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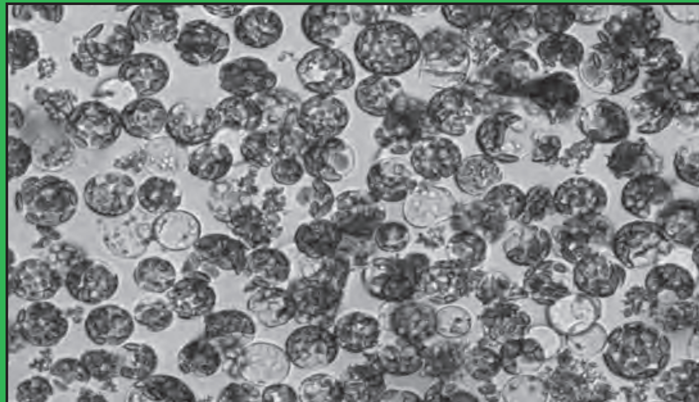
Plant Cell Culture Protocols

Edited by

Robert D. Hall



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Edited by

Robert D. Hall

CPRO-DLO, Wageningen, The Netherlands

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I

Introduction

1

An Introduction to Plant-Cell Culture

Pointers to Success

Robert D. Hall

1. Introduction

With the continued expansion of in vitro technologies, plant-cell culture has become the general title for a very broad subject. Although in the beginning it was possible to culture plant cells either as established organs, such as roots, or as disorganized masses, it is now possible to culture plant cells in a variety of ways: individually (as single cells in microculture systems); collectively (as calluses or suspensions, on Petri dishes, in Erlenmeyer flasks, or in large-scale fermenters); or as organized units, whether this is shoots, roots, ovules, flowers, fruits, and so forth. In the case of *Arabidopsis,* it is even possible to culture complete plants for generations from seed germination to seed set without having to revert to an in vivo phase.

In its most general definition, plant-cell culture covers all aspects of the cultivation and maintenance of plant material in vitro. The cultures produced are being put to an ever-increasing variety of uses. Initially, cultures were used exclusively as experimental tools for fundamental studies on plant cell division, growth, differentiation, physiology, and biochemistry *(1)*. Such systems were seen as ways to reduce the degree of complexity associated with whole plants, providing additional exogenous control over endogenous processes, to enable more reliable conclusions to be made through simpler experimental designs. However, more recently, this technology has been increasingly exploited in a more applied context, and successes in a number of areas have resulted both in a major expansion in the number of people making use of these techniques and also in an enhancement of the degree of sophistication associated with in vitro technology. Techniques for micropropagation and the

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production of disease-free plant stocks have been defined and refined to such an extent that they have become standard practice for a range of (usually vegetatively propagated) horticultural and ornamental crop plants, such as gerberas, lilies, strawberries, ferns, and so on, thus creating what is now a multimillion dollar industry.

Nevertheless, the discipline within this technology that will eventually have the greatest impact on both fundamental and applied plant science is that of genetic modification of plant cells. Although this methodology is effectively still only in its infancy, it is now already possible, using a range of different techniques, to modify genetically virtually every plant species that has been tested so far, albeit with widely divergent degrees of efficiency *(2)* Without doubt, this technology provides us with the most powerful single tool with which to study all aspects of plant-cell physiology, metabolism, and development by allowing the molecular dissection of individual components of the (sub)cellular organization of plants. In addition, the application of genetic modification techniques has already enabled us to produce crop plants with altered phenotypes, concerning e.g., herbicide resistance, insect resistance, and yield parameters *(2)*. Many additional applications are at the experimental/pre-commercial stage.

In simple terms, plant-cell culture can be considered to involve three phases: first, the isolation of the plant (tissue) from its usual environment; second, the use of aseptic techniques to obtain clean material free of the usual bacterial, fungal, viral, and even algal contaminants, and third, the culture and maintenance of this material in vitro in a strictly controlled physical and chemical environment. The components of this environment are then in the hands of the researcher, who gains a considerable degree of external control over the subsequent fate of the plant material concerned. An extra, fourth phase may also be considered where recovery of whole plants for rooting and transfer to soil is the ultimate goal.

