Novel Laboratory Exercises in Plant Tissue Culture: In Vitro Asymbiotic Germination of Orchid Seeds

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Summary. In vitro asymbiotic seed germination, subculture, and outplanting of orchids is presented as a laboratory exercise suitable for students of plant propagation or tissue culture. Dendrobium antennatum (Lindley), Phalaenopsis (Blume) white hybrid, or both, are used in this exercise because they flower predictably in the greenhouse, are reliable for seed production, and germinate and grow rapidly in vitro. The exercises can be used to instruct students in the skills involved in orchid seed sterilization, sowing, and culture, as well as instruct students in the unique features of orchid reproductive biology and symbiosis. A schedule is suggested for stock plant flower pollination, capsule harvest, seed sowing, and seedling subculture so that the necessary plant material is available for students to sow, subculture, and outplant seedlings during a single laboratory session.

Laboratory exercises designed to teach in vitro methods of propagation to students in introductory-level plant propagation or plant tissue culture courses naturally focus mainly on axenial (clonal) methods of in vitro culture, such as organogenesis, axillary shoot culture, and somatic embryogenesis. In vitro sexual propagation from seed is less commonly included as a student exercise, largely because it is relatively less important commercially. Orchids are an important exception to this generalization. In vitro germination of orchid seeds, also known as flaking, is important in the commercial production of hybrid orchids and of many species such as Paphiopedilum sp. Pitz. (Arditti and Ernst, 1993). In vitro asymbiotic germination is also an essential component of modern orchid breeding.

This exercise, presented along with sufficient background information via lecture or reading assignments (e.g., appropriate selections from Arditti, 1992; Arditti and Ernst, 1984; Hadley, 1982), can be used to teach the specific skills discussed below, but also to familiarize students with the unique historical, biological, and horticultural aspects of orchid reproduction. This exercise on in vitro asymbiotic orchid seed germination highlights an important event in the history of plant tissue culture, viz. Knudson's in vitro asymbiotic germination of Cattleya Lindl. and Laelia Lindl. orchid seeds (Knudson, 1922), which was the first successful in vitro propagation of any plant.

Asymbiotic germination, as practiced in this exercise, can be contrasted to the unique natural symbiotic seed germination strategy that has evolved in the Orchidaceae. Specifically, sexual reproduction of orchids in nature involves germination and survival of a relatively small fraction of the large number of extremely small seeds produced within each fruit (capsule). In contrast to the seeds of most higher plant species, orchid seeds are quite small (0.4 to 1.25 mm × 0.08 to 0.27 mm) (Arditti, 1992), consisting of a seed coat and a rudimentary embryo that essentially lacks food reserves directly available to the embryo to support germination and early seedling development. Germination in nature is entirely dependent on the formation of a mycorrhizal association with an appropriate fungus and, hence, is referred to as symbiotic germination. By digesting soil organic matter such as cellulose, the fungus makes soluble sugars available to the embryo (Hadley, 1982). Aside from its obligate mycorrhizal association, embryo development in orchids differs from that of non-orchid higher plants in that, at the point of seed maturity, the orchid embryo is simply a more-or-less spherical mass of undifferentiated cells without a root–shoot axis. Upon imbibition and rupture of the testa, the proembryo enlarges and functions in the absorption of water and minerals from the substrate, and organic compounds from its fungal associate. It usually is called a protocorm only after an apical shoot meristem differentiates, which occurs 4 to 5 weeks after sowing in the case of D. antennatum and the large-flowered Phalaenopsis hybrid used in this exercise. About 8 weeks after sowing, the first (adventitious) root meristem differentiates at the base of the first leaf (rather than from the protocorm). The developmental sequence of seed germination for both Dendrobium antennatum and Phalaenopsis is illustrated in Fig. 1.

Knudson was able to bypass the natural requirement for an appropriate mycorrhizal fungus and accomplish asymbiotic germination because he provided a simple sugar (sucrose) and other organic compounds in a tissue culture nutrient medium. In vitro orchid seed germination and early seedling development are obligately heterotrophic processes, in contrast to the facultative heterotrophic or photoautotrophic organic nutrition of non-orchid seedlings or other explants grown in vitro.

The student laboratory exercise described here includes three separate, but related, student activities, including: 1) sterilization and sowing of seed; 2) subculture of seedlings to fresh medium (2 months after sowing); and 3) outplanting of seedlings to the greenhouse (3 to 6 months after subculture). An additional necessary activity, which must be conducted in advance by the instructor, is flower pollination and harvesting of seed capsules. We stagger the various activities in order to have plant material at all three stages of development at one time, so that all three student activities can be executed during a single laboratory session. A typical time line of instructor/student activities for this exercise for either Dendrobium antennatum or Phalaenopsis white hybrid is illustrated in Fig. 2. The requisite intervals between activities can vary, of course, depending on the choice of orchid species and on greenhouse and laboratory temperatures and, to some extent, illumination.

For this exercise, we have specifically chosen D. antennatum and Phalaenopsis white hybrid (any large-flowered Phalaenopsis hybrid is suitable) for several reasons. Using both species allows for a comparison of the two common orchid growth habits, i.e., Dendrobium is sympodial, whereas Phalaenopsis is monopodial (Fig. 1) (Arditti, 1992). Seeds of the former are larger than the latter. In vitro germination and seedling growth are relatively rapid for both. Dendrobium antennatum naturally blooms year round, while Phalaenopsis normally flowers three times per year in north-temperate North America, but the latter can be induced to flower at other times by exposing stock plants to 20°C for 2 months followed by 2 to 3 months additional growing on at 25 to 30°C (Lee and Lin, 1984). Self- or cross-pollination of either orchid can be done successfully.

