Colchicine induction of Tetraploid Plants from Cultured Bulbscale Discs of the Easter lily Lilium longiflorum Thumb

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COLCHICINE INDUCTION OF TETRAPLOID
PLANTS FROM CULTURED BULBScale DISCS OF
THE EASTER LILY LILiUM LONGIFLORUm THUMB

By
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COLCHICINE INDUCTION OF TETRAPLOID
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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

The demands for genetic improvement of plants are rising and the methods to propagate superior plants are improving through tissue culture.

*Lilium* species are commercially propagated by scaling. The base of each bulbscale initiates a few bulblets. Recently, slices of bulbscales were successfully cultured *in vitro* and numerous bulblets were directly initiated from the explants, producing an abundance of plantlets. This practice of *in vitro* vegetative propagation of *Lilium* species not only increases efficiency of multiplying plantlets but also facilitates induction of polyploidy.

Polyploids are frequently demanded for genetic studies and also for their desirable characteristics. Except for the tiger lily, *Lilium tigrinum*, which is triploid, polyploid strains are not available in nature. It is therefore necessary to obtain them through artificial processes. Since Emsweller and Brierley (1940) first induced tetraploidy in *Lilium formosanum* by exposing the apical meristem of flowering stalk to colchicine solutions, several modifications have been applied in *in vivo* production of polyploid lilies. Nevertheless, the rate of success was not predictable. Recently Huang (1983) induced polyploids in *Lilium* plants through tissue culture, but the efficiency of his method was not reported.

The objective of this research was to evaluate a colchicine treatment method for inducing polyploidy in a bulbscale culture of *Lilium longiflorum*. 
LITERATURE REVIEW

*Lilium* species are commercially propagated by scaling. This technique makes it difficult to obtain large numbers of bulbs from disease-free stock in a short period of time. Several tissue culture methods have been developed to speed up propagation of *Lilium* species.

Sheridan (1968) induced calluses from stem apices of *Lilium longiflorum* Thunb, which were explanted on RM medium supplemented with 2 mg/liter of IAA. After three weeks of explantation, callus appeared at the edge of the tissue block in contact with the medium. Although stimulation produced callus from stem explants, the hormone IAA was found unessential for bud initiation. Explants placed on medium free of IAA initiated buds on the surface of the tissue blocks. Plantlets were formed thereafter. Calluses were occasionally proliferated after the formation of roots and shoots. On the other hand, in cultures on RM medium supplemented with IAA, calluses proliferated profusely. However, calluses grew more rapidly in liquid than on agar medium. Sheridan (1968) also found that kinetin at a concentration higher than 1 mg in combination with 2 mg IAA/liter stimulated cell growth in liquid medium. In cultures in which kinetin was omitted or its concentration lowered below 1 mg/liter, after 30 days an average increase of five-fold in fresh weight was noted. Totipotency of the cultured *Lilium longiflorum* cells was kept active on solid or in liquid medium even after six passages of subculturing.

Stenberg et al. in 1977 reported direct bud initiation from
leaves and floral parts cultured on MS medium supplemented with a combination of 5 or 10 mg/liter NAA and 0, 0.1, or 1 mg/liter kinetin. Although no difference in bud initiation between the two composite NAA concentrations was noted, the best hormonal combination for bud initiation was 10 mg/liter NAA and 1 mg/liter kinetin. Little root formation was seen in the cultures on this combination. Chen and Holden (1975) reported root-like structures appearing at the base of petal or anther filament of *Lilium philadelphicum* cultured on MS medium supplemented with 5 mg/liter NAA and 0.1, 0.5 or 1 mg/liter kinetin. This structure was then disorganized and became callus tissue, which initiated shoots ten weeks after explanting. The shoots continued to differentiate from the calluses on MS medium plus 1 mg 2,4-D and 1 mg kinetin/liter under light. Initiation of adventitious roots from the shoots was observed two weeks after transfer to MS basal medium devoid of hormones. However, floral parts or leaves used for explants in plant regeneration were not ideal since the supply of explants was somewhat limited.

