

The ontogeny of roots, flower spikes, and leaves were discussed briefly in an attempt to show the relationship of their ploidy to the

germ layers that give rise to these organs.

The size of the cells in the apex of the tetraploids was visibly larger than those of the diploid shoot apex. Stomatal number was determined to be the same for  $2n$  and  $4n$  leaves, but size differences were highly significant with the  $4n$  leaves having larger stomata. The number of stomata within a diploid leaf differed according to position on the leaf. It was found that the tip section of the leaf possessed more stomata than the base. It was also determined that the stomata at the tips were significantly smaller than those from the center of the leaf even in tetraploid leaves. The largest stomata are found on the epidermis of the sheath. Therefore, there is an inverse correlation between size and number according to the position on the leaf.

LITERATURE CITED

1. Baker, R. E. 1943. Induced polyploid, periclinal chimeras in Solanum tuberosum. Amer. Jour. Bot. 30(3): 187-194.
2. Blakeslee, A. F., and A. G. Avery. 1937. Method of inducing doubling of chromosomes in plants by treatment with colchicine. Jour. Heredity. 28(12): 393-411.
3. Bohn, G. W. 1947. Colchicine treatments for use with tomatoes. Jour. Heredity. 38(5): 157-160.
4. Braak, J. P., and A. E. Zeilinga. 1957. Production of a colchicine-induced tetraploid Asparagus. Euphytica. 6(3): 201-212.
5. Brues, A., and A. Cohen. 1936. Effects of colchicine and related substances on cell division. Biochem. Jour. 30: 1363-1368.
6. Clausen, R. E. 1941. Polyploidy in Nicotiana. Amer. Naturalist. 75: 291-306.
7. Cook, R. C. 1938. Tetraploid Zinnias. Jour. Heredity. 29: 187-188.
8. Dermen, Haig. 1931. Polyploidy in Petunias. Amer. Jour. Bot. 18(4): 250-261.
9. \_\_\_\_\_. 1938. A cytological analysis of polyploidy induced by colchicine and by extremes of temperature. Jour. Heredity. 29(6): 211-229.
10. \_\_\_\_\_. 1940. Colchicine polyploidy and technique. Bot. Rev. 6(11): 599-635.
11. \_\_\_\_\_. 1945. The mechanism of colchicine-induced cytohistological changes in Cranberry. Amer. Jour. Bot. 32(7): 387-394.
12. \_\_\_\_\_. 1947. Inducing polyploidy in peach varieties. Jour. Heredity. 38(23): 77-82.
13. \_\_\_\_\_. 1954. Colchicine in grapes. Jour. Heredity. 45(4): 159-172.

14. Dermen, Haig, and G. M. Darrow. 1938. Colchicine-induced tetraploid and 16-ploid strawberries. Proc. Amer. Soc. Hort. Sci. 36: 300-301.
15. \_\_\_\_\_, and D. H. Scott. 1938. A note on natural and colchicine-induced polyploids in peaches. Proc. Amer. Soc. Hort. Sci. 36: 299.
16. \_\_\_\_\_, and H. F. Bain. 1944. A general cytohistological study of colchicine polyploidy in Cranberry. Amer. Jour. Bot. 31(8): 451-463.
17. Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics. 11: 1-42.
18. Eigsti, O. J. 1938. A cytological study of colchicine effects in the induction of polyploidy in plants. Proc. Nat. Acad. Sci. 24: 56-63.
19. \_\_\_\_\_, and Pierre Dustin, Jr. 1955. Colchicine--Agriculture, Medicine and Biology. The Iowa State College Press. Ames, Iowa. 470 p.
20. Emsweller, D. L. 1940. Colchicine-induced polyploidy in Lilium longiflorum. Amer. Jour. Bot. 36(1): 135-144.
21. \_\_\_\_\_. 1947. The utilization of induced polyploidy in Easter lily breeding. Proc. Amer. Soc. Hort. Sci. 49: 379-384.
22. \_\_\_\_\_, and M. L. Ruttle. 1941. Induced polyploidy in Floriculture. Amer. Naturalist. 75: 310-326.
23. Engard, C. J. 1944. Morphological identity of the velamen and exodermis in orchids. Bot. Gaz. 105: 457-462.
24. Esau, K. 1953. Plant Anatomy. John Wiley & Sons, Inc. New York. 735 p.
25. Frazke, C. I., and I. G. Ross. 1952. Colchicine-induced variants in Sorgham. Jour. Heredity. 43(3): 107-115.
26. \_\_\_\_\_. 1957. A lineal series of mutants induced by colchicine treatment. Jour. Heredity. 48(2): 47-50.
27. Gaulden, M. E., and J. C. Carlson. 1951. Cytological effects of colchicine on the Grasshopper neuroblast in vitro with special reference to the origin of the mitotic spindle. Expt. Cell Res. Supple. 2. 416-433.