The success of this technology is to a great extent, dependent on abiding by a number of fundamental rules and following a number of basic protocols. For those who have no experience at all with in vitro technology, it is strongly recommended, prior to initiating a first research project, that some basic knowledge be gained by visiting a working lab, preferably one doing similar work to that which is planned. This will not only save time, but also will help to avoid many of the pitfalls that could arise. Researchers can then also make direct contact with an experienced scientist who may later act as mentor. To proceed, a straightforward, well-tested protocol can be used to become acquainted with the manipulations required to achieve a particular goal. Then, having gotten this protocol to work, the researcher can begin with the modifications needed to achieve the original goal. The aim of the rest of this chapter is to act as a refer-

ence giving some basic guidelines concerning how to initiate a research program based on in vitro technology for plant tissues. The remaining chapters in this book will then describe individual protocols for specific techniques in detail.

2. Materials

2.1. Plant Material

Probably the worst thing that any researcher can do when embarking on a new in vitro technique is to use material that is suboptimal. This not only means using the wrong species/variety/genotype, but also using the right material, but which has been grown under substandard conditions. Thus, choosing in vivo-grown material from plants that are diseased or too old, or have not been maintained in an active growth phase during their entire life should be avoided. With suboptimal material, problems can be encountered in obtaining sterile cultures, excessive variability in in vitro response can result, and at worst, a complete failure of the experiment may occur. For most applications, explants from very young plants will respond best. For this reason, in vitro germinated seedlings are a frequently favored choice. Seed is often also much more readily sterilized than softer plant tissues. This, therefore, maximizes the likelihood of obtaining explants that are not only healthy, but are also guaranteed to be free of undesirable contaminants. However, species producing small seed can give rise to problems in obtaining sufficient experimental material. Furthermore, seed from outbreeders can also be genetically heterogenous, entailing an undesired variation in in vitro response that otherwise might be avoided by using explants from a single, larger greenhouse-grown individual.

For specific applications, precise growth conditions may be essential, particularly with regard to the period directly before the plants are to be used. Similarly, even when plants are healthy and at the desired stage for use, it is often the case that only a specific part of these plants will give the best explants, e.g., a particular internode, the youngest fully expanded leaf, flower buds within a certain size range, and so forth. A good search of the literature and paying close attention to the recommendations of experienced researchers are always to be strongly recommended.

2.2. Equipment

A plant-cell culture laboratory does not differ greatly from most other botanical laboratories in terms of layout or equipment. However, the requirement for sterility dominates. Plant cell cultures require rich media, but are relatively slow-growing. This places them in great danger of being lost, within days, through the accidental introduction of contaminating microorganisms. Plant-

cell cultures also quickly exhaust their nutrient source, and therefore, sterile transfer to fresh media is a weekly to monthly requirement.

A cell-culture laboratory should be kept tidy, and dust-free with clean working surfaces. Some type of sterile culture transfer facility is essential. A laminar flow cabinet is preferable but a UV-sterilized transfer room or glove box, both of which are used solely for this purpose, and which are UV irradiated at all times when not in use, can also be employed effectively. Such facilities, when used for plant material, should never be used by colleagues for work on other organisms, such as yeast or *Escherichia coli.* It should also be held as a general rule that everything going into the sterile working area should already be sterile or, in the case of instruments, should be sterilized immediately on entry. This means also that in vivo grown plant material should only enter the transfer area after it has been submerged in the sterilizing solution.

The other equally important piece of equipment is the autoclave which is needed to sterilize glassware, media, and so forth. This should be of a size sufficient to cope with daily requirements. However, very large autoclaves should be avoided unless they are specifically designed for rapid heating and cooling before and after the high-pressure period to avoid long delays and also to prevent media being severely "cooked" as well as being autoclaved.