It has been well known that bulbscales in the genus *Lilium*, when isolated from parent bulb, readily regenerate bulblets, and this behavior enables horticulturists to propagate the plants in this genus. Therefore, explants excised from bulbscales would be amenable to culture on synthetic media.

Robb (1957) first cultured cylinders of *Lilium speciosum* bulbs on White's solid medium proliferated and differentiated into bulblets in 15-16 weeks. Plant regeneration occurred in far greater number when the explants were taken from the basal end of the bulbscale. Stimart and
Ascher (1978) reported that when bulbscale discs of *Lilium longiflorum* were cultured on RM medium supplemented with 0.03 mg/liter NAA in continuous darkness and in the photoperiods of 16 hours cool white fluorescence light and 8 hours dark at 25°C, the treatment of continuous darkness increased the number and size of bulblets initiated from the explants. The treatment of 16-hour light cycles suppressed bulblet formation but enhanced root weight, leafy structure formation and fresh weight of callus. They also noted that more and larger bulblets formed on sections from the basal part of the bulbscale and distal sections of bulbscale required NAA for growth, suggesting that there were physiological differences at different sites within a bulbscale.

Takayama and Misawa (1979) tested the effect of culture conditions on the differentiation of bulblets and roots from cultured bulbscales of *Lilium auratum* Lindl. They found that an optimum temperature of 20°C and an optimum pH of 6 increased root weight and length. Kinetin tended to stimulate the formation of bulblets while NAA induced roots. Sucrose at 90 or 120 g/liter favored callus formation. In *Lilium speciosum* various parts were used as explants. Bulbscale segments were found to be the best material for *in vitro* production of a large number of plantlets.

Takayama and Misawa (1980) noted that benzyladenine (BA) in MS medium stimulated bud initiation but inhibited root formation in bulbscale cultures of *Lilium auratum*. An addition of activated charcoal to the medium negated the effect of NAA on bulbscale differentiation, while the growth of bulblets was markedly stimulated. They also
reported that the physiological age of bulbs influenced the capability of organ formation; external bulbscales from old bulbs showed a low ability to form bulblets. On the other hand, bulbscales from young bulbs or internal bulbscales from old bulbs formed bulblets effectively.

Takayama and Misawa (1982a) further investigated the regulation of organ formation by NAA and kinetin on bulbscale culture of Lilium speciosum and Lilium auratum Lindl. The presence of NAA and kinetin in MS medium showed some synergistic effects on organogenesis. The effects of BA and kinetin were compared and the results indicated that BA has a stronger physiological effect on organ formation than kinetin and that their effects on Lilium auratum and Lilium speciosum were BA or kinetin-specific. The action of kinetin was affected by sucrose concentration and the strength of MS medium. High sucrose concentration negated the kinetin inhibition of root formation, while the high MS medium strength inhibited root formation. The significant role of sucrose concentration and MS medium strength on differentiation and growth could be related to osmotic regulation. They concluded that the optimum condition for multiple adventitious bulbscale formation in L. auratum and in L. speciosum was a basal medium containing 0.54 \( \mu \text{M} \) NAA in combination with 14 \( \mu \text{M} \) kinetin or 4.5 \( \mu \text{M} \) BA.

Chen et al (1983) succeeded in culturing leaves and bulbscales of Lilium formosanum on MS medium. It was noted that kinetin promoted bud formation but retarded root initiation.

Takayama and Misawa (1982b) presented a scheme for in vitro propagation of Lilium species. This scheme comprised three processes:
1) establishment of aseptic culture on MS medium; 2) multiplication of propagula in liquid medium; and 3) preparation for reestablishment of plants in soil.

Conventional methods of polyploid induction by colchicine in lilies have been reported by many workers. Emsweller and Brierley (1940) first succeeded in induction of tetraploidy in Lilium formosanum. When the flowering stalks of the plants were six to eight inches high, the top leaves were all trimmed back in order to expose the apical meristem. The stem tips were then immersed in various concentrations of aqueous colchicine solution. Bulblets were later initiated in the axils of the old leaf stubs. Some of the plants derived from the bulblets were tetraploid with thick leaves, large stomates, flowers and pollen grains.

