CONIFERS: CULTURE AND GENETIC ENGINEERING

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INTRODUCTION

Plant tissue and cell culture techniques have improved greatly over the last few decades. Consequently, for many herbaceous species, industrial application has become possible, primarily as a means to achieve large-scale clonal propagation. Except in a few cases, where conifers have been mass propagated clonally by rooting of stem cuttings obtained from seedlings (1), most large-scale planting of conifers is still carried out with sexually produced seedlings. The main reason that clonal propagation of conifers has only found limited application is the fact that cloning of older specimens that have proven their quality is generally not possible using traditional rooting-cuttings techniques. Therefore, sexual reproduction still provides the bulk of the planting material for reforestation. However, recent advances in *in vitro* technology have changed the outlook for clonal propagation of conifers. In particular, the development of the technology to clone zygotic embryos *in vitro* promises, as explained later, great gain in the genetic quality of planting stock, and industrial application of this technology has already started (2,3). The benefits and drawbacks of cloning have been outlined by Park *et al*. (4).

BASIC TECHNIQUES

Basal Media

General Considerations. The nutrient medium is a key element in cell and tissue culture. However, media design is difficult because of the many complex interactions of nutrients in solution (5) and, consequently, media are often still not fully optimized. This is particularly the case for nutrient media used for conifers, and, therefore, many conifer species are still difficult to maintain long term *in vitro*. The media developed to date are often narrow in their applicability. Many media work well only for a limited number of species and genotypes because cultured cells and tissues can vary greatly in their nutritional and growth regulator requirements. Furthermore, nutritional demands generally change during development. Callus growth often needs higher mineral concentrations than shoot or embryo initiation, whereas conifer somatic embryo initiation, proliferation, maturation, and germination each need a different nutrient environment to proceed properly. Clearly, nutrient media have to be optimized for species, genotype within species, and each developmental stage during the culture process. Basal media and their various modifications have been described in detail for all plant tissue cultures (6) and for tree species specifically (5,7), and much of the nonreferenced information below has been taken from these sources.

The most popular media for conifers are Murashige and Skoog (MS), Litvay *et al*. (LV), Schenk and Hildebrandt (SH), Greshof and Doy (GD), von Arnold and Erickson (LP), and Gupta and Durzan (DCR) (6). These media differ greatly from each other: MS and LV are high ionic-strength media (95.8 and 104.2 mM, respectively). Strength of the medium can have a considerable effect on culture behavior. For example, in cultures of *Pinus ponderosa* cotyledons, high salt media promoted callus growth, whereas low salt media stimulated adventitious shoot formation (8). A medium for conifers developed by Teasdale (9) is low in potassium and ammonium and high in phosphorus, and contains iron in molar excess over the chelating agent. This medium, besides promoting *in vitro* growth in general, stimulates root formation. Smith (10) used a medium low in calcium but high in sodium, copper, and zinc. An interesting effect of this medium is that, in a variety of species tested (*Pinus radiata*, *Pinus taeda*, *Pinus elliotii*, and *Pseudotsuga menziessii*), it allows initiation and maintenance of somatic embryogenesis (SE) in the absence of normally required growth regulators such as auxins and cytokinins. This is important because some growth regulators, such as the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) can persist in the tissue long after its presence is required (11).

Major Components of Basal Media.

Nitrogen. In most tissue culture media, nitrogen is largely provided in the form of nitrate. Other sources are ammonium salt, amino acids, and complex products such as casein hydrolysate. It is generally recognized that a proper balance of nitrate and ammonium is important in stimulating morphogenesis and embryogenesis. However, ammonium requires careful scrutiny because it can easily become toxic. For some conifer species, *in vitro* development progressed properly on medium that contained glutamine in place of ammonium. Others, in

Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology, edited by Michael C. Flickinger

contrast, showed little growth on media devoid of ammonium. Ammonium disappears rapidly from culture media, lowering the pH of the medium in the process. It is difficult to buffer against this.

Nitrogen is also supplied as amino acids. Sometimes several are used but generally only one is selected, most frequently glutamine and less often arginine or asparagine. Risser and White (12) found that glutamine alone was as effective in *Picea glauca* callus cultures as a mixture of 18 amino acids. Khlifi and Tremblay (13) demonstrated that glutamine on its own, in the absence of inorganic nitrogen, is sufficient for maturation of *Picea mariana* somatic embryos. Unfortunately, glutamine is chemically unstable and cannot be autoclaved. It degrades rapidly once incorporated in the medium, even if kept refrigerated (14). Glutamine was less effective in *P. glauca* somatic embryo proliferation than casein hydrolysate (15). For some conifer species, casein hydrolysate is beneficial only when inorganic nitrogen is present at suboptimal levels in the medium.