Although specialized techniques have specific equipment requirements (noted in the relevant chapters), in addition to the sterile transfer and autoclaving facilities, the following are generally needed to perform basic cell-culture procedures:

1. Tissue-culture-grade chemicals with appropriate storage space at room temperature, 4°C and –20°C.
2. Weighing and media preparation facilities: Balances to measure accurately mg to kg quantities should be available.
3. A range of sterilization facilities In addition to the autoclave, a hot-air sterilizing oven is useful. Sterile filters (0.22-µm) are required for sterilizing heat-labile compounds. If large volumes of sterile liquids are required, a peristaltic or vacuum pump is also to be strongly recommended
4. A source of (double) distilled water.
5. Stirring facilities that allow a number of different media to be made simultaneously
6. A reliable pH meter with solutions of HCl and KOH (0.01, 0 1, 1.0, and 10 *M*) to adjust the pH accurately
7. Culture vessels either of (preferably borosilicate) glass or disposable plastic, tubes, Erlenmeyer flasks, jars, and so on.
8. Plastic disposables, e.g. Petri dishes (9, 6, 3 cm), filter units, syringes, and so forth, as well as plastic bottles of various sizes for freezing media and stock solutions for long-term storage

9. Sealants, e.g., aluminium foil, Parafilm/Nescofilm, clingfilm/Saranwrap.
10. Basic glassware (measuring cylinders, volumetric flasks), dissection instruments, hot plate/stirrer, gas, water, and electricity supply, microscopes, and so forth.
11. Microwave: Although not essential, the ability to make solidified media in "bulk" and remelt it for pouring when required not only saves time, but also avoids the risk of undesired condensation building up in culture vessels (especially Petri dishes) on prolonged storage.

2.3. Washing Facilities

The importance of cleaning glassware in a tissue-culture laboratory should never be underestimated. Furthermore, incorrect rinsing is equally as bad as incorrect washing. Traces of detergent or old media can cause devastation the next time the glassware is used. If not to be washed immediately, all glassware should be rinsed directly after use and should not be allowed to dry out. Therefore, keep a small amount of water in each vessel until it is cleaned. Certain media components (e.g., phytohormones), which are only poorly soluble in water when dried onto the inside of a flask, may not be removed by the normal washing procedures, but can redissolve the next time the vessel is autoclaved and contaminate the medium. For this reason, flasks used to make or store concentrated stocks of medium components should not be used for any other purpose.

New automatic washing machines can be programmed to wash at temperatures approaching 100°C, rinse extensively with warm and then cold water, and finally demineralized water before even blow-drying! However, if such equipment is not available, washing by hand is equally as good, if a little time-consuming. In this case, glassware should be soaked overnight in a strong detergent before being thoroughly scrubbed with a suitable bottle brush and then rinsed two to three times under running tap water and finally at least once with demineralized water. All glassware should then be dried upside down before being stored in a dust-free cupboard until required. It is generally recommended that glassware be thoroughly washed in an acid bath on a regular basis.

2.4. Media

There is a small number of standard culture media that are widely used with or without additional organic and inorganic supplements (*see* Appendix; *3–7*). However, next to these, there is an almost unending list of media that have been reported to be appropriate for specific purposes *(8)*. Protoplast culture media, for example, can have a wide variation in composition, reflective of the often critical conditions required by these highly sensitive and fully exposed cells. However, even these are to a large extent derived from one of the standard recipes. Plant-culture media generally consist of several inorganic salts, a

(small) number of organic supplements (e.g., vitamins, phytohormones), and a carbon source. In addition to these standard components, the specific needs of particular species or tissues, or the precise conditions required to initiate a desired in vitro response dictate which additional supplements are required. Today, with the wealth of knowledge concerning a very divergent list of plant species that has been built up over the last 20 years and that is readily available in the literature, the choice of medium with which to begin for a particular plant should be made only after referring to previous publications on the same or related species.