Later, Emsweller and Ruttle (1941) and Emsweller and Lumsden (1943) immersed freshly detached bulbscales of Lilium longiflorum in colchicine solution prior to scaling. A small percentage of bulblets initiated from the treated scales developed into tetraploid plants.

Tetraploid plants were also induced in L. formosanum by immersing bulbscales in colchicine solution or applying colchicine solution to the growing point in the connecting region of the plumule and the radicle of germinating seedlings (Iizuka and Ikeda, 1968). They further obtained hypo-, hyper-, and eu-tetraploids from 4x x 4x and 4x x 2x hybrids. These derivatives were valuable from the ornamental point of view because of their large, heavy-textured crown and short stems.

Earlier, Sagawa (1958) obtained triploid plants from reciprocal
crosses between diploid and tetraploid clones of the Easter lily _L. longiflorum_. These hybrid plants possessed similar gigantic characteristics as tetraploids of _L. formosanum_.

In a breeding program evaluating tetraploid Easter lilies, Emsweller and Uhring (1960) noted that varietal autotetraploids were both self- and cross-sterile and inferior to the diploids from which these tetraploids were derived. On the other hand, tetraploids induced from intervarietal hybrids were slightly cross-fertile. Subsequent generations produced more seeds and the seedling progenies were more variable than any produced by diploids. They further noted that continued breeding and selection on the tetraploid level have produced seedlings superior to any diploids available at that time.

Many instances of _in vitro_ induction of chromosome doubling have been reported in the literature. In haploid cell cultures, chromosome doubling is an indispensable procedure in restoration of diploid number to produce isogenic plants (Narayanaswamy and Chandy, 1971). Although occurring occasionally in somatic tissue culture (Ramulu et al 1976), polyploidization may be induced effectively by colchicine treatment of cultured cells.

Heinz and Mee (1970) experimented with sugarcane cells suspended in liquid medium containing different concentrations of colchicine. Intensive endoreduplications of chromosomes were noted in treated cells. Most dividing cells had doubled the chromosomes number. Of 28 plants randomly selected for chromosome counts, 18 were proven to be polyploids. No polyploid was observed in the control.
Orton and Steidl (1980) treated callus cultures originating from hybrid embryos of the interspecific cross *Hordeum vulgare* × *Hordeum jubatum* with colchicine and noted that 40% of the plants regenerated were tetraploids of high fertility.

Hermsen *et al.* (1981) studied chromosome doubling through adventitious shoot formation from cultures of diploid potato hybrids. It was noted that 85% of the plantlets regenerated had their chromosomes doubled or quadrupled.

Chen and Goeden-Kallemeyn (1979) obtained tetraploid and octaploid plants from colchicine treated diploid callus of daylily. Differentiating callus tissues of *Hemerocallis flava* L. was placed on morphogenetic induction medium containing 0, 10, 20 or 40 mg colchicine/liter in the dark at 12°C for 3 days and recuperated for one week under the same conditions without colchicine. Over 50% of the plantlets initiated from the colchicine-treated calluses were completely tetraploid. All plantlets propagated from control explants had a diploid number of 22 chromosomes. In their experiments the most effective concentration of colchicine for production of tetraploids was 20 gm/liter.

Espino and Vazquez (1981) exposed leaf cultures of *Saintpavlia ionantha* to colchicine or caffeine on MS medium and then transferred them onto a medium free of the mutagen. The incidence of polyploidy from colchicine treatment was higher than from caffeine treatment. In both instances cytochimeric and octoploid plantlets were identified by nuclei size difference.
Lyrene and Perry (1982) treated blueberry shoots with colchicine after seedlings were established on Knops medium. Shoots were obtained and treated by either explanting on modified Knops agar medium containing 0.001 to 0.2 percent colchicine or exposing to 0.2 percent colchicine for 1 to 14 days in liquid medium. They noted that growth and survival of explants were reduced in higher colchicine concentrations in solid or liquid medium. However, their preferred method was in liquid medium on a rotating wheel for 24 hours with 0.20 percent colchicine.

