

# Basal Medium and Sucrose Concentration Influence Regeneration of Easter Lily in Ovary Culture

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**Abstract.** Influences of culture media and sucrose concentrations on plant regeneration from Easter lily (*Lilium longiflorum* L. cv. Ace) ovary tissues were investigated. Pistils excised from unopened flower buds (3–5 cm long) were sectioned and cultured on either B-5 medium or Murashige and Skoog (MS) medium containing 2%, 5%, or 10% sucrose, with 1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA. Callus formation was most prolific on MS medium containing 5% sucrose. Shoot differentiation was higher on MS medium than on B-5 medium. Rooted plants were transferred into soil medium and grown in a greenhouse. Root tip smears showed that 35% of the regenerated plants showed a variation in chromosome numbers from 10 to 25 per cell, while the rest of the regenerants showed the normal 2n = 2x = 24 chromosomes per cell. The mixoploid condition also existed in different root cells of the same regenerated plant. Chemical names used: 2,4-dichlorophenoxyacetic acid (2,4-D); 6-benzylaminopurine (BA).

Easter lily is an economically important greenhouse crop, ranking fourth in total sales among the potted flowers in the U.S. market (Miller, 1992). Propagated vegetatively by bulbs, Easter lily has only a few cultivars (<10) commercially sold. Development of new cultivars exhibiting altered traits such as shorter plant heights and different flower colors would potentially increase the marketability of this crop. For improvement of Easter lily through genetic manipulation, a plant regeneration system must be developed and utilized. Ovary cultures have been used for plant regeneration from *Lilium formosanum* (Hayashi et al., 1986) and production of interspecific hybrids between *Lilium longiflorum* and *L. xelegance* (Kano et al., 1988). Ovary culture may also be used to obtain haploid plants which can be induced to homozygous doubled haploids. Effect of growth regulator concentrations on callus formation in *Lilium longiflorum* was also studied by Tribulato et al. (1997) and Wickremesinhe et al. (1994). The objectives of this study were to compare different culture media and sucrose levels on the regeneration of Easter lily via ovary tissue cultures and to determine ploidy levels of regenerated plants.

## Materials and Methods

*Plant materials and media preparation.* Easter lily (*Lilium longiflorum* L. cv. Ace)

bulbs supplied by Pacific Bulb Growers Assoc. Res. Farm (Brookings, Ore.) were cold-treated at 7 °C for 6 weeks and then planted and grown in 15-cm plastic pots containing peat-lite medium (Sunshine Mix #1; SunGro Horticulture, Bellevue, Wash.) in the greenhouse. Unopened flower buds (3 to 10 cm long) were excised and dipped in 10% household bleach (0.525% sodium hypochlorite) for 10 min under a laminar-flow hood. Ovaries extracted from the surface-sterilized buds were cut into two longitudinal sections and cultured on agar medium, with the cut-surface placed down and slightly embedded. Two halves of each ovary were cultured in a jar as a treatment and each treatment was replicated three times. Two trials were run for each set of media utilized. All variables were held constant except one per trial. Cultures were initially placed in a dark incubator at ≈22 °C. After 3 to 4 weeks, the callus cultures were transferred to fresh media and moved to the light at room temperature. After an additional period of 3 to 4 weeks, calli formed from these cultures were transferred to shooting media. Shoot formation became evident anywhere from 6 to 20 weeks after cultures were transferred to shooting media.

Media used in this experiment were pre-packaged and purchased from Carolina Biological Supply Co. (Burlington, N.C.). Sucrose was used as the carbohydrate source in all cultures. Growth regulators were taken from stock solutions prepared prior to this experiment. pH of all media was adjusted to 5.8 with 1.0 N HCl and 0.1 or 1 N NaOH before autoclaving. Media were solidified using either agar tablets (3%) or 8 g·L<sup>-1</sup> Phytigel (Sigma Chemical Co., St. Louis). The media were autoclaved at 121 °C at 20 psi (58.6 kg·cm<sup>-2</sup>) for 20 min. Media

were stored at 6 °C until use. Culture initiation was done on media (20 mL/jar) placed in baby-food jars (5 cm diameter × 5 cm tall; Gerber, Minneapolis). Shooting media were placed in Magenta boxes with dimensions of 6.5 cm × 6.5 cm × 8 cm.

