have to be prepared 1 to 2 months prior to this exercise and supplied to the student at the onset of the experiment. The indirect organogenesis medium includes 8.8 μM (2 mg·liter⁻¹) BAP plus 2.5 μM (0.5 mg·liter⁻¹) IBA. Media is dispensed into 25 × 150-mm borosilicate glass culture tubes at 20 ml·liter⁻¹ each, capped, and autoclaved at 1.2 kg·cm⁻² for 15 min at 121°C. If limited space and media are problems, sterile, disposable petri plates (55 × 150 mm) can be used for the first 4 weeks instead of culture tubes.

Protocol

Leaf disinfection and preparation.

This exercise can be completed in an open laboratory with or without the use of a laminar flow hood. To decrease the amount of contamination, it is important to explain and demonstrate aseptic procedures to the students prior to this laboratory. All work surfaces need to be wiped with 70% ethanol and allowed to dry. Young leaves from the second and third axillary branches of established *Torenia* plants are selected. Leaves are cleaned gently in tepid tap water, dipped for 10 to 20 s in 70% ethanol, and placed in 0.5% sodium hypochlorite for 15 min, followed by three rinses with sterile deionized water, holding the leaves in the last rinse until ready for use. Sterile forceps or scalpels must be used to hold plant material after disinfection. To hasten results and/or to eliminate the previous steps, use leaves from aseptic cultures.

Leaves are then cut into 0.5 × 0.5-cm pieces after discarding the leaf margins. The leaf sections are placed on the appropriate medium with the abaxial side down. Each student should culture at least four explants per medium. If contamination is possible, the students should wrap culture vessels with PVC film or Parafilm™. Culture vessels should be labeled with the date and individual's initials.

Scheduling. Cultures are incubated at 21 to 23°C under a 16-h photoperiod provided by cool-white fluorescent lamps at 50 to 60 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Cultures should be observed at least weekly, comparing the explant development on each medium and looking for the initiation of shoots, leaves, and callus. For more-precise measurements of callus production, fresh weight values can be taken aseptically on a weekly basis and, at the end of the semester, graphed or plotted over time.

Callus and shoot initials will be visible after 2 weeks of culture if sterile leaves were the initial explant, and in 3 to 4 weeks from the first culture if in vivo leaves were used. In another 2 weeks, shoots and callus will be large enough to separate and subculture. Callus tissue can be subcultured back onto the callus induction medium or onto the indirect organogenesis medium. If callus is placed on the latter medium, shoots will be visible in 4 weeks and large enough to subculture in another 2 weeks.

Transferring from culture. After shoots are large enough to handle, they divided and

subcultured, prepare *Torenia* for outplanting by rooting individual plantlets in vitro on MS medium with no growth regulators. After 2 to 4 weeks, plantlets are potted in a greenhouse medium and place under intermittent mist to acclimatize for 2 to 3 weeks. To complete this exercise in one semester or term, the outplanting exercise may need to be restricted to those plantlets that are produced through direct organogenesis.

Instructional information

Students should be required to keep a laboratory notebook during this exercise to assist them with their laboratory write-up. Details about the experiment should be recorded, as well as the dates of subculture, the duration of the experiment, numbers of cultures, visual observations over the duration of the experiment, and conclusions that were drawn. The write-up at the end of the experiment should also include the objectives of the laboratory, a description of procedures that were used other than those described for the lab exercise, a discussion about the sources of contamination that were observed, and a discussion of why the results were obtained.

This laboratory easily lends itself to modification, and can lead to several discussion topics that can be prompted by asking questions such as:

- Where does callus originate?
- Does the culture of explants on their abaxial surface affect organogenesis differently than if they were cultured on their adaxial surface?
- What effect does the size of the explant have on organogenesis?
- How does the auxin:cytokinin ratio in a medium determine the type of organogenesis?
- What is the difference between adventitious shoots and axillary shoots?

