Plant Cell Culture

Essential Methods

Michael R. Davey and **Paul Anthony**

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Preface

More than a century has passed since the first attempts were made to culture isolated plant cells in the laboratory, the number of publications confirming the substantial progress achieved in this area of research, especially during the last four decades. In many ways, plant cell culture *per se* has been overshadowed by the recent, phenomenal progress achieved in recombinant DNA technology. Nevertheless, the ability to culture cells and tissues in the laboratory through to the regeneration of fertile plants provides an important base for several technologies. For example, the mass production of elite plants is exploited extensively in present-day commercial enterprises, while techniques such as the generation of haploid plants, *in vitro* fertilization, embryo rescue and somatic hybridization are available to assist the plant breeder in generating hybrid plants. Similarly, the transfer into plants of specific genes by transformation also provides an important underpin to well established techniques of plant breeding, emphasizing the requirement for close liaison between breeders and cell technologists. Many of the approaches associated with the culture of plant cells in the laboratory demand an experienced eye, particularly in the selection of cultures that are most likely to retain and express their totipotency. Consequently, cell culture is, in many respects, as much an art as a science. However, what is remarkable is the ability of individual cells to multiply and to differentiate into intact plants when given the correct environmental conditions in the laboratory. Although cell-to-plant systems have been described for many plants, including some of our most important crops, there are dicotyledons and, in particular, monocotyledons, that are still recalcitrant to regeneration under *in vitro* conditions. These remain a challenge to researchers involved in plant cell culture.

We have had to be selective in the topics that are included in this volume. Consequently, we have focused on aspects of micropropagation, pathways of plant regeneration, mutagenesis, cryopreservation, secondary products, and the technologies associated with hybrid plant production and genetic manipulation. The chapters each provide a general background to the specific areas with appropriate methodology. Whilst the protocols are presented with reference to specific examples, the procedures can be modified accordingly for new material. Our contributors have been asked to provide precise details, however seemingly trivial, of the methods presented, to focus in the 'Troubleshooting' sections on some of the common problems often encountered, and to give detailed advice for the avoidance of such difficulties.

In general, such information is not included in research papers in learned journals. We thank all of the contributors for their patience and understanding during the preparation and extensive editing of the manuscripts. We hope they have also benefited from the experience of providing the detailed protocols that are in routine use in their laboratories.

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1
Plant Micropropagation

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1.1 Introduction

The technique of plant tissue culture is used for growing isolated plant cells, tissues and organs under axenic conditions (*in vitro*) to regenerate and propagate entire plants. 'Tissue culture' is commonly used as a blanket term to describe all types of plant cultures, namely callus, cell, protoplast, anther, meristem, embryo and organ cultures [1]. It relies on the phenomenon of cell totipotency, the latter being the ability of single cells to divide, to produce all the differentiated cells characteristic of organs, and to regenerate into a whole plant. The different techniques of culturing plant tissues may offer certain advantages over traditional methods of propagation. Growing plants *in vitro* in a controlled environment, with in-depth knowledge of the culture conditions and the nature of the plant material, ensures effective clonal propagation of genetically superior genotypes of economically important plants. Tissue cultures represent the major experimental systems used for plant genetic engineering, as well as for studying the regulation of growth and organized development through examination of structural, physiological, biochemical and molecular bases underlying developmental processes. Micropropagation has become an important part of the commercial propagation of many plants [2–6] because of its advantages as a multiplication system [7–9]. Several techniques for *in vitro* plant propagation have been devised, including the induction of axillary and adventitious shoots,

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the culture of isolated meristems and plant regeneration by organogenesis and/or somatic embryogenesis [10–12].

Fertile plants can be regenerated either by the growth and proliferation of existing axillary and apical meristems, or by the regeneration of adventitious shoots. Adventitious buds and shoots are formed *de novo*; meristems are initiated from explants, such as those of leaves, petioles, hypocotyls, floral organs and roots.

This chapter summarizes the application of the most commonly used *in vitro* propagation techniques for trees, shrubs and herbaceous species that can be implemented on a continuous basis throughout the year.