*Influence of basal medium and sucrose concentration.* The effects of MS medium (Murashige and Skoog, 1962) and Gamborg's B-5 medium (Gamborg et al., 1968) on shoot formation were compared, with sucrose (5%) and growth regulator concentrations (1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA) held constant. Cultures were maintained under a 16-h day/8-h night cycle under cool-white fluorescent lamps with 80 μmol·m<sup>-2</sup>·s<sup>-1</sup> at 22 °C. Regenerated shoots were counted 60 d after culture initiation. The numbers of shoots formed per explant (total number of shoots divided by the number of replications on a particular medium) were calculated. The numbers of shoots per viable explant (total number of shoots divided by the number of ovaries which actually grew) were also calculated. Height, numbers of buds, and total numbers of leaves of 10 randomly selected regenerated plants were also observed for comparison to 10 randomly selected control plants. The effect of sucrose concentration on callus formation was tested using MS media with 1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA with either 2%, 5%, or 10% sucrose as a nutrient source. Fresh weights of the ovary explants were taken initially and after 21 d of incubation. Percent increase in fresh weight was calculated for each explant by taking the change in fresh weight divided by the initial fresh weight. Data from each experiment were analyzed by the Student–Newman–Keuls multiple range test for significant differences between the mean percentage of fresh weight increases.

*Plant establishment in soil medium.* Plants regenerated from ovary cultures were divided and transferred to peat-lite medium (Sunshine Mix #1) contained in plastic cell packs (72 cells per tray). Each tray was covered with a transparent plastic lid to retain a humid environment and kept at room temperature under the light (80 μmol·m<sup>-2</sup>·s<sup>-1</sup>). Once the plants reached 3 to 6 inches in height, they were individually transferred to 6-inch plastic containers and grown to flowering stage in the greenhouse.

*Chromosome counts.* Root tips of regenerated plants were excised and prepared for chromosome observations through a series of steps. First, root tips were placed in an ice bath at 2 °C for 24 h. Next, the root tips were fixed in 1 part glacial acetic acid : 3 parts ethanol for 3 to 4 h and rinsed three times with distilled water. The root tips were hydrolyzed in 1 N HCl at 60 °C for 10 min and rinsed three times with distilled water. Root tips were placed in Fielgen's stain for at least 50 min, rinsed again, and placed in an enzyme solution consisting of 0.2% cellulase and 0.2% pectinase (Sigma Chemical Co.). Root tips were stained with acetocarmine for microscope observations of chromosomes. Chromosomes which were easily observed in cells were counted using a light microscope (×1000 magnification). Meta-phase chromosomes were counted in six root tip cells of each regenerated plant.

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## Results and Discussion

The effect of basal medium on bulblet formation was measured by the total number of shoots produced from a particular ovary. Ovary explants placed on MS medium produced more shoots per viable explant than those placed on B-5 medium (Fig. 1) when determined 60 d from culture initiation. On both media, shoot formation appeared to begin in the interior tissue of the ovary. The effects of sucrose concentration in MS medium on callus production were measured by changes in fresh weight. Medium containing 5% sucrose allowed the largest callus production (Table 1). Fig. 2 shows the size of initial buds used and the progression of events from callus production to flowering stage. By far, the most regenerated plants (116 of 258 total) were grown on media containing MS plus 5% sucrose, 1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA.

