D.J. Gray. 1989. Somatic embryo ontogeny in suspension cultures of orchardgrass. Crop Sci. 29:448–452.

Gray, D.J. 1990. Somatic cell culture and embryogenesis in the Poaceae, p. 25–57. In: M.J. Kasperbauer (ed.). Biotechnology in tall fescue improvement. CRC Press, Boca Raton, Fla.

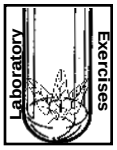
Gray, D.J., B.V. Conger, and G.E. Hanning. 1984. Somatic embryogenesis in suspension and suspension-derived callus cultures of *Dactylis glomerata*. Protoplasma 122:196–202.

Gray, D.J. and B.V. Conger. 1985. Time-lapse light photomicrography and scanning electron microscopy of somatic embryo ontogeny from cultured leaves of *Dactylis glomerata*(Gramineae). Trans. Amer. Microsc. Soc. 104:395–399.

Gray, D.J. and A. Purohit. 1991. Somatic embryogenesis and development of synthetic seed technology. Crit. Rev. Plant Sci. 10:33–61.

Trigiano, R.N., D.J. Gray, B.V. Conger, and J.K. McDaniel. 1989. Origin of direct embryos from cultured leaf segments of *Dactylis glomerata.* Bot. Gaz. 150:72–77.

Laboratory Exercises Illustrating Organogenesis and Transformation using Chrysanthemum Cultivars



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Summary. A tissue culture laboratory exercise illustrating regeneration of whole plants from leaf segments of chrysanthemum by organogenesis is described. Using simple, common media, shoots can be generated in 5 weeks and rooted after an additional 3 weeks. Acclimatization of plants can be accomplished in a simple mistbed in the greenhouse. The exercise is adaptable to depict genotype differences among cultivars, optimization of shoot induction, effects of growth regulators, and experimental design. Callus is typically not formed during shoot formation; however, co-cultivation of leaf segments with a virulent strain of *Agrobacterium tumefaciens* produces callus with a strain of disarmed *A. tumefaciens* harboring NPTII construct affects regeneration of plants resistant to kanamycin.

Chrysanthemum (*Dendranthema grandiflora* Tzvelev.) is a popular floricultural species worldwide and is grown as a garden or pot crop. Chrysanthemums typically are propagated commercially via stem cuttings, but also have been micropropagated successfully by adventitious shoot formation (organogenesis) from a variety of tissue and callus cultures (Earle and Langhans, 1974; Fujii and Shimizu, 1990; Lu et al., 1990; Roest and Bokelmann, 1975; Slusarkiewicz-Jarzina et al., 1982.). Although encountered infrequently in exercises for plant tissue culture classes, chrysanthemum can be used to demonstrate many methodologies and concepts.

Aside from illustrating a technique or concept, the most-important attribute that a laboratory exercise for a tissue culture class can have is reliability—the "experiment" must yield the desired results most of the time. The exercise also must be straightforward for inexperienced students (a few simple steps), inexpensive, capable of being completed in a short amount of time, and perhaps most important, easily prepared by the instructor. We have developed laboratory exercises illustrating organogenesis and transformation using leaf sections of chrysanthemum that have all of the above-mentioned characteristics. Furthermore, the exercises are versatile—in addition to organogenesis from leaves, variations of it may be used to demonstrate the following concepts: 1) differences in the ability of genotypes to regenerate shoots and roots; 2) the effects of plant growth regulator combinations on shoot and root formation; 3) pulse treatments for initiation and growth of shoots; 4) simple transformation of cells with a "wild-type" *Agrobacterium tumefaciens*; and 5) transformation of cells with a disarmed vector resulting in the recovery of transformed shoots. Variations 1 through 4 have been conducted successfully for 3 years by introductory classes (10 to 19 students), whereas variation 5 has been completed by small advanced classes (four or less students) and by independent study students. Each of the above-mentioned experiments is detailed below, including operations, timetables, and expected results.

General considerations

All of the cultivars of chrysanthemum mentioned below may be obtained from Yoder Brothers, Barberton, Ohio, as rooted or unrooted cuttings. We suggest growing the following cultivars for the exercises: 'Adorn', 'Goldmine', 'Hekla', 'Iridon', and 'Rave'. Plant three to five cuttings in each of at least five 10-cm-diameter plastic pots. The growth of the donor plants is important—if possible, cultivate the plants in the laboratory or growth room illuminated with ≈100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent and incandescent light for 16 h/day at 25 to 28C. Alternately, plants grown in the greenhouse under shade and with cooling will provide suitable explant materials. Pinch longer shoots often to encourage branching, and fertigate regularly to maintain vigorous growth [see May and Trigiano (1991) for details of growing conditions].