28. Havas, L. J. 1940. A colchicine chronology. Jour. Heredity. 31: 115-117.
29. Hawkes, J. G. 1942. Some effects of the drug colchicine on cell division. Jour. Genetics. 44: 11-22.
30. Inoue, S. 1952. Effect of colchicine on the microscopic and submicroscopic structure of the mitotic spindle. Expt. Cell Res. Supple. 2. 305-311.
31. Johansen, D. A. 1940. Plant Microtechnique. First Edition. McGraw-Hill Book Co. New York. 522 p.
32. Kamemoto, H. 1950. Polyploidy in Cattleyas. Amer. Orchid Soc. Bull. 19: 366-373.
33. \_\_\_\_\_. 1958. Polyploidy in Vanda. Proc. Second World Orchid Conf. pp. 51-55.
34. \_\_\_\_\_. 1959. The origin and significance of polyploidy in Vanda. Pacific Orchid Soc. Bull. 16(3,4): 77-95.
35. \_\_\_\_\_, and L. F. Randolph. 1949. Chromosomes of the Cattleya tribe. Amer. Orchid Soc. Bull. 18: 366-369.
36. Kosaki, K. 1958. Preliminary investigations on the cytogenetics of Dendrobium. Proc. Second World Orchid Conf. pp. 25-29.
37. Levan, A. 1938. Effect of colchicine on root mitosis in Allium. Hereditas. 24: 471-486.
38. \_\_\_\_\_. 1939. The effect of colchicine on meiosis in Allium. Hereditas. 25: 9-26.
39. MacLeod, R. A. 1947. Some effects of colchicine on orchids. Amer. Orchid Soc. Bull. 16(6): 336-337.
40. Moore, E. T. 1947. The use of colchicine in orchids. Amer. Orchid Soc. Bull. 16(9): 512-513.
41. Muller, H. J. 1954. The nature of genetic effects produced by radiation. Radiation Biology. McGraw-Hill Book Co. New York. 1(part 1): 351-473.
42. Muntzing, A. 1936. The evolutionary significance of autopolyploidy. Hereditas. 21: 253-378.
43. \_\_\_\_\_, and E. Runquist. 1939. Notes on some colchicine-induced polyploids. Hereditas. 25: 491-495.

44. Newcomer, E. H. 1941. A colchicine-induced tetraploid Cosmos. Jour. Heredity. 32: 161-164.
45. \_\_\_\_\_, 1941. A colchicine-induced homozygous tomato obtained through doubling clonal haploids. Proc. Amer. Soc. Hort. Sci. 38: 610-611.
46. O'Mara, J. G. 1939. Observations on the immediate effects of colchicine. Jour. Heredity. 30: 35-37.
47. Popham, R. A. 1960. Variability among vegetative shoot apices. Bull. of the Torrey Bot. Club. 87(2): 139-150.
48. Randolph, C. F. 1932. Some effects of high temperature on polyploidy and other varieties in Maize. Proc. Nat. Acad. Sci. 18: 222-229.
49. \_\_\_\_\_. 1950. Effects induced in Maize by X-rays and the Bikini test atomic bomb. Jour. of Cellular and Comparative Physiology. 35, Suppl. I: 103-117.
50. Ross, J. G., C. J. Franzke, and L. A. Schuh. 1954. Studies on colchicine-induced variants in soybean. Jour. Heredity. 46(1): 10-15.
51. Rotor, G. B., Jr. 1958. Colchicine as a tool in orchid hybridization. Proc. Second World Orchid Conf. pp. 159-170.
52. Satina, S., and A. F. Blakeslee. 1941. Periclinal chimeras in Datura stromonium in relation to development of leaf and flower. Amer. Jour. Bot. 28(10): 862-871.
53. Satina, S., A. F. Blakeslee, and A. G. Avery. 1940. Demonstration of the three germ layers in the shoot apex of Datura by means of induced polyploidy in periclinal chimeras. Amer. Jour. Bot. 27(10): 895-905.
54. Shanti, Batra. 1952. Induced tetraploidy in Muskmelons. Jour. Heredity. 43(3): 141-148.
55. Snedecor, G. W. 1948. Statistical Methods. 4th Ed., Iowa State College Press. Ames, Iowa. 485 p.
56. Sparrow, A. H. 1942. Colchicine-induced univalents in diploid Antirrhinum majus. Science. 96: 363-364.
57. Storey, W. B. 1952. Chromosome numbers of some Vanda species and hybrids. Amer. Orchid Soc. Bull. 21: 801-806.

58. Storey, W. B. 1953. The pentaploidal origin of Vanda Nora Potter. Pacific Orchid Soc. Bull. 11: 17-25.
59. Thompson, R. C., and W. F. Kosar. 1939. Polyploidy in lettuce induced by colchicine. Proc. Amer. Soc. Hort. Sci. 36: 641-644.
60. Wada, B. 1949. Further studies on the effect of colchicine upon the mitosis of the stamen-hair in Tradiscantia. Cytologia. 15: 88-95.
61. Warmke, H. E., and A. F. Blakeslee. 1939. Induction of single and multiple polyploidy in Nicotiana by colchicine treatment. Jour. Heredity. 30(10): 419-432.