B-5 and N-6 media were both superior to MS medium for callus production from filaments with anthers in *Lilium longiflorum* Thunb. (Arzate-Fernandez et al., 1997). The sucrose concentration utilized in that study was 9%. The current study examined sucrose concentration effects on callus growth, and 5% was superior to both 10% and 2%. For ovary-slice culture of *Lilium formosanum* Wallace, 8% sucrose was superior to 3% (Hayashi et al., 1986). In addition, for the culture of immature embryos of *Lilium longiflorum*, 3% sucrose was determined to be the best. Anthers of *Lilium longiflorum* produced callus on medium with 3% sucrose and 9% sucrose, but 15% sucrose resulted in shriveling of the tissues (Kim and Sung, 1990).

The major difference between the two culture media is the form and amount of nitrogen. MS medium contains higher concentrations of ammonium (20.6 meq·L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>), nitrate (39.4 meq·L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>), and calcium (6 meq·L<sup>-1</sup> Ca<sup>2+</sup>) compared to B-5 medium (2 meq·L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>, 24.7 meq·L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>, 2 meq·L<sup>-1</sup> Ca<sup>2+</sup>). The total ionic concentration of macronutrients is also higher in MS medium (94.9 meq·L<sup>-1</sup>) than in B-5 medium (60.3 meq·L<sup>-1</sup>). A recent study showed that shoot multiplication from shoot and leaf disc cultures of sugarbeet was enhanced when cultures were grown with higher nitrate and ammonium levels than those found in standard MS medium (Tsai and Saunders, 1999). Reasons for the higher rate of shoot formation on MS medium in the current study may include

Table 1. Effect of sucrose concentration on callus production from *Lilium longiflorum* 'Ace' ovary tissues cultured on MS medium containing 1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA.

Sucrose concn (%)	Mean fresh wt increase (g/explant)
2	1.22 ± 0.31 a <sup>c</sup>
5	2.40 ± 0.62 b
10	1.90 ± 0.28 ab
Linear	NS
Quadratic	***

<sup>c</sup>Mean separation by the Student–Newman–Keuls multiple range test (n = 30).

ns, \*\*\*Nonsignificant, or significant at the P = 0.01 level, respectively.

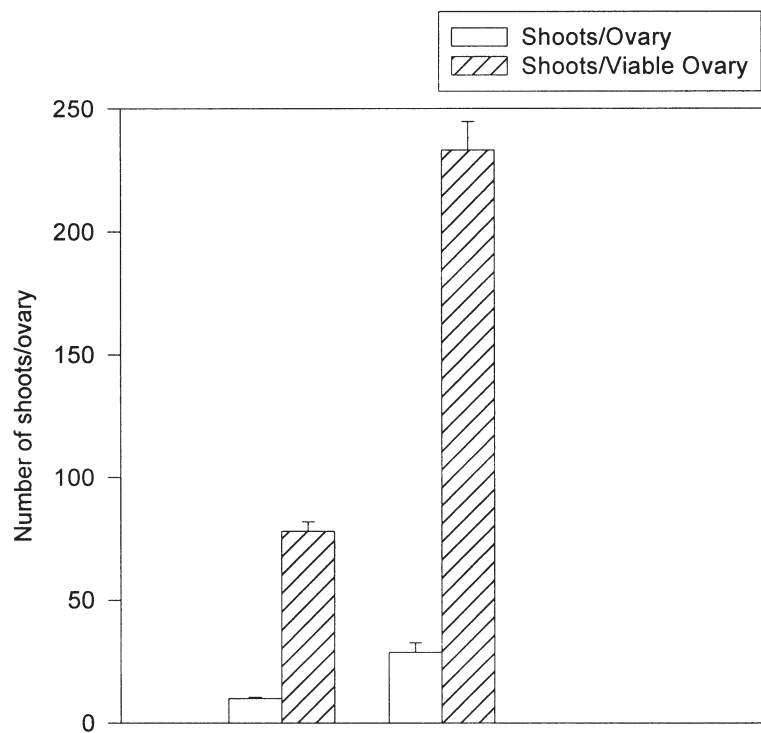


Fig. 1. Mean number of shoots/explant and shoots/viable explant in excised ovaries of *Lilium longiflorum* 'Ace'. Ovaries were sliced in half and cultured in vitro on B-5 or MS basal medium with 5% sucrose, 1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA. (The total number of shoots counted from MS media was ≈500, while the total number of shoots from B5 media was ≈300.) Bars indicate standard error.