Calcium, Magnesium, and Boron. The calcium concentration in media is often low because of its poor solubility in water. Its availability is even further reduced if gellan gum is used as the gelling agent, because bivalent ions like calcium and magnesium are needed to solidify this compound. Calcium deficiency is, therefore, common *in vitro*, the most frequently observed symptom of it being shoot tip necrosis (16). Low levels of calcium are not always deleterious. Half-strength LV medium, which even at full strength is very low in calcium, supported somatic embryo initiation and maturation in, among others, *Picea* spp. (13,17,18), *Larix* spp. (19), and *Pinus* spp. (19,20). The LV medium is high in boron and magnesium, which, in part, compensates for the low calcium level. There is a strong interaction between calcium, boron, and magnesium in cell suspension cultures of *P. radiata*, which indicates that there is an acceptor molecule that binds both calcium and boron. Furthermore, magnesium competitively displaces calcium on this binding site (21).

Potassium and Phosphate. Potassium is the most abundant cation in cells. It is involved in osmotic control, glycolysis, and photosynthesis, and regulation of cytoplasmic pH. However, an oversupply of potassium can inhibit root growth. Increasing the phosphate level to a level higher than that in MS sometimes stimulates conifer shoot formation and elongation. Phosphate is removed from the medium rapidly and, therefore, deficiencies can quickly arise.

Microelements. Few studies have been carried out to determine microelement requirements. Microelement needs are difficult to determine precisely. Microelements can leach into the medium from the glass of the culture vessels and are often present in low concentration in the water used in media preparation. They also occur in substantial amounts if agar is used to solidify the medium. Microelements interact in a complex manner among themselves and with other nutrients.

Iron is generally used with the chelating agent sodium ethylenediaminetetraacetic acid (EDTA). However, EDTA can be toxic and thus should be used with caution. In *P. radiata* suspension cultures, NaFeEDTA was optimal at a concentration well below that is used for other species (22). Excess EDTA can complex zinc and thus cause zinc deficiency.

Manganese occurs in high concentration in some conifer culture media. Its uptake in conifer tissues is inhibited by copper, whereas manganese itself inhibits iron uptake. Tissues grown on agar are rarely deficient in copper because agar contains high levels of that element. *P. taeda* and *P. radiata* cell suspension cultures required little copper, presumably because photosynthesis and lignin biosynthesis are inactive in these cultures (22).

Vitamins. Most culture media contain the vitamins added to MS medium. These are niacin, pyridoxine, thiamine, and myoinositol, all of which are relatively heat stable and thus autoclavable. Other vitamins are not essential in most conifer cultures.

Growth Regulators. Of the several classes of growth regulators, the most commonly used ones are auxins, cytokinins, and abscisic acid (ABA). These are all involved in the various phases of adventitious shoot development and embryogenesis. Among auxins commonly used in conifer tissue cultures there are the stable synthetic ones, such as 2,4-D, naphthaleneacetic acid (NAA) or indolebutyric acid (IBA). Auxins are active in cell division and elongation. The most common cytokinins are benzylaminopurine (BA) and kinetin (K). Together with the auxins, they control meristem formation. The growth inhibitor ABA is used primarily for developmental regulation in somatic embryos to bring about morphological and physiological maturation. The plant growth regulator requirements for the initiation of SE are different for several *Pinus* species (23).

Carbohydrates and Osmotica. The most common carbohydrate used is sucrose, which is easily absorbed and metabolized by cells. Carbohydrates have many functions in tissue culture. At low concentration, they serve as the main energy source for the growing tissues. At higher concentrations, they control water uptake into cells. Different stages of development, for example, SE, have different water requirements. This is primarily controlled by adjusting the osmotic water potential of the media with carbohydrate. Other means of controlling water availability to the cells are by adjusting the concentration of gelling agent (20) or by adding metabolically inactive osmotica such as polyethylene glycol (24).

Plant Material

Conifer cultures are generally initiated from immature or mature zygotic embryos. Much less commonly used are cotyledons of germinating embryos or primordial shoots excised from seedlings or trees. The disadvantage of using embryos or cotyledons is that, at this early stage of development, we do not yet know what characteristics the tree

will have at later stages in its life cycle. This generally cannot be assessed properly until the tree has reached about half of its rotation age. Unfortunately, a large-scale practical technology to micropropagate conifers at that age does not yet exist. Therefore, practically all of the current applications are with juvenile, as yet untested conifer material.