Perry and Lyrene (1984) further noted that the survival rate of treated blueberry explants decreased with increasing colchicine concentration and treatment duration. Differential responses to colchicine treatments were also noted among species tested.

In another experiment, Goldy and Lyrene (1984) obtained 4x and 8x plants from treated blueberry explants with colchicine in liquid medium. It was also noted that one plant had 4x - 8x chimera.

Recently, Huang (1983) applied 0.05% colchicine solution to cotton-balls into the center of intervarietal and interspecific lily hybrid plantlets. He also immersed bulblet scales which initiated from lily cultures in colchicine solution and then recultured. In both instances polyploids were obtained. Gigas characteristics such as thicker and wider leaves, deeper green color with larger stomata, and a doubled chromosome number were noted. Later, larger flowers were also noted. However, he actually induced polyploidy in vivo then cultured the already polyploid tissue.
MATERIAL AND METHODS

Bulbs harvested from three year old plants of the Easter lily *Lilium longiflorum* Thumb cv 'Nellie White' were used in this research. The bulbs were dug out from the garden in September, 1983 and stored in a refrigerator at about 5°C for 4 weeks. For preparing explants outer scales were discarded and inner, healthy ones were striped off and cleaned in non-toxic detergent solution for 10 minutes. The scales were then rinsed in running water for 30 minutes, dipped in 70% ethanol for 10 seconds and disinfected in 10% commercial bleach for 15 minutes. After being rinsed three times in sterile water, the lower half of a scale was sliced at a thickness of approximately 2-3 mm. Three pieces of bulbscales were inoculated into each culture flask unless otherwise stated. Each culture flask contained 40 ml of culture medium.

MS formula (Murashige and Skoog, 1962) supplemented with 0.1 mg NAA and 3 mg KN/liter, proven to be a bud initiation medium (Chen et al., 1983) was used to initiate the culture for mitotic activation. All media were adjusted to pH 5.6 and solidified with 8 gm agar per liter. The cultures were incubated in the dark at 25°C.

Three bulbscale discs were sampled at 5-day intervals for 20 days after being explanted on the bud initiation medium for mitotic survey. These discs were then fixed in Nawashin solution for 24 hours, dehydrated with ethanol-butanol series and embedded in paraffin blocks. Serial sections were made along the tip-base axis of the bulbscale discs at 8 μM thick. The sections were stained with safranin...
and fast-green combination and mounted with synthetic resin before being examined under the microscope.

Ten and 20 days after explanting, the bulbscale discs were transferred onto the MS medium further supplemented with 0, 10, 20 and 40 mg colchicine/liter for 2 and 4 days respectively, before being returned to the MS medium free of colchicine, for bud initiation. At mid-point of colchicine treatment, the bulbscale discs were inverted to insure that both sides of discs received colchicine. The cultures were incubated in the dark at 25°C until adventitious buds were visually noted. The bud-forming discs were then transferred onto MS medium supplemented with 0.1 mg NAA/liter and incubated at 25°C in lighted growth chamber at a 16 hr photoperiod (cool-white fluorescent light at 1 KI(x) for completing plantlet development.

Ploidy identification was made by somatic chromosome counts, stomate and pollen grain measurements. Somatic chromosomes were prepared by squashing root-tips, which were pre-treated with cool water at 5°C for 24 hours, fixed in acetic ethanol (1:3) for 24 hours, macerated in 1N HCl at 60°C for 10 minutes and stained with Feulgen reagents. Three root-tips were collected from each plantlet and chromosomes were counted from five cells in each root for determining the ploidy in the germ layer L₃.