For satisfactory and reproducible results, use only young, partially expanded, light green, 2- to 4-cm-long leaves for explant tissue. Surface-sterilize whole leaves using 5% Clorox (0.26%

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NaOCl) for 5 min (or 10% Clorox for material grown in the greenhouse) with constant agitation, followed by three rinses with sterile distilled water. Place a leaf in a sterile 100 × 15-mm plastic petri dish and drain excess water by tilting and gently tapping the dish on the flow hood table. Using a #10 scalpel blade, excise four or five 0.5-cm^2 sections from the midrib area of the leaf. Place the sections with the abaxial surface (underside) of the leaf in contact with the medium.

The culture medium is composed of Murashige and Skoog (MS) basal salts [Murashige and Skoog (1962), or can be purchased from Sigma (St. Louis)] amended with 88 mM sucrose, 0.55 mM *myo*-inositol, 2.9 μM thiamine•HCl, and 8 g of agar/liter. Depending on the type of experiment, the medium is poured into either 60 or 100 × 15-mm plastic petri dishes. Typically, one or two leaf sections are placed in each of the 60-mm dishes, whereas two to four sections are cultured in each 100-mm dish. Growth regulator combinations will be provided for each variation of the experiment. For student exercises, we usually prepare at least four dishes of each treatment for each student.

Variation 1: Differences between genotypes in shoot and root regeneration

One of the basic concepts in the tissue culture of plants is that not all genotypes will respond equally under similar cultural conditions. In fact, some cultivars of chrysanthemum will not produce either shoots or roots in vitro in response to any treatment thus far devised. This exercise was developed to illustrate this concept, and is probably the easiest of the variations to conduct with large classes. Use all five of the cultivars mentioned; if materials are limited, use only 'Adorn', 'Goldmine', and 'Iridon'. This experiment also provides the basic protocol for the other four variations of the exercise.

Day 1. Culture two leaf sections in each 60-mm dish containing MS medium (as above) supplemented with 11.5 μM filter-sterilized indole-acetic acid (IAA) and either 0.1 or 1.0 μM benzyladenine (BA). Also include treatments of IAA only, BA only, and no growth regulators. Incubate dishes in a 25C incubator with 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of fluorescent light for 2 weeks. At the end of this period, explants exposed to IAA and BA should have distorted shapes, and sparse, light green crystalline callus may have formed around the cut edges; darker green meristematic zones or small buds also may be apparent on 'Iridon', 'Hekla', and 'Rave' explants. This ends the initiation phase of the exercise (Stages 0 and I of traditional micropropagation)

Day 15. Transfer all explants to MS medium lacking growth regulators and incubate under the same conditions as described previously for 3 weeks. This is the "shoot elongation" phase of the experiment (Stage II of micropropagation).

Day 36. Explants of 'Hekla', 'Iridon', and 'Rave' incubated initially on growth regulators should have produced shoots and some roots directly without an intervening callus. Shoots should have formed primarily on the cut margin of the explant. The most shoots (and least number of roots) should be on 'Iridon' explants and decreasing numbers on 'Hekla' and 'Rave'. 'Goldmine' and 'Adorn' explants should have produced primarily roots and an occasional shoot. None of the explants should have produced appreciable amounts of callus, and those initially incubated on either only IAA, BA, or medium lacking growth regulators should have formed a few roots (especially 'Goldmine' and 'Adorn') and few, if any, shoots. If whole-plant regeneration is desired, then excise shoots >1 cm and transfer to GA7 Magenta boxes or similar vessels containing MS medium lacking growth regulators. Incubate as before for 3 weeks (Stage III of micropropagation).

Day 57. At this time, shoots should have elongated and developed roots. Plantlets may be transferred to soil and placed under intermittent mist for 2 to 3 weeks and then grown as a normal greenhouse crop (Stage IV of micropropagation).

Variation 2. The effects of plant growth regulators on formation of shoots and roots

One of the first steps in developing a micropropagation protocol is to determine the type (or combination) and concentration of plant growth regulators that will provide the desired response. Leaf explants of chrysanthemum are very responsive to both the type and concentration of cytokinin in the initiation medium. Using the cultivars Hekla, Iridon, and Goldmine, follow the procedure provided in variation 1, except prepare MS media containing 11.5 μM IAA in combination with the following concentrations and types of cytokinin or cytokinin-like compounds: 0.01, 0.1, 1.0, and 5.0 μM kinetin, 2iP, BA, and thidiazuron. There are a total of 17 treatments including IAA only. Incubate for 2 weeks as above and then transfer to MS medium lacking growth regulators. All treatments except IAA only will produce shoots on explants of 'Iridon' and 'Hekla'; primarily roots should form from explants of 'Goldmine'. The students should count the number of roots and shoots produced in each treatment and determine which treatment produced the most shoots. This experiment is amenable to several experimental plans, including balanced and unbalanced incomplete blocks, randomized complete-block, and completely randomized designs. If statistical evaluation is desired, we suggest that the number of shoots per experimental unit be transformed using the function the square root of the number plus 1 to stabilize (normalize) variation.