1.2 Methods and approaches

1.2.1 Explants and their surface disinfection

Small pieces of plants (explants) are used as source material to establish cells and tissues *in vitro*. All operations involving the handling of explants and their culture are carried out in an axenic (aseptic; sterile) environment under defined conditions, including a basal culture medium of known composition with specific types and concentrations of plant growth regulators, controlled light, temperature and relative humidity, in culture room(s) or growth cabinet(s). The disinfection of explants before culture is essential to remove surface contaminants such as bacteria and fungal spores. Surface disinfection must be efficient to remove contaminants, with minimal damage to plant cells. This chapter focuses on the general procedures for developing *in vitro* cultures, illustrated by protocols for specific plants and explants.

PROTOCOL 1.1 Surface Disinfection of Explants

Equipment and Reagents

- Autoclave
- Laminar flow cabinet
- Ultraviolet lamp
- Scalpels, forceps, scissors, rest for supporting axenic instruments (Duchefa), glass beakers (100 ml), glass Petri dishes (100 × 15 mm), white cotton gauze[a] (15 × 15 cm), magnetic mini-stirrer (ScienceLab) and stirring bars, filter paper (Whatman, Standard Grade; 10 mm diameter circles), aluminium foil, funnel and suction flask, glass beakers (100 ml–1 l in volume).
- Unifire Gasburner (Uniequip), glass bead sterilizer (Duchefa) or alcohol lamp
- Distilled water: 350 ml aliquots in 500 ml bottles
- Tween 20 (Sigma)
- Ethanol: 95 and 70% (v/v)
- NaClO or $Ca(ClO)_2$: 0.5–5% or 3–7% (w/v) aqueous solutions, respectively (Chemos GmbH)

- $HgCl_2$ (Sigma): 0.1–0.2% (w/v) aqueous solution[b]
- H_2SO_4: 96% (v/v) solution[c]
- Bacteriocidal soap
- Culture vessels with sterile culture medium (See Protocol 1.2 for preparation of culture medium).

Method

1 Place several filter papers into each of the glass Petri dishes. Wrap the Petri dishes, glass beakers, scissors, scalpels, forceps, funnel, white gauze and suction flask in aluminium foil.

2 Disinfect the material from Step 1 and bottles of distilled water in an autoclave at 120 °C, 118 kPa (1.18 bar) steam pressure for 20 min.

3 Disinfect the laminar flow cabinet by exposing the work bench to ultraviolet illumination for 3 h. Spray the work surface of the cabinet with 95% (v/v) ethanol; allow to dry.

4 Remove the epidermis from stem segments and scale leaves from buds of woody species[d].

5 Wash the explants under running tap water for 5 min.

6 Wash hands thoroughly with bacteriocidal soap before commencing work.

7 Disinfect the explants in the laminar flow cabinet. Place the explants in a beaker (autoclaved). Wash the explants (by stirring on magnetic mini-stirrer) in 70% (v/v) ethanol (2 min) and 5% (w/v) NaClO, containing 20 drops per litre of Tween 20 (15–30 min). After immersion in each solution, wash the explants 3 times with sterile distilled water for 3, 5 and 10 min; discard the washings

8 After surface disinfection, keep the plant material in distilled water in Petri dishes in the laminar flow cabinet to prevent drying.

9 Before preparing the explants, disinfect the forceps and scalpels using a glass bead sterilizer, Unifire Gasburner, or by flaming using the alcohol lamp for 10–15 s.

10 Remove the cut ends of the explants[e] (e.g. apical or axillary buds, leaves, petioles, flowers, seedling segments) with a sterile scalpel before placing the explants on the culture medium.

Notes

[a]Place small plant parts, such as tiny seeds or buds, into gauze bags to facilitate manipulation during disinfection.

[b]Mercuric chloride ($HgCl_2$) is a highly effective surface sterilant but is extremely toxic. Local regulations must be enforced with its use. The duration of surface disinfection in 0.1% (w/v) aqueous solution is 1–3 min for leaves and stems of herbaceous plants, 8–10 min for nodal and apical segments of woody plants, and 10–20 min for seeds.

[c]Use for 4–5 min to disinfect seeds with a hard testa.

[d]Removal of the epidermis from the stem segments and scale leaves from buds may increase the disinfection efficiency in woody species.

[e]Cut the ends of the explants in the laminar flow cabinet on sterile filter papers or on a sterile white tile.