Stomate sizes were measured from a fingernail polish film formed on the lower epidermis of the leaf with a disc micrometer. Five stomates were measured for identification of ploidy in the epidermal or germ layer L₁. For pollen measurement, pollen grains were collected
immediately after anthesis and stained with acetocarmine. Five pollen grains were measured for determining the ploidy level in layer L2.
RESULTS

Serial sections cut parallel to the tip-base axis of *Lilium longiflorum* bulbscale discs were made at 5-day intervals after explanting to determine the ideal time for colchicine treatment. Starch grains were easily detected in storage parenchyma cells after five days of culturing (Fig. 1A). No mitotic activity was shown at this stage. After ten days of incubation, numerous cells with large nuclei were localized at the surface of the bulbscale disc (Fig. 1B). However, sections made twenty days after incubation showed different stages of bulblet development. Figure 1C shows cells were dividing anticlinally and periclinaly, suggesting that reorganization of a tissue was taking place. In some discs initiation of buds was noted. As shown in Figure 1D, an apical dome with a leaf primordium was clearly seen.

Chromosome counts, measurement of stomate lengths, and diameters of pollen grains revealed that differences clearly existed between diploid and tetraploid lilies (Table 1, Fig. 2 A-F). The chromosome counts made from root-tip cells of plantlets evolved from untreated bulbscale discs were 24, whereas those for tetraploids were 48 (Fig. 2 A-B). As indicated in Table 1, the average stomate length for diploids was $103.60 \pm 0.50 \mu M$ (Fig. 2C) whereas that for tetraploids was $144.00 \pm 0.73 \mu M$ (Fig. 2D). The mean pollen grain diameter for the diploid lily was $97.95 \pm 0.30 \mu M$ (Fig. 2E) as compared to $140.44 \pm 0.25 \mu M$ for the tetraploid (Fig. 2F). Thus, stomate length, pollen diameter, and chromosome counts were the criteria used for detecting ploidy in germ layers L1, L2 and L3, respectively.
Figure 1: Serial sections of *L. longiflorum* bulbscale disc made at different times after culturing.

(A) Made 5 days after explanting. (200x)
(B) Made 10 days after explanting. (200x)
(C) Made 20 days after explanting. (200x)
(D) Made 20 days after explanting. (70x)
Table 1: Stomate and pollen sizes of diploid and tetraploid Easter lilies (µM)

<table>
<thead>
<tr>
<th>Chromosome Number</th>
<th>Stomate Length Mean</th>
<th>Stomate Length S.E.</th>
<th>Pollen Diameter Mean</th>
<th>Pollen Diameter S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>103.60</td>
<td>0.50</td>
<td>97.76</td>
<td>0.30</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>144.00</td>
<td>0.73</td>
<td>140.44</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figure 2: A comparison of chromosome numbers (A and B) (800x), stomate sizes (C and D) (200x), and pollen grain sizes (E and F) (200x) between diploid (left) and tetraploid (right) Easter lily plants.
Based upon stomate sizes, pollen grain diameters, and root-tip chromosome counts made in each of the plantlets regenerated from colchicine-treated bulbscale discs, the results of treatments are summarized in Tables 2 and 3. As shown in Table 2, the colchicine concentration at 10 mg/liter in the medium did not affect the survival of discs exposed for two days as compared to the discs which were grown on colchicine-free medium. However, the media containing higher colchicine concentrations reduced the survival rate of discs. The survival percentages of discs grown on the media containing 0 and 10 mg colchicine/liter were 89.7% and 88.8%, respectively, whereas discs grown on the media containing 20 and 40 mg colchicine/liter had the same survival percentages of 69.4%. The capacity of bulblet initiation as represented by the average number of plantlets produced by each bulbscale disc varied from 2.5 plantlets per disc in the 40 mg/liter treatment to 4.2 plantlets per disc in the 20 mg/liter treatment. On the other hand, the treatments where bulbscale discs were mitotically activated for 10 days and exposed to colchicine for two days showed a considerable effectiveness in polyploidization. The relative frequencies of tetraploid plantlets occurred in 10, 20 and 40 mg colchicine/liter treatments were 19.5, 30.8 and 25.4, respectively (Table 2). Among the tetraploids cytologically examined, three plantlets or 3.5% in the 10 mg/liter treatment were cytochimeras. Two of these plantlets were sectorial cytochimeras containing both diploid and tetraploid cells in the root-tips or L3 (Fig. 3 A-B). The third one had haploid and diploid pollen grains or L2 (Fig. 3 D), but diploid in
Table 2: Induction of polyploidy in bud-initiating bulkscale discs on MS solid medium containing 3 mg KN, 0.1 mg NAA/liter and various concentrations of colchicine for two days after 10 days of culturing.