Variation 3. Pulse treatments for initiation and growth of shoots

Most micropropagation protocols are based on a treatment of explants with growth regulators (initiation period), followed by incubation on medium that either lacks or has reduced concentrations of growth regulators. Such treatments usually are referred to as pulse treatments. This concept is demonstrated easily using the cultivar Iridon and varying the time the explants are incubated on MS medium containing 11.5 μM IAA and either 0.1 or 1.0 μM BA. The treatments for this experiment are 0, 1, 3, 5, 7, 10, 14, 21, and 35 days on the above media, followed by MS medium without growth regulators for the remainder of the 35-day period. Shoots should not form on explants from treatments 0, 1, 3, and 5 days; whereas 10 to 17 days of incubation fosters the development of many shoots. Fewer shoots are formed on explants from the 21-day treatment, and although some shoots develop on explants from the 35-day treatment, they usually are stunted and vitrified.

Variation 4. Simple transformation of cells using "wild-type" *Agrobacterium tumefaciens*

There has been increased interest in the transformation of ornamental plants in recent years, and chrysanthemum is no exception (de Jong et al., 1993; Lowe et al., 1993; van Wordragen et al., 1991; 1992). The plant regeneration system described above (variation 1) is suited ideally for transformation experiments involving *A. tumefaciens* for the following reasons: Shoots are formed directly (without an intervening callus phase); the shoots develop from the cut edges of the explant, which are easily infiltrated with the vector; and the plant tissue has high regenerative capacity.

A recent report (van Wordragen et al., 1991) and experiments in our laboratory have indicated that whole chrysanthemum plants are susceptible to infection by a number of "wild-type" *A. tumefaciens* strains. Galls have been induced successfully on stems of 'Iridon', 'Hekla', and 'Goldmine' using *A. tumefaciens* strains A281 (Hood et al., 1986) and A208. Strain A281 incited larger galls in less time compared to strain A208.

Using the protocol below, it is easy to achieve transformation of cells of leaf explants. Successful transformation is indicated by production of copious amounts of callus that normally is not formed on leaf explants of these cultivars during shoot formation (see variation 1).

Day 1. Culture leaf sections of any cultivar (two per 60-mm dish) on MS medium containing 1.0 μM BA and 11.5 μM IAA for 2 or 3 days in conditions described under variation 1.

Day 2. Grow *A. tumefaciens* strain (A281 supervirulent is good) in 50 ml of AB medium (White and Nester, 1980) in a 125-ml Erlenmeyer flask on a rotary shaker at 100 rpm for 24 h.

Day 3. Adjust the suspension of bacteria to 10^5 or 10^6 colony-forming units with AB broth and place in a sterile 50-ml beaker. Immerse one-half of the leaf section explants from a single leaf in the suspension of bacteria and remove excess fluid by

blotting on sterile, dry, filter paper; submerge the remaining sections in AB medium without bacteria and blot dry. Return the leaf sections to their original dishes and incubate as before for 2 days. Unused bacterial suspension, instruments, and filter paper should be autoclaved before discarding.

Day 5. Filter-sterilize MS liquid medium containing 100 mg•liter^{-1} cefotaxime into two sterile 50-ml flasks. Vigorously agitate the leaf sections in the solution, remove, and blot dry with sterile filter paper. Use different beakers for bacteria-treated and bacteria-free sections. Incubate on MS medium amended with growth regulators and 100 mg•liter^{-1} cefotaxime (Sigma, St. Louis) for 9 days.

Day 14. Repeat steps at day 5 and reculture on MS medium without growth regulators, but containing 100 mg•liter^{-1} cefotaxime, for 21 days.

Day 35. Leaf sections of 'Iridon' and 'Hekla' not treated with bacteria should produce plentiful shoots, as in other experiments, and those of 'Goldmine' should have scanty callus along the cut edges, no shoots, and a few roots. Leaf sections of both 'Iridon' and 'Goldmine' treated with bacteria should have patchy areas of heavy growth of yellow-green callus; roots and shoots should not be present. Transfer callus to MS medium without growth regulators supplemented with 100 mg•liter^{-1} cefotaxime every 3 weeks. Callus should be autotrophic for plant growth regulators, and can be maintained for indefinite periods. Caution: All plant material and petri dishes exposed to *A. tumefaciens* should be autoclaved before discarding.