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Classroom Exercises in the Study of Orchardgrass Somatic Embryogenesis

D.J. Gray¹, R.N. Trigiano²,
and B.V. Conger³

Additional index words. cell culture, *Dactylis glomerata*, plant regeneration

Summary. Orchardgrass is a member of the Poaceae, a family characterized by monocotyledonous-type embryos. This species is unique in that somatic embryogenesis can be induced from the cultured leaves of potted plants, which can be maintained conveniently in the greenhouse. Both embryogenic callus and isolated somatic embryos develop directly from cultured leaf sections. Embryogenic callus can be maintained indefinitely. Somatic embryos exhibit typical embryo morphology and germinate readily into plants. Exercises designed to lead students through aspects of culture initiation, maintenance, and plant regeneration are described.

Members of the Poaceae constitute the single most economically important group of plants. This family includes the nutritious cereal grain crops such as

¹CFREC–IFAS, University of Florida, 5336 University Avenue, Leesburg, FL 34748.

²Department of Ornamental Horticulture and Landscape Design, University of Tennessee, POB 1071, Knoxville, TN 37901-1071.

³Department of Plant and Soil Science, University of Tennessee, POB 1071, Knoxville, TN 37901-1071.

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corn (*Zea mays* L.), oats (*Avena sativa* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.), as well as forage grasses such as annual ryegrass (*Lolium multiflorum* Lam.), tall fescue (*Festuca arundinacea* Schreb.), and the wheatgrasses (*Agropyron* spp.). The cereals are important sources of complex carbohydrates, oils, and proteins, whereas the forages are important in dairy and meat production. Sugarcane (*Saccharum officinarum* L.), another member of this family, is an important source of sucrose.

Much research emphasis in the Poaceae has gone toward developing biotechnological approaches to crop improvement. One area of biotechnology that received early attention by researchers was that of in vitro plant regeneration. The grasses and cereals initially were regarded by researchers to be difficult or impossible to regenerate from tissue and cell cultures. However, continuous effort since the early 1980s led to significant advancements, such that plant regeneration has been obtained for all grass and cereal species for which regeneration has been attempted (Gray, 1990).

Somatic embryogenesis is the most common mode of regeneration reported in the Poaceae (Gray, 1990). Embryogenic culture systems are of use in the classroom, not only for illustrating plant regeneration, but also for studying embryo development and morphology. Because the family Poaceae is contained within the Monocotyledoneae, a taxonomic division based on embryo morphology, their zygotic embryos are distinctly different from dicotyledonous embryos. In many poaceous plants, the induction of somatic embryogenesis is not simple and reliable enough to be used for classroom exercises. Many species require use of immature zygotic embryos as explants, which can only be obtained by careful cultivation and pollination of source plants. Other species tend to produce somatic embryos that have relatively abnormal morphologies when compared to zygotic embryos. However, a few poaceous species, notably orchardgrass (*Dactylis glomerata* L.), are much easier to manipulate.

These laboratory exercises cover the methods used to produce somatic embryos from cultures of orchardgrass. Selected clones of this species are highly embryogenic and can be maintained in the greenhouse as potted plants. These factors account for its convenient use. Leaf tissues are used as explant material for initiating cultures from which morphologically correct somatic embryos are produced. Thus, the orchardgrass embryogenic system is ideal for demonstrating monocotyledonous somatic embryogenesis. The exercises are arranged to lead students through successful culture establishment, observations of embryogenic callus formation and somatic embryo development, including the gradient embryogenic response that is typical of this culture system, culture maintenance, and plant regeneration. Each exercise begins with a background discussion, then provides specific instructions to accomplish each procedure and con-

cludes with the specific data to be taken along with questions for students to answer based on their observations. To provide the instructor (and students) with answers to the questions, a discussion of expected results follows the exercise section of this paper. For illustration, previous publications on orchardgrass that provide detailed descriptions and photographs are referenced at appropriate points in the exercises. It is suggested that instructors obtain copies of the publications from their local science library or via interlibrary loan. These exercises assume that students were trained previously in axenic culture techniques and basic methods of plant tissue culture.