For this student exercise, we use seed collected from a mature capsule, at or just prior to dehiscence. On the other hand, seeds of some orchid species, such as Cymbidium goeringii (Nagashima, 1993) and many interspecific Phalaenopsis hybrids germinate better if plated out before maturity. In such cases, a form of embryo (ovule) rescue (“green podding”) commonly is practiced, in which an intact green seed capsule is surface-sterilized and immature seeds are extracted and placed in culture. An additional...
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Advantage of "green pod" culture is that an intact capsule is more easily surface-sterilized than loose mature seed. Nonetheless, in this exercise we prefer to use loose seeds collected from mature capsule for several reasons. Students learn the useful, but slightly more-challenging, technique involved in sterilization of loose seeds compared to an entire capsule. Fewer capsules and fewer stock plants are required because a single mature Phalaenopsis capsule, for example, will provide 50,000 to 100,000 seeds. The seeds from only one such capsule are sufficient for as many as 50 students, whereas at least one capsule per student would be required for "green pod" culture. Finally, mature seeds can be stored for 6 months or longer in a desiccator at room temperature, whereas immature seeds in or from green capsules lose viability rapidly.

Seed sterilization and sowing

Each student is given \( \approx 1000 \) ripe seeds, equivalent to \( \approx 250 \mu l \) loosely packed in a capped shell vial. Sterilization involves soaking seeds in a scintillation vial or a 50-ml Erlenmeyer flask containing \( \approx 30 \) ml of a solution of 0.5% sodium hypochlorite (w/v) plus one drop of Tween 20. The solution should be swirled by hand about once each minute for 12 to 15 min. Working under a laminar flow hood, sterile seeds are separated from the solution by filtering the contents of the flask, by vacuum or by gravity, through a presterilized filter paper disc in a Buchner or conical glass funnel. Seeds then are rinsed with sterile distilled water five or six times. A sterile inoculation loop is used to scrape seeds from the filter paper and streak them as evenly as possible onto the surface of the germination medium. The medium is Knudson C, as described by Arditti (1992), which is available commercially (K4003, Sigma, St. Louis), preformulated with 20 g•liter\(^{-1}\) sucrose. The pH is adjusted to 5.5 prior to addition of 7 g•liter\(^{-1}\) agar. After autoclaving, 20 ml of this medium is poured into 9-cm disposable plastic petri dishes. A sowing density of \( \approx 300 \) seeds per petri plate is appropriate, although seeds are too small to be counted accurately during sowing. Students are instructed to prepare two identical plates for each (or either) species. Petri plates should be sealed with Parafilm and placed in a culture room at 24°C under illumination from cool-white fluorescent tubes at about 70 \( \mu \)mol•m\(^{-2}•s^{-1} \) PAR. Weekly or biweekly, over the next 8 weeks, students can observe germination through the petri dish lid by means of a dissecting microscope. Typically, 95% of the seeds germinate. Students may be instructed to observe and sketch stages of development, as illustrated in Fig. 1.

Subculture

Using 8-week-old pregerminated seedlings, students, again working under a laminar flow hood, can transfer seedlings from germination plates to baby food jars or other relatively tall vessels containing a medium identical to that used for germination. Spacing between seedlings should be \( \approx 15 \) mm.

Outplanting

Three to 4.5 months after subculture, when seedlings have four fully developed leaves and are \( \approx 5 \) to 6 cm tall, they are ready for acclimatization and outplanting to the greenhouse. Good choices for the potting medium are either straight sphagnum moss or a mixture of four parts fine fir bark to one part coarse perlite. We transplant 10 seedlings...
A Laboratory Exercise to Demonstrate Direct and Indirect Shoot Organogenesis using Internodes of *Myriophyllum aquaticum*

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**Summary.** Only a few plants are suitable for reliably demonstrating rapid direct and indirect shoot organogenesis in vitro. A laboratory exercise has been developed using internodes of *Myriophyllum aquaticum*, an amphibious water garden plant. Stock shoot cultures are established and maintained in vitro from nodal explants cultured on agar-solidified medium consisting of half-strength Murashige & Skoog salts (MS) and 30 g•liter⁻¹ sucrose. Students use these cultures as the source of internode explants. Explants are cultured on agar-solidified full-strength MS with 30 g•liter⁻¹ sucrose, 100 µg•liter⁻¹ myo-inositol, and 0.4 mg•liter⁻¹ thiamine•HCL and factorial combinations of 0 to 10 µM 2iP and 0 to 1.0 µM NAA. Adventitious shoot development occurs directly from the explant epidermis within 4 days and is promoted in media supplemented with 2iP alone. Cytokinin-supplemented media amended with NAA induce organogenic callus formation, but reduce 2iP promotion of direct shoot organogenesis. After 4 weeks, shoot organogenesis on the various media is quantified and can be analyzed statistically. Chemical names used: N-(3-methyl-2-butenyl)-1H-purin-6-amine (2iP); α-naphthaleneacetic acid (NAA).

**Additional index words.** adventitious shoot formation, plant tissue culture, teaching, aquatic plant.

**Literature Cited**


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Florida Agricultural Experiment Station Journal Series no. R-03292.

**HortTechnology** - Jan. / Mar. 1992