It can be seen, from the standard media recipes listed in the Appendix, that the micro- and macroelements and organic supplements can vary considerably. The species to be used will generally determine which medium to choose and, of course, the aim of the experiment (e.g., callus production, plant regeneration, somatic embryogenesis, anther culture, and so on) will determine which additional supplements are required. This is especially so for the phytohormones, which can play an extremely important role in determing the response of plant cells/tissues in vitro. Indeed, in many cases, it is only the number, concentration, type, and balance of the phytohormones used that distinguishes one experimental design from another. Of the macrocomponents, the source of nitrogen (N) is often considered to be of particular influence. Most media have N in the form of both nitrate and ammonia, but the ratio of one to the other can vary enormously to the extremes that one of the two sources is absent. Alternatively, both sources can be omitted and replaced by organic N sources in the form of amino acids, as in the case of AA medium *(9)*. Although many media are composed as a fine balance to promote and maintain cell growth in vitro, temporary divergence from using the usual media components is often employed to direct growth and morphogenesis in particular directions. For example, by limiting or removing the N or phosphate source, secondary metabolite production can be stimulated, and through the qualitative and quantitative manipulation of the sugar supplement, organogenesis or embryogenesis may be induced.

Briefly, the importance of the different media components can be given as follows:

1 Inorganics
   a Macronutrients. Ca, K, Mg, N, P, and S are included in anion and/or cation form and are generally present at m*M* concentrations All are essential for sustained growth in vitro
   b. Micronutrients: B, Co, Cu, Fe, I, Mo, Mn and Zn are generally included at μ*M* concentrations Ni and Al may also be included, but the miniscule amounts required are possibly already present as contaminants in, e.g., agar.

2. Organics
   a. Vitamins: Generally, thiamine (vitamin B_1), pyridoxine (vitamin B_6), nicotinic acid (vitamin B_3) and myoinositol are included, but only thiamine is considered to be essential. The others have growth-enhancing properties. The concentrations of each can vary significantly between the different media compositions (*see* Appendix).
   b. Amino acids: Some cultured plant cells can synthesize all amino acids, none are considered essential. However, some media do contain certain amino acids for their growth-enhancing properties, e.g., glycine in MS media *(3)*. However, high concentrations of certain amino acids can prove toxic. Crude amino acid preparations (e.g., casamino acids; ***10***) can also be used (e.g., for protoplast culture), but their undefined nature makes them less popular.
   c. Carbon source: Generally, most plant-cell cultures are nonautotrophic and are therefore entirely dependent on an external source of carbon. In most cases, this is sucrose, but occasionally glucose (e.g., for cotton cultures) or maltose (e.g , for anther culture) is preferred.
   d. Phytohormones: The most commonly used phytohormones for plant-cell culture are the auxins and cytokinins. However, for specific applications with certain species, abscisic acid or gibberellic acid may be also used. Auxins induce/stimulate cell division in explants and can also stimulate root formation. Both natural (indole-3-acetic acid, IAA) and synthetic (e.g., indole-3-butyric acid, IBA; 1-naphthalene acetic acid, NAA; 2,4-dichlorophenoxyacetic acid, 2,4-D; *p*-chlorophenoxyacetic acid, pCPA) forms are used.

      Although the synthetic forms are relatively stable, IAA is considered to be rapidly inactivated by certain environmental factors (e g., light). In addition, auxin-like compounds, such as Dicamba and Picloram, can be used to the same effect. Cytokinins play an influential role in cell division, regeneration, and phytomorphogenesis, and are believed to be involved in tRNA and protein synthesis. Although the natural form, Zeatin (or Zeatin riboside) is available commercially and is widely used for certain applications, the synthetic cytokinins (benzyladenine, BA, or 6-benzylaminopurine, BAP; kinetin, K; and isopentyl adenine, 2-iP) are more generally used. Other compounds, such as Thidiazuron and phenylurea derivatives, also have cytokinin activity with the former, for example, gaining increasing popularity for woody species. Gibberellin (usually GA_3) is occasionally used to stimulate shoot elongation in cultures that contain meristems or stunted plantlets. Abscisic acid (ABA) is sporadically used, but its mode of action is unclear. In some cases, it is used for its inhibitory and, in some cases, for its stimulatory effect on cell-culture growth and development.