Variation 5. Recovery of transformed shoots

This experiment is similar to the protocol used in variation 4, except that *A. tumefaciens* "wild-type" A281 is replaced with EHA105, a disarmed A281 (lacking genes encoding growth regulators) and harboring a gene (and promotor) for neomycin phosphotransferase (NPTII). This gene, when incorporated in the plant genome, will confer resistance to kanamycin (Sigma, St. Louis), an antibiotic. The exercise should be designed with a treated group (co-cultivation) and a control group (without bacteria). Experiments in our laboratory have shown that untransformed cells of chrysanthemum are sensitive to as little as 5 mg•liter^{-1} kanamycin, and that 'Iridon' is the best cultivar for this exercise. A note of caution—as described in Variation 4, all materials, including plants and explants, that come in contact with *A. tumefaciens* should be autoclaved prior to discarding.

Days 1–5. Same as in variation 4.

Day 14. Transfer all explants to MS medium without growth regulators and supplemented with 100 mg•liter^{-1} cefotaxime and 50 mg•liter^{-1} kanamycin and incubate for 21 days as before.

Day 35. Shoots should be formed at this time. Shoots originating from untransformed cells will be "bleached" white; whereas putatively transformed shoots will remain dark green. Some of these will be escapes, and all green shoots should be transferred to fresh medium containing cefotaxime and kanamycin for 3 weeks. If these shoots are escapes, the new growth will be white; if the NPTII gene has been incorporated, then the shoots will remain dark green. From our experience, about 2% to 5% of the shoots formed will be transgenic. More exhaustive and conclusive tests can be completed for the presence of the NPTII gene in the plants (e.g., Reiss et al., 1984).

A variation of the above experiment is to use the following four treatments: 1) co-cultivation with bacteria; 2) without bacteria, medium lacking cefotaxime and kanamycin; 3) without bacteria, including cefotaxime; and 4) without bacteria, including kanamycin. White and green shoots are obtained with treatment 1. Green shoots are produced in treatments 2 and 3, and generally more shoots are produced in treatment 3. Green and white shoots are produced in treatment 4, but, when green shoots are transferred to fresh medium containing kanamycin, all shoots will become white.

Literature Cited

de Jong, J., W. Rademaker, and M.F. Van Wordragen. 1993. Restoring adventitious shoot formation on chrysanthemum leaf explants following cocultivation with *Agrobacterium tumefaciens*. Plant Cell, Tissue & Organ Cult. 32:263–270.

Earle, E.D. and R.W. Langhans. 1974. Propagation of *Chrysanthemum* in vitro. I. Multiple plantlets from shoot tips and the establishment of tissue cultures. J. Amer. Soc. Hort. Sci. 99:128–132.

Fujii, Y. and K. Shimizu. 1990. Regeneration of plants from achenes and petals of *Chrysanthemum coccineum*. Plant Cell Rpt. 8:625–627.

Hood, E.E., G.L. Helmer, R.T. Fraley, and M.D. Chilton. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of TiBo542 outside of T-DNA. J. Bacteriol. 168:1291–1301.

Lowe, J.M., M.R. Davey, J.B. Power, and K.S. Blundy. 1993. A study of some factors affecting *Agrobacterium* transformation and plant regeneration of *Dendrathema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). Plant Cell, Tissue & Organ Cult. 33:171–180.

Lu, C-Y., G. Nugent, and T. Wardley. 1990. Efficient, direct plant regeneration from stem segments of chrysanthemum (*Chrysanthemum morifolium* Ramat. cv. Royal Purple). Plant Cell Rpt. 8:733–736.

May, R.A. and R.N. Trigiano. 1991. Somatic embryogenesis and plant regeneration from leaves of *Dendranthema grandiflora*. J. Amer. Soc. Hort. Sci. 116:366–371.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Reiss, B., R. Sprengel, H. Will, and H. Schaller. 1984. A new sensitive method for qualitative and quantitative analysis of neomycin phosphotransferase in crude cell extracts. Gene 30:217–223.

Roest, S. and G.S. Bokelmann. 1975. Vegetative propagation of *Chrysanthemum morifolium* Ram. in vitro. Scientia Hort. 3:317–330.

Slusarkievicz-Jarzina, A., M. Zenkterler, and B. Podlewska. 1982. Regeneration of plants from leaves of *Chrysanthemum morifolium* Ram. cv. Bronze Bornholm in in vitro cultures. Acta. Soc. Bot. Poloniae. 51:173–178.

van Wordragen, M.G., J. de Jong, H.B.M. Huitema, and H.J.M. Dons. 1991. Genetic transformation of *Chrysanthemum* using wild type *Agrobacterium tumefaciens* strains; strain and cultivar specificity. Plant Cell Rpt. 9:505–508.

van Wordragen, M.F., P.B.F. Ouwerkerk, and H.J.M. Dons. 1992. Agrobacterium rhizogenes mediated induction of apparently untransformed roots and callus in chrysanthemum. Plant Cell, Tissue and Organ Cult. 30:149–157.

White, F.F. and E.W. Nester. 1980. Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. J. Bacteriol. 141:1134–1141.