<table>
<thead>
<tr>
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<th>Colchicine concentrations (mg/liter)</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>No. discs treated</td>
<td>39</td>
</tr>
<tr>
<td>Discs survived</td>
<td>35</td>
</tr>
<tr>
<td>%</td>
<td>89.7</td>
</tr>
<tr>
<td>No. plants reg.</td>
<td>102</td>
</tr>
<tr>
<td>Ave. No. plants</td>
<td>2.9</td>
</tr>
<tr>
<td>Tetraploid plants</td>
<td>0</td>
</tr>
<tr>
<td>%</td>
<td>0</td>
</tr>
<tr>
<td>Cytochimeral</td>
<td>0</td>
</tr>
<tr>
<td>plants</td>
<td>3.5</td>
</tr>
</tbody>
</table>

(1) Two plants are sectorial chimeras having both 2x and 4x cells in root-tips or L3, and one plant has 1x and 2x in pollen grains or L2.

(2) Periclinal chimeras (tetraploid in epidermal layer only).

(3) One plant has both 2x and 4x cells in root-tips or L3, tetraploid in stomata or L1 and pollen grains or L2.
Table 3: Induction of polyploidy in bud-initiating bulbscale discs on MS solid medium containing 3 mg KN, 0.1 mg NAA/liter and various concentrations of colchicine for four days after 20 days of culturing.

<table>
<thead>
<tr>
<th>Colchicine concentrations (mg/liter)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. discs treated</td>
<td>40</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Discs survived</td>
<td>40</td>
<td>39</td>
<td>34</td>
<td>37</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>76.5</td>
<td>66.7</td>
<td>72.5</td>
</tr>
<tr>
<td>No. plants regenerated</td>
<td>101</td>
<td>75</td>
<td>63</td>
<td>125</td>
</tr>
<tr>
<td>Ave. No. Plants per disc</td>
<td>2.5</td>
<td>1.9</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Tetraploid plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>%</td>
<td>0</td>
<td>0</td>
<td>6.3</td>
<td>19.2</td>
</tr>
<tr>
<td>Cytochimeral plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>0</td>
<td>0</td>
<td>2(1)</td>
<td>9(2)</td>
</tr>
<tr>
<td>%</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

(1) One plantlet has both 2x and 4x cells in root-tips L3 and tetraploid in L1 and L2.

(2) One plantlet contained 2x and 4x in the root-tip or L3 and diploid in epidermis or L1. Had five flowers; one had diploid pollen grain, one had mixoploid pollen grain, and the rest had diploid pollen grain.
the other layers. The 12 periclinal cytochimeral plantlets, or 11.54% of the total examined in the 20 mg colchicine/liter treatment were tetraploid in the epidermis or L₁ but diploid in L₂ and L₃. The third one was a sectorial cytochimera in L₃ but tetraploid in L₁ and L₂. However, all 102 plantlets regenerated from discs previously not exposed to colchicine were diploid.

As shown in Table 3, colchicine-containing medium reduced survival rates of the discs. The survival rate of discs grown on the colchicine-free medium was 100%, whereas discs grown on the media containing 10, 20 and 40 mg colchicine/liter had the survival rates of 76.5%, 66.7% and 72.5%, respectively. However, colchicine-free medium did not affect survival of bulbscale discs. The capacity of bulblet initiation as represented by the average number of plantlets produced by each bulbscale disc varied from 1.9 plantlets per disc in the 20-mg colchicine/liter treatment to 3.4 plantlets per disc in the 40-mg colchicine/liter treatment.