      Altering the qualitative and quantitative balance of the phytohormones included in a culture medium, and especially in relation to the auxin/cytokinin balance is one of the most powerful tools available to the researcher to direct in vitro response. In many cases, making the correct choice, right from culture initiation, is all-determining.

e. Others: In the past, a wide range of relatively indefinable supplements have been used for plant-cell culture ranging from protein hydrolysates to yeast extracts, fruit (e.g., banana) extracts, potato extracts, and coconut milk. However, the use of such components, through their unknown composition combined with our improved knowledge of cellular requirements in vitro, together with the increasing availability of components, such as zeatin, is now greatly reduced. Coconut milk, however, is still widely used for protoplast culture and is now commercially available.

3. Antibiotics: Both synthetic and naturally occurring antibiotics can be used for plant-cell culture. These play an essential role, for example, in eliminating *Agrobacterium* species after cocultivation in transformation experiments or in providing selection pressure for stably transformed cells. However, for standard practices, the use of antibiotics is usually avoided, since these can have unknown physiological effects on cell development. Low levels are nevertheless often used in the more risky/expensive large-scale operations, e.g., in fermenters and in micropropagation programs.
4. Gelling agent: It is becoming increasingly evident that not only the concentration, but also the type of agent used to make solid media influences the in vitro response of cultured plant tissue. Both natural products extracted from seaweeds (e.g., agar, agarose, and alginate) and their more recently emerged substitutes (e.g., Gelrite, Phytagel), obtained from microbial fermentation, can be used. Each has its advantages and disadvantages, and the choice is usually determined by the species and the application. Agars and agaroses generally produce gels that are stable for prolonged periods and are considered not to bind media components excessively. Products with various degrees of purity are available, and low-gelling temperature types can even enable the embedding of sensitive cells, such as protoplasts. On the other hand, Gelrite/phytagel produces a rigid gel at much lower concentrations than agar or agarose. They are also almost transparent, which makes it easier, e.g., to identify contamination at an early stage. These gels do, however, tend to liquify in long-term cultures owing to pH changes or the depletion of salts necessary for crosslinking. Higher concentrations of antibiotics (e.g., kanamycin) may also be required in Phytagel/Gelrite solidified media in comparison to those solidified with agar/agarose.

In most countries, the most commonly used media are now commercially available (e.g., from Sigma, Duchefa) at competitive prices, saving a lot of time and effort. Furthermore, when the exploratory work is completed and a specific modified medium has been designed for use, some companies (e.g., Duchefa, Haarlem, The Netherlands) will even make this medium to order.

2.5. Culture Facilities

It is to be strongly recommended that plant-cell cultures be incubated under strictly controlled and defined environmental conditions. Although certain cultures (e.g., shoot cultures) will have a set of optimum conditions for growth, they may continue to survive and grow under other, suboptimal conditions.

Other cultures, however, e.g., protoplast or microspore cultures require very precise treatments. Deviations from this, by 1–2° in temperature can mean complete experimental failure. Facilities are therefore required that allow good and reliable regulation of light quality and intensity, photoperiod, temperature (accuracy to ±1°C), air circulation, and in certain countries, humidity. The space available should also be sufficient to allow the execution of experiments under uniform conditions. The choice of facility is often difficult. Several small incubators give flexibility, but generally increase variability in culture conditions and can also prove expensive. A large walk-in growth room in which can be placed not only shelves, but also rotary shakers, bioreactors, and so on, reduces flexibility, but is generally more economical. The extra equipment then no longer needs expensive stand-alone, controlled environment units. However, the failure (through an electrical fault, power cut, and so forth) of such a large growth room could be disastrous, and therefore, safety features should always be included, so that technical personnel can immediately be warned, 24 h/d, when the environmental conditions seriously deviate from the chosen settings.