Materials and methods

A special clone of orchardgrass, 'Embryogen-P' (Conger and Hanning, 1991), is the most-convenient source of explant tissue and is available from B.V. Conger (Dept. of Plant and Soil Science, The Univ. of Tennessee, PO Box 1071, Knoxville, TN 37901-1071). The clone should be maintained in greenhouse pots in high-quality potting mix, with regular fertilizer applications, and kept in vigorous growing condition. Before becoming root-bound, plant number can be increased by splitting entire plants, including the root ball, and transplanting into two or three new pots. Insect and fungal pests should be controlled; however, culture response may be decreased for up to 6 weeks after chemical spraying, especially with systemic fungicides. Therefore, stock plant health and vigor are the most-important factors in successful embryogenic culture initiation of orchardgrass.

Equipment required to accomplish the exercises include an analytical balance, autoclave, laminar flow culture hood, pH meter, and a stereomicroscope with light source. The following also are required: sterile distilled water, 50% commercial bleach with a drop of surfactant (such as Triton-X100™, Tween 20™, or Ivory Liquid™ soap) added, sterile 150-ml beakers covered with aluminum foil, scalpel with a supply of disposable #10 blades, fine forceps, Bunsen burner, quench bottle containing 95% EtOH for cooling instruments, and Parafilm™.

Chemicals and supplies are available from Sigma (St. Louis). The culture medium used is Schenk and Hildebrandt (SH) basal salt mixture and vitamin powder with 30 µM dicamba (synthetic auxin growth regulator), 3% sucrose, and 0.7% tissue culture-tested agar. This medium is hereafter referred to as SH30, whereas medium lacking dicamba is designated as SH0. The pH is adjusted to 5.4 before autoclaving. The cooling medium then is poured, 30 ml, into each 15 × 100-mm sterile petri dish.

Exercises

Exercise 1—culture establishment.

Orchardgrass is a forage grass in which the body of a potted plant consists of dense clumps of

tillers. New tillers arise by adventitious budding from the base of previously developing tillers. Each tiller is actually a complete plant in itself, with a root-shoot axis and several flattened, interfolded clasping leaves. Grass leaves grow from a pronounced basal meristem, such that the youngest part of a given leaf is the portion nearest to the shoot apical meristem. Youngest leaves are those nearest to the center. Thus, the youngest, most meristematically active leaf tissues are those nearest the bases of the innermost leaves.

To initiate cultures, remove individual tillers from a rapidly growing plant. Dissect individual leaves carefully so as to prevent damage and identify the innermost two leaves. Remove the basal-most 4 cm of these leaves and separate the two halves of each longitudinally. Each leaf base should yield two strips of blade tissue ≈ 4 cm long × 4 mm wide. Place bleach solution into a sterile beaker and prepare three additional beakers with sterile distilled water for rinsing. Surface-disinfect the leaf halves by gently agitating in the bleach solution for 2 min. Sterile forceps may be used to accomplish agitation. Gently rinse the leaf halves three times in sterile water, then remove one leaf-half at a time and cut on a hard, sterile surface (such as a petri dish) with a fresh, sharp scalpel blade. Cut the basal most part of each leaf half crosswise into seven approximately square sections (approximate size = 4 × 4 mm) and discard the remaining portion of each leaf-half. Immediately plate the sections on SH30 medium. All of the dissection steps should be accomplished as rapidly as possible.

Arrange the sections from each leaf-half in separate rows such that basal sections are at one end of a given row and the most-distal sections are at the other end. Sections from four leaf-halves should be placed in each petri dish. Seal each dish securely with several layers of parafilm.

Incubate cultures at 23°C in dark and evaluate daily for contamination. Transfer noncontaminated sections to fresh medium as needed. Forceps should be sterilized between moving of each section when transferring noncontaminated sections to avoid spreading of inconspicuous contamination to clean sections.