Bulbscale discs cultured for 20 days in the bud initiation medium and then exposed to colchicine in the medium for four days showed polyploidization only in the two higher concentration treatments (Table 3). All 176 plantlets regenerated from discs treated with 0 and 10 mg colchicine/liter were diploid. Of 63 plantlets regenerated from 20 mg colchicine/liter treatment, 4 or 6.3% were tetraploid. One of these was a sectorial cytochimera in the root-tip or L₃, but tetraploid in L₁ and L₂. Another plantlet was a periclinal cytochimera which had tetraploid stomates or L₁ but diploid in L₂ and L₃. Of the 125 plants regenerated
from the 40-mg colchicine/liter treatment, fifteen were pure tetraploid. Among those eight plants were periclinal cytochimeras having tetraploid stomata or L₁ and diploid L₂ and L₃ (Fig. 3 C). One plant was a sectorial cytochimera in root-tips or L₃. Its stomate size fitted to that of a tetraploid. The plant bore five blossoms; one was mixoploid, one was tetraploid and the other three were diploid (Fig. 4).

Morphologically, tetraploid plants had fewer but thicker leaves than diploid (Fig. 5), whereas sectorial cytochimera plants had longer and thicker leaves than diploid (Fig. 3 E). On the other hand, the flower size and corolla tube of tetraploids were bigger than the flower size and corolla tube of diploid *Lilium longiflorum* plants.
Figure 4: A sectorial cytochimera of *Lilium longiflorum* bearing five flower buds.
Figure 5: Diploid and tetraploid Easter lily plants regenerated from bulbscale cultures.

(A) A diploid plant at rosette stage.

(B) A tetraploid plant at rosette stage.

(C) Size difference of corolla in a diploidy on the left and tetraploidy on the right.

(D) Difference of length in the corolla tube between a diploidy on the left and a tetraploidy on the right.
DISCUSSION

Tetraploid and mixoploid plants were regenerated from *Lilium longiflorum* bulbscale discs grown on MS medium in the presence of different concentrations of colchicine. No spontaneous chromosome variations were observed in the plants regenerated from the bulbscale discs grown on the colchicine-free medium which contained 3 mg kinetin and 0.1 mg NAA/liter. Sheridan (1974, 1975) showed that long-term callus cultures of *Lilium longiflorum* seemed to remain cytologically stable at the diploid level. Goeden-Kallemeyn and Chen (1978) also reported no deviation in chromosome number in daylily plants evolved from calluses subcultured on MS medium supplemented with 1 mg 2,4-D and 1 mg kinetin/liter.

To the contrary, D'Amato (1977) noted that some differentiated cells in the explants might undergo endoreduplication before entering mitosis, a process which produced polyploidy. Ramulu, et al. (1976) obtained diploid, tetraploid and cytochimeras from cultured anthers and stem internodes of *Lycopersicum peruvianum* plants grown on MS media containing various concentrations of kinetin. Brossard (1976) also reported that kinetin influenced ploidy levels of tobacco pith grown in vitro.

Unlike shoot-tip cultures, callus and suspension cultures showed cytological and genetic variability (Sunderland, 1977). Thorpe (1981) stated that chromosome variability in vitro could appear as early as the first subculture, but occurred more often in long-term cultures. However, in this study, among the 203 plants regenerated from discs
cultured on the colchicine-free medium, no chromosome variations were noted, indicating that in bulbscale cultures nuclear stability was assured.

In both experiments, the survival rates observed in colchicine-treated explants were lower than those in the colchicine-free medium (Tables 2 and 3). This could be due to the toxic effects of colchicine. Lyrene and Perry (1982) and Perry and Lyrene (1984) also reported that colchicine reduced the survival rate in treated blueberry explants.

Abrupt variations in number of plantlets regenerated in different colchicine treatments appeared to be due to an error on sampling bulbscale discs for explanting. Since the more older bulbscales were explanted for one treatment, the less the plantlets would be regenerated. Takayama and Misawa (1980) found that cultures of older bulbscales produced fewer plantlets. Stimart and Ascher (1978) also reported that age difference affected regeneration capability of bulbscales discs in a culture. In addition, they also found an effect of disc position on bulblet initiation. Explants from the distal part initiated fewer bulblets than those from the basal part of a bulbscale.