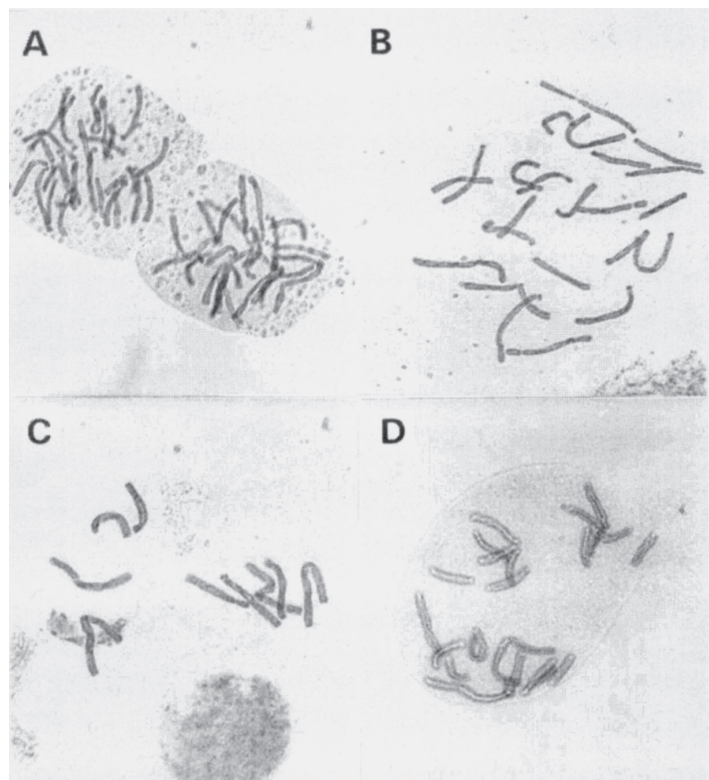


Fig. 2. Regeneration of *Lilium longiflorum* 'Ace' through ovary tissue cultures: (A) and (B) = the sizes of flower buds and pistils, respectively, used for ovary excision; (C) = callus proliferation and shoot organogenesis on MS medium containing 5% sucrose, 1 mg·L<sup>-1</sup> 2,4-D and 2 mg·L<sup>-1</sup> BA; (D) = shoot proliferation on the same regeneration medium; (E) = a potted regenerated plant; and (F) = regenerated plants at flowering stage.