1.2.2 Culture media and their preparation

Culture media contain macroelements, microelements, vitamins, other organic components (e.g. amino acids), plant growth regulators, gelling agents (if semisolid) and sucrose. Gelling agents are omitted for liquid media. The composition of the culture medium depends upon the plant species, the explants, and the aim of the experiments. In general, certain standard media are used for most plants, but some modifications may be required to achieve genotype-specific and stage-dependent optimizations, by manipulating the concentrations of growth regulators, or by the addition of specific components to the culture medium. Commercially available ready-made powdered medium or stock solutions can be used for the preparation of culture media. A range of culture media of different formulations, and plant growth regulators are supplied by companies such as Duchefa and Sigma-Aldrich. Murashige and Skoog medium (MS) is used most extensively [13]. A procedure for the preparation of MS medium supplemented with plant growth regulators for raspberry micropropagation [14] is given in Protocol 1.2.

PROTOCOL 1.2 Preparation of Culture Medium

Equipment and Reagents

- Culture vessels: 25 × 150 mm sterile plastic disposable culture tubes with screw-caps (Sigma-Aldrich), Full-Gas Microbox culture jars (jar and lid OS60 + ODS60; Combiness), Erlenmeyer 'Pyrex' flasks 125 ml capacity (Sigma-Aldrich) or Petri dishes (60 × 15 mm or 100 × 15 mm; Greiner Bio-One). Glass Petri dishes, if used, must be disinfected by autoclaving or dry heat treatment
- Autoclave
- Laminar flow cabinet
- Refrigerator/freezer
- Distilled water (water purification system)
- Electronic heated stirrer
- Analytical balances
- pH meter
- Microwave oven
- Pipettes and measuring cylinders

- Beakers, 100 ml and 1–2 l, 100 ml flasks, funnels, aluminium foil
- PP/PE syringes without needles, capacity 50 ml (Sigma-Aldrich)
- Acrodisc syringe membrane filters (25 mm, 0.2 μm pore size; Sigma-Aldrich)
- 1 M HCl and KOH
- MS packaged powdered medium, including macro and microelements and vitamins (Duchefa)
- Plant growth regulators for raspberry micropropagation: benzylaminopurine (BAP) and β-indolebutyric acid (IBA; Duchefa)
- Other plant growth regulators: auxins – naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D); cytokinins – kinetin, zeatin, 6-γ-γ-(dimethylallylamino)-purine (2-iP), thidiazuron (TDZ); gibberellins – gibberellic acid (GA_3); abscisic acid (ABA); organic components – sucrose, plant agar, citric acid, ascorbic acid (Duchefa)
- Plant preservative mixture – PPM (Plant Cell Technology, Inc.).

Method

1 To prepare 1 l MS medium, dissolve 4.406 g powdered medium in 500 ml of double distilled water in a 2 l beaker.

2 Prepare separate stock solutions of each plant growth regulator.

3 Add heat stable supplements to the medium before autoclaving, such as 30 g sucrose, 8 g agar, the desired plant growth regulators in a specific volume of stock solution (e.g. 5 ml BAP and 5 ml IBA) to reach the required final concentrations (1 mg/l BAP and 0.1 mg/l IBA for raspberry micropropagation). Adjust the medium to the final volume (1 l) by adding double distilled water[a].

4 Adjust the pH of the medium to 5.6–5.8 with 1 M HCl or KOH[b] and heat in microwave oven until the gelling agent is dissolved.

5 Autoclave the medium at 1 kg/cm (15 psi) at 121 °C for 20 min[c].

6 Dispense the medium into the culture vessels (15 ml per culture tube, 50 ml per Erlenmeyer bank, 50 ml per Full-Gas Microbox culture jar, 30 ml per 9 cm Petri dish) in the laminar flow cabinet. Close the vessels.

Preparation of Stock Solutions

1 Prepare separate stock solution for each plant growth regulator. Weigh the plant growth regulators to obtain a quantity 20 times the quantity given in the formulation for the medium (e.g. 20 mg BAP and 2 mg IBA), and dissolve in 100 ml distilled water[d].

2 Dissolve auxins (NAA, IAA, IBA and 2,4-D) in 1 ml ethanol and make up to 100 ml with distilled water.

3 Dissolve cytokinins (kinetin, zeatin, BAP, 2-iP) and ABA in 1 ml 1 M NaOH or 1 M KOH; make up to 100 ml with distilled water.