The occurrence of cytochimeral plants were observed in all of the colchicine treatments that generated polyploidy. A periclinal cytochimera might have resulted from the colchicine effect only on the epidermal layer or L₁ of a developing bulblet. An evolution of sectorial cytochimeras could have been the result of only a part of the germ layer being affected by colchicine. Earlier, Chen and Goeden-Kallemeyn (1979) observed maxoploid plants regenerated from a colchicine-treated daylily
callus. Peck and Arisumi (1968) and Arisumi (1964; 1972; 1973) also obtained cytochimeras after colchicine treatment of daylily plants. Histological examination revealed that serial sections of bulbscale discs made 10 days after explanting had fewer cells mitotically active than those made 20 days after culturing. In the latter case, even buds were observed (Figure 1). The different developmental stages in the discs taken 20 days after culturing could be due to the age difference of the discs. If the disc should have responded to the colchicine treatment at the bulblet forming stage, a cytochimera might have been developed. Ramulu et al. (1976) reported that the origin of regenerated plantlets could be from few cells rather than from one cell.

Pure polyploidy was induced in Lilium by colchicine treatment through conventional methods (Emsweller and Lumsden, 1943; Emsweller, 1949 and Sagawa, 1958). Due to high toxicity of the chemical, the mortality of the treated plants was usually high; therefore, the success rate was generally low. Recently, Huang (1983) obtained polyploid lilies using in vitro techniques, but the success rate was not reported.

In these experiments, the frequency of occurrence of tetraploid plants evolving from colchicine-treated bulbscale discs were considerably high. This indicated that the treatments were effective, especially in the bulbscale discs treated 10 days after culturing. The procedure is outlined in the following flow chart (Figure 6).
Figure 6. A flow chart illustrating the production of tetraploid plants by colchicine treatment of cultured bulb scale discs of *Lilium longiflorum*.

Bulbscale disc cultures on MS medium supplemented with 3 mg kinetin and 0.1 mg NAA/liter (morphogenetic induction medium) for 10 days

转移至相同的形态发生诱导介质，同时添加秋水仙素，持续2天

转移回不含秋水仙素的形态发生诱导介质

植株发育

四倍体鉴定
SUMMARY

Tetraploidy in cultured bulbscales of *Lilium longiflorum* Thumb cv 'Nellie White' bulbscale segments was induced by colchicine. Two to three mm taken from near the bulbscale bases were mitotically activated on Murashige and Skoog's MS medium supplemented with 0.1 mg NAA and 3 mg kinetin/liter. Ten and 20 days after explanting, the discs were transferred onto the MS medium containing 0, 10, 20 and 40 mg colchicine/liter. Ten day explants were treated for 2 days and 20 day explants for 4 days, before being returned to the MS medium, free of colchicine, for bud initiation. At the mid-point of treatment, the discs were turned upside down to insure that both sides of the discs received colchicine treatment. The cultures were incubated in the dark at 25°C until adventitious buds were visually noted. The bud-forming discs were then transferred onto MS medium containing 0.1 mg NAA/liter and incubated in a lighted growth chamber at 25°C for completing plantlet development. Ploidy identification was made by root-tip chromosome counts, stomate and pollen grain measurements taken from regenerated potted plants.

In the treatments where bulbscale discs were mitotically activated for 10 days and exposed to colchicine for two days showed considerable effectiveness in polyploidization. The percentage of tetraploid plants appearing in 10, 20, and 40 mg colchicine/liter treatments were 19.5, 30.8 and 25.4%, respectively. On the other hand, bulbscale discs cultured for 20 days in the bud-initiating medium before being exposed for four days showed polyploidization only in the two
higher concentration treatments. Of 63 plants regenerated from 20-mg/liter colchicine treatment, 4 plants or 6.3% were tetraploid; whereas in 40-mg/liter treatment, 24 plants or 19.2% of 125 plants were tetraploid. Some of the plants were cytochimeras. All 203 plants regenerated from the bud-initiating medium without colchicine treatments in the two experiments had the diploid number 2n-24.
LITERATURE CITED