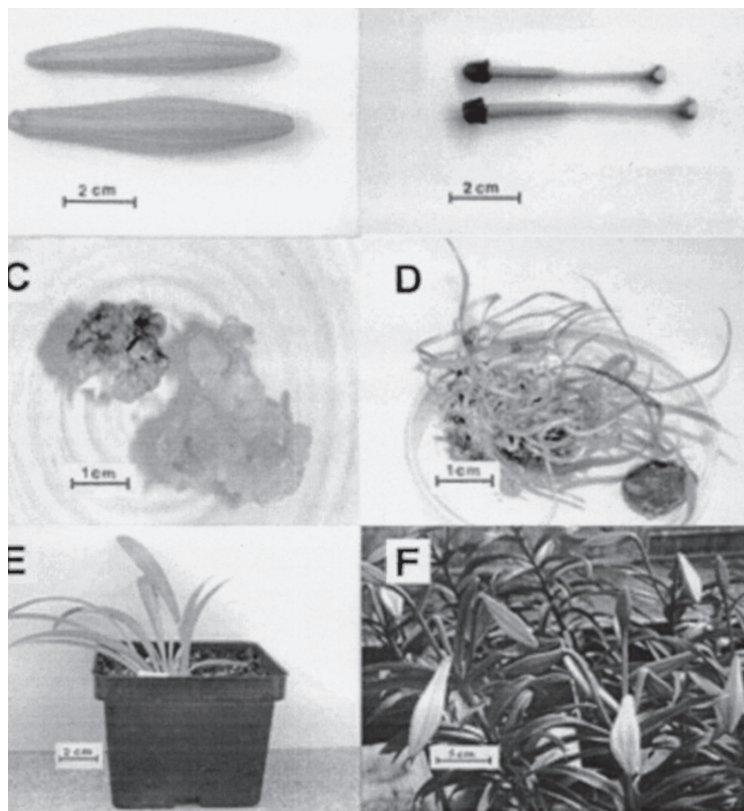


Fig. 3. Variation in chromosome numbers in root tip cells of *Lilium longiflorum* 'Ace' regenerated from ovary tissue cultures: (A) = cells of a diploid plant grown from control bulb ( $2n = 24$ ); (B) = root cells of a regenerated diploid plant ( $2n = 24$ ); (C) = mixoploid cells ( $2n = 10$ ) of a regenerated plant; and (D) = mixoploid cells ( $2n = 20$ ) of a regenerated plant.

less energy spent in reducing  $\text{NO}_3^-$  to  $\text{NH}_4^+$  by tissues grown on MS than on B-5 medium. The higher  $\text{Ca}^{++}$  concentration in MS medium could also have contributed to increased shoot production, since calcium is an important structural cell wall component and has numerous other roles in plant cells (Galston et al., 1980).

Chromosome numbers of regenerated plants were compared to root tip cells of control plants, which had 24 chromosomes. Of 258 plants regenerated from  $\approx 50$  different calli in this experiment, 168 plants (65%) exhibited the diploid number ( $2n = 2x = 24$ ) of chromosomes, while 90 plants (35%) exhibited a mixoploid condition (Fig. 3). In mixoploid plants, different cells of the same root tip had different numbers of chromosomes when four different samples were counted for each plant. This differs from data presented by Qu et al. (1988) who observed a higher percentage of mixoploid than diploid Easter lily plants regenerated from anther cultures. The range of chromosome numbers observed in our regenerated plants was hypohaploid ( $2n = 10$ ) to hyperdiploid ( $2n = 25$ ), as earlier observed by Bennici (1979). Further studies are

needed to determine whether the plants that exhibited the diploid number of chromosomes are from somatic cell origin and true diploids, or whether they were doubled haploids. Cells in mixoploid plants may have proliferated from more than one cell of the ovary tissues. Chromosome numbers were not obviously related to length of time in culture or type of media used (data not shown).

Regenerated plants, when potted in commercial mix (Sunshine Mix #1), grew and flowered normally in the greenhouse as the bulb-grown plants. However, regenerated plants of the first generation somaclones were shorter in stem lengths and had fewer leaves as compared to the control plants grown from bulbs produced in the greenhouse. Average heights ( $n = 10$ ) of the regenerated and control plants were  $29 \pm 3$  cm and  $49.0 \pm 1.3$  cm, respectively. The regenerated plants had an average of  $37 \pm 3$  leaves, while the control plants had an average of  $52 \pm 2$  leaves. Most of the regenerated plants had only one or two flower buds, while three or more flower buds produced by the control plants. Had regener-

ated plants been grown for several cycles, as the control bulbs were, the height and number of leaves may have been more similar.

These experiments demonstrated that sucrose concentration and basal medium affect both callus production and shoot formation of cultured ovary tissues. Ovary culture in Easter lily may not be a useful means of asexual propagation, since there is a wide variety of ploidy levels in regenerated plants. However, ovary culture in Easter lily could potentially be used as a means of genetic manipulation and cultivar development. The possibility of using ovary culture in obtaining doubled haploid lines has yet to be studied, because involvement of the female gamete as the origin of plant regeneration has not been substantiated.

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