4 Store the stock solutions in 100 ml flasks in a refrigerator (not frozen) for not more than 2 months[e].

Filter Sterilization of Heat Sensitive Compounds

1 Wrap a funnel and 100 ml flask in aluminum foil and autoclave.

2 Fill the PP/PE syringe with the solution of heat labile constituents (e.g. zeatin, 2-iP, IAA, GA_3, citric acid, ascorbic acid). Mount an Acrodisc syringe membrane filter on the syringe and filter the solution into the funnel and into a sterile flask. Dispense the filter sterilized solution into convenient aliquots (e.g. 10–20 ml) in sterile, screw-capped vessels. Perform this operation in a laminar flow cabinet. Store the filter sterilized solutions at −20 °C.

Notes

[a]Heat labile constituents, such as some growth regulators and organic compounds (e.g. zeatin, 2-iP, IAA, GA_3, citric acid, ascorbic acid), should not be autoclaved but filter sterilized before adding to the autoclaved culture medium after the medium has cooled to 40–50 °C in the laminar flow cabinet.

[b]The pH of the culture medium is usually adjusted to 5.6–5.8. For acid-loving species, a lower pH is required (4.5 or less).

[c]To minimize contamination by micro-organisms, a broad-spectrum biocide/fungicide for plant tissue culture [Plant Preservative Mixture (PPM); Plant Cell Technology, Inc.] may be added to the medium at a concentration of 2–20 ml/l, which effectively prevents or reduces microbial contamination. Some plant species are more sensitive to PPM than others. Rooting in less tolerant plant species may be partially inhibited. In this case, the explants should be exposed to PPM for only a limited time.

[d]Cytokinins (BAP, kinetin, 2-iP, zeatin) are added to the culture medium to induce axillary or adventitious shoots. Auxins (2,4-D, NAA, IAA) induce callus formation. IBA is generally used to induce adventitious roots. GA_3 or polyamines added to the medium will promote shoot elongation.

[e]Culture media should be used within 2 to 4 weeks of preparation and may be kept for 6 weeks before use, if refrigerated.

1.2.3 Stages of micropropagation

The following distinct stages are recognized for the micropropagation of most plants:

Stage I: Establishment of axenic cultures – introduction of the surface disinfected explants into culture, followed by initiation of shoot growth. The objective of this stage is to place selected explants into culture, avoiding contamination and providing an environment that promotes shoot production [15]. Depending on the type of explant, shoot formation may be initiated from apical and axillary buds

(pre-existing meristems), from adventitious meristems that originate on excised shoots, leaves, bulb scales, flower stems or cotyledons (direct organogenesis), or from callus that develops at the cut surfaces of explants (indirect organogenesis). Usually 4–6 weeks are required to complete this stage and to generate explants that are ready to be moved to Stage II [16]. Some woody plants may take up to 12 months to complete Stage I [15], termed 'stabilization'. A culture is stabilized when explants produce a constant number of normal shoots after subculture [16].

Stage II: Multiplication – shoot proliferation and multiple shoot production. At this stage, each explant has expanded into a cluster of small shoots. Multiple shoots are separated and transplanted to new culture medium [16]. Shoots are subcultured every 2–8 weeks. Material may be subcultured several times to new medium to maximise the quantity of shoots produced.

Stage III: Root formation – shoot elongation and rooting. The rooting stage prepares the regenerated plants for transplanting from *in vitro* to *ex vitro* conditions in controlled environment rooms, in the glasshouse and, later, to their ultimate location. This stage may involve not only rooting of shoots, but also conditioning of the plants to increase their potential for acclimatization and survival during transplanting. The induction of adventitious roots may be achieved either *in vitro* or *ex vitro* in the presence of auxins [17–19]. The main advantage of *ex vitro* compared to *in vitro* rooting is that root damage during transfer to soil is less likely to occur. The rates of root production are often greater and root quality is optimized when rooting occurs *ex vitro* [20–23].

Stage IV: Acclimatization – transfer of regenerated plants to soil under natural environmental conditions [16]. Transplantation of *in vitro*-derived plants to soil is often characterized by lower survival rates. Before transfer of soil-rooted plants to their final environment, they must be acclimatized in a controlled environment room or in the glasshouse [24, 25]. Plants transferred from *in vitro* to *ex vitro* conditions, undergo gradual modification of leaf anatomy and morphology, and their stomata begin to function (the stomata are usually open when the plants are in culture). Plants also form a protective epicuticular wax layer over the surface of their leaves. Regenerated plants gradually become adapted to survival in their new environment [26].