Determine the number of sections from each leaf weekly (i.e., innermost vs. outermost) that became contaminated. Chart the relative contamination rate as a function of leaf position in the tiller. Did younger leaves become more, or less, contaminated than older leaves over time?

Exercise 2—the gradient embryogenic response. The embryogenic response in orchardgrass is related to leaf position within a tiller (i.e., the innermost, or youngest, leaf vs. the outermost, or oldest, leaf), as well as the position of cultured leaf sections relative to the shoot apical meristem (i.e., proximal or distal). Two distinct embryogenic responses are possible (Conger et al., 1983): 1) either an embryogenic callus develops, or 2) somatic embryos grow directly from the

leaf surface. Embryogenic callus is white-to-yellowish in color and friable-to-wet in consistency. Often, adventitious roots develop from the leaf sections but never lead to plant regeneration. Somatic embryos, which are described in more detail below, appear as small (≈ 1 mm), white, organized structures, either embedded in callus or growing directly from the leaf section surface.

Plate leaf sections on SH30 medium as described above such that the sections from each leaf half are kept together in a single row and arranged in sequential order from the most-proximal to the most-distal section. Place the sections from four leaf halves in each dish (i.e., four rows of sections/dish). Mark each leaf from each tiller so that the identity of the innermost vs. the outermost of two leaves per tiller is maintained. Culture and screen for contamination as described above.

Determine the number of sections from each location within each leaf half that produces embryogenic callus and/or direct embryos at weekly intervals for 8 weeks. Chart the occurrence of embryogenic callus and somatic embryos as a function of time and position of the section in the original tiller. Do sections from younger or older leaves or sections from proximal or distal locations within a leaf produce more callus or direct embryos? Which sections respond most rapidly? How do observed embryogenic responses of individual sections correlate with the meristematic zones in intact orchardgrass leaves?

Exercise 3—maintenance of embryogenic callus. Embryogenic callus of orchardgrass can be maintained by monthly transfer of callus and/or somatic embryos to fresh SH30 medium. Embryogenic callus is very heterogeneous and continuously sectors into embryogenic and nonembryogenic portions. For maintenance of long-term embryogenic cultures, it is important to transfer only embryogenic sectors (Gray et al., 1984).

Isolate embryogenic callus and somatic embryos from leaf cultures. Dissect callus under a stereomicroscope and identify various types of callus and tissue. Separate dry, friable-type callus from wet, mucilaginous-type callus. Identify and separate sectors with numerous root primordia, as well as clumps of large somatic embryos and masses of small somatic embryos embedded in a watery matrix (Gray et al., 1984). Separate the callus and tissues by type and plate small clumps of each, five clumps per dish, onto SH30 medium. Also, be sure to plate single, isolated somatic embryos in order to test their response. Incubate in the dark for 4 weeks, as before.

Determine what callus types and/or tissues gave rise to embryogenic callus. Which of these produced the most embryogenic callus and somatic embryos? Where did embryogenic callus arise from plated somatic embryos? What type of tissue isolated from embryogenic callus would be best to use in culture maintenance? Why does embryogenic callus continually sector?

Exercise 4—observations on mono-

cotyledonous somatic embryos. Orchardgrass embryogenic cultures produce monocotyledonous somatic embryos that are of the poaceous type. The typical embryo possesses a scutellum and a coleoptile. While the somatic embryos are small, it is possible to see morphological details using a stereomicroscope. Abnormal embryos are also common. For references, consult Conger et al. (1983, 1989), Gray et al. (1984); Gray and Conger (1985), and Trigiano et al. (1989).

Observe somatic embryos developing from callus and directly from leaf cultures. Correlate specific embryos observed with those illustrated in referenced publications. Identify the scutellum and coleoptile. Observe abnormal embryos. What types of abnormalities are most common? How might such abnormalities have occurred during embryo development?