In incubators without lighting, obtaining uniform conditions is realtively easy. However, when light is introduced into a culture room, variation almost inevitably arises. Not every culture vessel can be placed at an equal distance from the light source. Limited space also often necessitates piling Petri dishes two or three deep. Furthermore, even with the best air circulation, local temperature differences at culture/shelf level can be significant. Although little can be done about this, it is certainly important to be aware of these inequalities. Consequently, it is recommended to carry out related experiments in the same place in the culture room if at all possible. The most uniform provision of light in a culture room is through fluorescent tubes placed above the shelves. However, since space usually has to be used efficiently, shelves are usually stacked above each other. This often results in significant localized increases in temperature on the upper shelves. This is not only undesirable, but also can result in the frequently occurring problem of Petri dish condensation. This can be so extreme that the explants end up sitting in a pool of liquid, which can prove highly detrimental to culture development/survival. Insulating materials placed above the lights or channeled air flows along shelves can help to some extent, but the latter may increase the risk of contamination. The problem is immediately solved if the lights are placed vertically on the walls behind the shelving, but this entails the disadvantage of a significant variation in light intensity across each shelf. The importance of these different factors to the plant material to be used and the nature of the work to be done determines which type of facility should be chosen and how it should be organized.

3. Methods

3.1. Sterilization of Equipment

1 Transfer facilities: On installation, transfer areas (laminar flow cabinets, inoculation rooms, glove boxes) should be thoroughly decontaminated using a suitable disinfectant and, then, if the material allows, 70% ethanol (**Note:** any object made of perspex should never be exposed, however brief, to alcohol, since it will become brittle and crazed). New flow cabinets should be left running overnight to clean the filters thoroughly before being brought into circulation Once in use, it should become standard practice for every user to spray down the transfer area with 70% alcohol both before and after use Furthermore, for transfer rooms and glove boxes, which are sterilized by UV light, an exposure of at least 15 min between each user is required to ensure complete decontamination.

2 Glassware Before sterilizing open glassware (e g., beakers, Erlenmeyer flasks, and so on), these should be capped with a double layer of aluminum foil to ensure that sterility is maintained after treatment Glassware with screw caps should always have these loosened half a turn before treatment to prevent high pressures building up, which can lead to the vessel exploding. Glassware can routinely be autoclaved at 121°C at a pressure of 15 psi for 15 min Alternatively, dry heat can be used at 160°C for 3 h The latter should, however, be avoided when plastic caps are used (e g., for closing culture tubes), since these cannot withstand the prolonged high temperatures Dry heat sterilization is also to be recommended for glassware destined for use with protoplast media The osmolality of these media is often very critical, and even small amounts of condensation, which can result from autoclaving, can prove detrimental

3 Instruments: We routinely flame the lower parts of instruments (e g., scalpels, forceps, and so on) in the laminar flow cabinet directly before use These are then always allowed to cool before bringing into contact with plant tissue. Between manipulations, the instruments are stored with their working surfaces submerged in 70% ethanol in a glass vessel (e g., a 100-mL measuring cylinder or beaker) kept in the transfer area for this purpose. The alcohol is replaced at least once a day. Instruments and other metal objects can also be sterilized using dry heat after first wrapping them in aluminum foil or heavy brown paper. Autoclaving is to be avoided, since the combination of elevated temperatures and steam quickly leads to corrosion

4 Heat-labile components. Certain plastics (e g., PVC, polystyrene) and other materials may not tolerate the high temperatures generally required for sterilization If it is not known what material a component is made of or if it is unclear whether a known material is autoclavable, it is always unwise to gamble Check with a single item first if possible Otherwise, use the alternative of a chemical method (e g., immersion for several minutes in 70% ethanol or in one of the solutions listed below for plant material) or UV radiation However, the latter is only suitable if the UV rays can penetrate to all surfaces of the object concerned

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