1.2.4 Techniques of micropropagation

Cultures of apical and axillary buds

Currently, the most frequently used micropropagation method for commercial mass production of plants utilizes axillary shoot proliferation from isolated apical or axillary buds under the influence of a relatively high concentration of cytokinin. In this procedure, the shoot apical or axillary buds contain several developing leaf primordia. Typically, the explants are 3–4 mm in diameter and 2 cm in length. Development *in vitro* is regulated to support the growth of shoots, without adventitious regeneration.

PROTOCOL 1.3 Propagation by Culture of Apical and Axillary Buds

Equipment and Reagents

- Culture facilities – culture room or plant growth cabinet with controlled temperature, light and humidity; culture vessels
- Laminar flow cabinet, ultraviolet lamp
- Scalpels, forceps, scissors, a rest for holding sterile tools (Duchefa), 50 ml beakers
- Unifire Gasburner (Uniequip), glass bead sterilizer (Duchefa) or glass alcohol lamp
- Ethanol 70% and 95% (v/v); Tween 20 (Sigma); NaClO (Chemos GmbH); $HgCl_2$ (Sigma)
- Bacteriocidal soap
- Murashige and Skoog medium (MS-Duchefa)
- Anderson's Rhododendron medium (AN-Duchefa)
- Plant growth regulators and organic components: BAP, 2-iP, zeatin, TDZ, adenine sulfate, NAA, IAA, IBA, sucrose, agar
- Distilled water
- Activated charcoal (Duchefa)
- Commercial plastic multi-pot containers (pot diam. 40 mm) with covers
- Peat, perlite, vermiculite

Method

Explant selection and disinfection:

1 Select the explants as single-node segments, preferentially from juvenile[a], rejuvenated plants[b,c], or *in vitro*-derived plants.

2 For commercial large-scale micropropagation, it is preferable to use pathogen-indexed stock plants as a source of explants.

3 See Protocol 1.1 for surface disinfection of explants.

Establishment of cultures:

1 Place isolated disinfection apical and axillary buds, from which the upper scale leaves have been removed, on culture medium (MS-based medium for *Lavandula dentata* L. and AN medium for *Vaccinium corymbosum* L.). See Protocol 1.2 for preparation of culture media. Carry out these operations in a laminar flow cabinet after UV and ethanol disinfection (See Protocol 1.1).

2 Add cytokinins to the medium to induce axillary shoots: BAP (0.01–5 mg/l), 2-iP (0.01–10 mg/l), zeatin (2–15 mg/l), TDZ (0.01–10 mg/l), adenine sulfate (40–120 mg/l). Add auxins (NAA, IAA, IBA) in low concentrations (0.01–0.1 mg/l) to

the medium to support shoot growth[d]. Optimize experimentally the cytokinin and auxin types and concentrations for each species[e].

3 Culture the explants for 4 weeks on cytokinin-containing medium in the growth cabinet at 23 ± 2 °C with a 16 h photoperiod (50 μmol/m^2/s; white fluorescent lamps).

Shoot multiplication:

1 Separate *in vitro* regenerated axillary shoots and transfer the shoots onto the appropriate culture medium (MS medium for *L. dentata* and AN medium for *V. corymbosum*) supplemented with the same or a reduced cytokinin concentration.

2 Cut the regenerated shoots into one-node segments and culture on cytokinin-supplemented medium to stimulate shoot proliferation.

3 Repeat the procedure depending on the number of shoots required. Some of the regenerated shoots *in vitro* can be retained for use to provide an axenic stock of explants for further multiplication.

Rooting of regenerated shoots:

Root the regenerated shoots by two approaches:

1 *Ex vitro* rooting by 'pulse treatment' – immerse the stem bases of 15–20 mm long regenerated shoots into an auxin solution (e.g. IBA at 1–10 mg/l) in 50 ml beakers for 3–7 days, followed by planting in commercial plastic multi-pot containers with soil or a mixture of peat, perlite and vermiculite (equal volumes). Cover the containers and shoots to maintain soil and air humidity.

2 *In vitro* rooting on culture medium supplemented with IBA at a concentration of 1 mg/l and activated charcoal at 1–10 g/l[f]. Reduction of the components of the culture medium to half strength, darkness during culture[g] and inoculation with mycorrhizal fungi[h], may stimulate rooting.