Exercise 5—somatic embryo germination and plant recovery. Orchardgrass somatic embryos germinate readily and in a manner similar to that of zygotic embryos when placed on SH0 medium and incubated in light (cool-white fluorescent) at 25°C (Gray and Conger, 1985). Small rooted plants are removed from petri dishes, transferred quickly (to avoid dehydration) to moist growing medium mix in small pots, which are enclosed in plastic bags and cultured as above. When plants begin to grow vigorously, they are transferred to greenhouse pots and maintained in the same manner as stock plants (Conger et al., 1983).

Transfer well-developed somatic embryos, five per dish, to SH0 medium and incubate as above. Alternatively, transfer small clumps of embryogenic callus to the same medium. Observe each for signs of germination.

Count the number of shoots and roots present after 2, 4, 6, and 8 days to determine whether shoots or roots emerge first. Identify the developing pigmented coleoptiles. Count the number of shoots per callus; this provides an indication of the relative number of well-developed somatic embryos per callus. Determine the percentage of somatic embryos that produce acclimated plants. What is the first sign of germination and when does it occur?

Discussion

This discussion is meant to describe the results expected from completing the exercises successfully. For broader background, including theoretical considerations, students are encouraged to consult the indicated publications concerning orchardgrass, as well as review articles (Gray, 1990; Gray and Purohit, 1991).

A measure of bacterial and fungal contamination growing on the medium or from cultured leaf sections is expected. While up to 50% of sections may become contaminated, a more-normal contamination rate is 10%. It is important to follow directions for flaming instruments between sections

to eliminate, rather than spread, contamination.

The gradient embryogenic response is demonstrated by correlating the relative position in a leaf from which a given section is obtained. After 4 to 6 weeks, basal sections produce embryogenic callus. The callusing response diminishes progressively in sections from increasingly more-distal positions of the leaf, being replaced by a direct embryogenic response and, eventually, no embryogenic response at all. The differential embryogenic response is related to meristematic activity in the explant tissue. Leaf sections with abundant undifferentiated meristematic cells tend to produce embryogenic callus. As meristematic activity is decreased, embryogenic response is reduced such that only direct somatic embryos develop on sections with limited meristematic activity. Sections in which meristematic activity is too low or has ceased do not exhibit an embryogenic response.

Embryogenic calli or individual embryos are transferred to fresh SH30 medium to maintain and increase the embryogenic line. When transferring callus, it is important to isolate only sectors that are producing somatic embryos. The watery matrix material that contains masses of small somatic embryos frequently produces the most-vigorous and -prolific embryogenic callus. Embryogenic callus continues to sector into embryogenic and nonembryogenic callus-types, primarily due to the recallusing of existing somatic embryos. The somatic embryos are anatomically complex and produce a number of different callus types, including embryogenic and rhizogenic (root-forming) callus, when recultured on SH30.

When viewed with a stereomicroscope, small, white somatic embryos that are morphologically similar to zygotic embryos either appear in embryogenic callus or emerge directly from uncalledus leaf sections. The embryos possess a distinct scutellum and a notch from which a coleoptile develops and enlarges during germination. Direct embryos are frequently attached to the leaf by a distinct suspensor. Individual embryos are induced to germinate into plants by transfer to medium lacking dicamba. Resulting plants are easily acclimated to greenhouse conditions.

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Laboratory Exercises Illustrating Organogenesis and Transformation using Chrysanthemum Cultivars

R.N. Trigiano¹ and
R.A. May²

Additional index words. acclimatization, *Agrobacterium*, cytokinins, leaves, regeneration, tissue culture

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 $\approx 100 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ fluorescent and incandescent light for 16 h/day at 25 to 28°C. 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%

¹Institute of Agriculture, Department of Ornamental Horticulture and Landscape Design, The University of Tennessee, Knoxville, TN 37901-1071.

²Department of Horticulture, Michigan State University, East Lansing, MI 48824-1325.