Examples

Micropropagation of *Lavandula dentata* by culture of apical and axillary buds (27).

1 Excise stem segments (each 2–3 cm in length) bearing apical or lateral axillary buds from 5-year-old plants between September and December.

2 Disinfect the stem segments by immersion in 70% (v/v) ethanol for 30 s, and sodium hypochlorite (NaClO) solution (1 g/l) containing 0.01% (v/v) Tween-20 for 20 min; rinse thoroughly with sterile distilled water.

3 Culture the dissected apical and lateral buds vertically on MS culture medium supplemented with sucrose (30 g/l), agar (6 g/l; Merck), cytokinin (BAP; 0.5 mg/l) and auxin (IBA; 0.5 mg/l) at pH 5.6–5.8.

4 Maintain the cultures in the growth cabinet at 25 ± 2 °C under a 16 h photoperiod (50 μmol/m^2/s; white fluorescent illumination).

5 Root the isolated shoots on MS medium supplemented with 0.5 mg/l NAA.

Micropropagation of *Vaccinium corymbosum* by culture of apical and axillary buds [17].

1 Harvest branches with dormant buds from mature donor plants during February and at the beginning of March; cut the branches into single-node segments.

2 Disinfect the segments with apical and axillary buds by washing under running tap water for 1 h, followed by immersion in 70% (v/v) ethanol for 2 min. Transfer the cuttings into 300 ml 0.1% (w/v) mercuric chloride with three drops of Tween for 6 min. Wash the explants thoroughly with sterile distilled water (three changes, each 15 min). Retain all the washings and discard according to local regulations for toxic chemicals.

3 Culture the isolated dormant apical and axillary buds, from which the upper scales are removed after disinfection, on AN medium supplemented with sucrose (30 g/l), Phytoagar (8 g/l) and zeatin (2 mg/l), at pH 4.5–5.0.

4 Maintain the cultures in the growth cabinet at $23 \pm 2\,^{\circ}C$ with a 16 h photoperiod ($50\,\mu mol/m^2/s$, white fluorescent illumination).

5 For further proliferation of *in vitro* regenerated axillary shoots, culture the shoots on the same medium with zeatin (0.5 mg/l) with subculture every 5 weeks.

6 Root the regenerated shoots (each 15–20 mm in height) *ex vitro* by dipping (2–3 min) into IBA solution (0.8 mg/l), followed by planting in commercial plastic multi-pot containers (pot diam. 40 mm) filled with peat-based compost, or *in vitro* on AN medium with IBA (0.8 mg/l) and activated charcoal (0.8 g/l).

Notes

[a]The branches from the basal part of the crown, near to the trunk and highest order of branching, are more juvenile than others in the crown of the plant. More juvenile are epicormics, shoots originating from spheroblasts, severely pruned trees, stump and root sprouts [28].

[b]Rejuvenation may be initiated by grafting scions from mature trees onto juvenile rootstocks. Use explants for culture from trees 1–3 years after grafting [29].

[c]Keeping the cut branches in the sterile liquid medium without growth regulators or in water, in a growth cabinet for 4–5 days, may force the plant material into growth.

[d]Synthetic auxins are more stable and most effective. They include IBA and NAA at 0.1–10 mg/l, 2,4-D at 0.05–0.5 mg/l and the natural auxin IAA (1–50 mg/l). IBA is the most effective auxin for adventitious root induction.

[e]Prepare the MS culture medium with several combinations of growth regulators and grow the same type of explant (dormant bud) for 5 weeks. During testing for the optimal culture medium, change only one factor at a time in the composition of the medium. In order to determine appropriate cytokinin type and concentration for shoot induction, combine different concentrations (0.5, 1, 2, 3 and 5 mg/l) of cytokinins with 0.05 mg/l auxin. Evaluate the number of regenerated shoots and select the most efficient cytokinin concentration. Use the most efficient cytokinin concentration in combination with different auxin concentrations (0.05, 0.1, 0.2, 0.5) to determine the optimal auxin concentration.

[f]For some plants, such as *Sequoiadendron giganteum* and *Fraxinus excelsior*, rooting is optimal by maintaining the shoots in auxin-supplemented medium (induction medium) for 1–5 days, followed by transfer to an auxin-free medium for root formation.

[g]Some plants form roots more rapidly in the dark during auxin treatment.

[h]Mycorrhizae are a close relationship between specialized soil fungi (mycorrhizal fungi) and plant roots. Mycorrhizae may stimulate the rooting of some species [30–34].

Meristem and single- or multiple-node cultures (shoot cultures)

Meristems are groups of undifferentiated cells that are established during plant embryogenesis [35]. Meristems continuously produce new cells which undergo differentiation into tissues and the initiation of new organs, providing the basic structure of the plant body [36]. Shoot meristem culture is a technique in which a dome-shaped portion of the meristematic region of the stem tip is dissected from a selected donor plant and incubated on culture medium [37]. Each dissected meristem comprises the apical dome with a limited number of the youngest leaf primordia[a], and excludes any differentiated provascular or vascular tissues. A major advantage of working with meristems is the high probability of excluding pathogenic organisms, present in the donor plant, from cultures[b]. The culture conditions are controlled to allow only organized outgrowth of the apex directly into a shoot, without the formation of any adventitious organs, ensuring the genetic stability of the regenerated plants.

The single-or multiple-node technique involves production of shoots from cultured stem segments, bearing one or more lateral buds, positioned horizontally or vertically on the culture medium[c]. Axillary shoot proliferation from the buds in the leaf axils is initiated by a relatively high cytokinin concentration[d]. Meristem and node cultures are the most reliable for micropropagation to produce true-to-type plants[e].

PROTOCOL 1.4 Propagation by Meristem and Nodal Cultures

Equipment and Reagents

- Culture facilities (culture room or plant growth cabinet) with automatically controlled temperature, light, and air humidity; sterile disposable Petri dishes (60 and 100 mm; Greiner Bio-One), Full-Gas Microbox culture jars (jar and lid OS60 + ODS60; Combiness)
- Laminar flow cabinet, ultraviolet lamp
- Stereomicroscope
- Unifire Gasburner (Uniequip), glass bead sterilizer (Duchefa) or glass alcohol lamp
- Scalpel, needles, fine tweezers, rest for holding sterile tools (Duchefa)
- Detergent Mistol (Henkel Ibérica, SA), ethanol 70% and 95% (v/v); Tween 20 (Sigma); NaClO (Chemos GmbH); $HgCl_2$ (Sigma)
- 'Keep Kleen' disposable vinyl gloves (Superior Glove Works Ltd.)

- Bacteriocidal soap
- Plant growth regulators and organic components: BAP, GA_3, IBA, myoinositol, sorbitol, thiamine, nicotinic acid, glycine, phloroglucinol, agar, sucrose, ribavirin (Duchefa)
- Double distilled water
- Activated charcoal (Duchefa)
- Quoirin and Lepoivre medium (QL; Duchefa)
- Driver and Kuniyuki medium (DKW; Duchefa)
- Filter paper bridges made from Whatman filter paper[f]

Method

Explant selection and disinfection:

1 Select the explants, single-or multiple-node segments, preferentially from juvenile, rejuvenated plants, *in vitro* derived plants, or branches with dormant buds in the case of woody species.

2 Disinfect the explants according to Protocol 1.1. *In vitro*-derived plants should already be axenic.

Meristem cultures:

1 Isolate the meristems under the stereomicroscope in the laminar hood. Remove the upper leaves from each bud. Hold shoot segments with each bud and carefully remove the remaining leaves and leaf primordia one by one using dissection instruments. Disinfect the equipment (needle, scalpel and tweezers) regularly during this procedure using the gasburner. Excise each meristem (0.1 mm in diam.; 0.2–0.5 mm high) with one to two leaf primordia and transfer to the surface of semi-solid QL culture medium [38].

2 Culture the isolated meristems on semi-solid QL medium, or in the same liquid medium by placing the meristems on semisubmerged filter paper bridges. Use a similar composition of growth regulators as for bud cultures. Determine the optimal types and concentrations of growth regulators for each species.

Nodal cultures:

1 Culture the nodal explants in a vertical or horizontal position on cytokinin-enriched medium (see Protocol 1.3).

2 Avoid inserting the explants too deeply into the medium and submerging the nodes.

3 Culture for 4 weeks on cytokinin-containing medium.

See Protocol 1.3 for shoot multiplication and rooting.