Surface Disinfection, Excision of Explants, and Culture

Explants, free of microorganisms, are easily obtained if they are enclosed by protective layers such as bud scales or seed coats. These surrounding tissues can be harshly disinfected without damage to the explant. The outer layers can then be removed aseptically from the explant. Far more difficult to disinfect are tender new shoots and roots. A problem with excision is wounding and the resulting production of toxic phenolics by oxidation. This damage can be alleviated by use of antioxidants during excision or if excision is carried out under water.

Incubation Environment

Most conifer cultures are kept at constant temperature between 20 and 25°C. As there appears to be little advantage to varying the temperature according to diurnal patterns, this is rarely done. It has recently been shown that maturation of somatic embryos of *P. glauca* x *engelmanni* at lower than usual temperatures strongly alters the cold tolerance of the embryos (25). For rooting, a lower temperature (17–20°C) is sometimes recommended. Light requirements vary with culture type. In most cases, somatic embryos are initiated and partially matured in darkness. Once cotyledons develop, 16/24 h of low intensity (approximately 50 μmol m^{-2} s^{-1}) fluorescent light is generally applied. Photosynthesis at these intensities is minimal and an easily absorbed and metabolized carbohydrate in the medium is, therefore, required.

CLONAL PROPAGATION

Organogenesis and Embryogenesis

In earlier years, most efforts were focused on propagation by first inducing adventitious shoot formation, primarily from cotyledons, and then roots by a process called *organogenesis*. Success on a commercial scale has so far been limited to a few species, most notably *P. radiata*. A more effective method, developed over the last two decades, is SE, which differs from organogenesis in that the propagules are embryos rather than rooted shoots. SE cultures are initiated from immature or mature zygotic embryo explants on medium high in auxin, most commonly 2,4-D. This initiates a cell mass composed of immature embryos that grows rapidly by cleaving of the embryos for as long as the auxin is applied. Over the course of a number of subcultures, hundreds of immature embryos are produced per gram of fresh mass tissue. Initiation rates are generally highest if zygotic embryos excised from immature seed are used as explants (23,26). Cleavage is arrested and maturation follows when immature embryos are transferred to medium without auxin but containing higher concentrations of osmotica and the growth regulator ABA (24). Treatments that improve physiological maturation of the embryos are increased gelling agent concentration (20) and desiccation of the embryos before germination (24). Germination and transfer to soil generally proceed without difficulty.

Strategies for Applying SE in Industry

SE has worked well for many *Picea* and *Larix* species but less well for *Pinus* and *Abies*. Outside of the Pinaceae, there are only a few species that responded. However, the ease with which SE could be mastered in some species has led several forest industries to employ a genetic improvement strategy that involves a combination of breeding, SE, and cryopreservation (26). SE is initiated from zygotic embryos excised from seed of superior families created by breeding. Once in the proliferation phase, part of each SE mass is used for the production of clonal plants, which are then field tested. The remainder of each SE mass is transferred to liquid nitrogen for long-term storage (cryopreservation). Once the field tests show which are the best clones, the corresponding cryopreserved SE masses are thawed and used for production of clonal plants. High production can be achieved either by further SE or by producing a few plants by SE, which are subsequently mass cloned by rooting of cuttings (4). This latter scenario is preferred when SE lines do not produce mature embryos in sufficient numbers to be of practical use. The advantage of the breeding–SE–cryopreservation strategy is as follows. Breeding creates superior families, but does not allow selection within families. SE and cryopreservation can capture the best of this largely nonadditive within-family variation, resulting in considerable genetic gain. In a combined breeding–SE experiment with *P. glauca* and *P. taeda*, it was found that initiation of SE is under strong additive genetic control (17,27). In *Picea abies*, this was less the case (28). For *Picea* and species of other conifer genera, where SE initiation is under strong additive genetic control, one could routinely include one parent with a high capacity for SE in each sexual cross, thus obtaining seed families that are all responsive to SE.

Clonal Propagation of Mature Conifer Trees

As already indicated, the combination of induction of SE in zygotic embryos, cryopreservation, and field testing is a powerful tool in improving the genetic makeup of planting material. However, the fact that it takes many years of field testing before the long-term value of the clones can be assessed is a drawback. This delay in clone choice could be avoided if the technology existed for true-to-type cloning of selected, superior individuals found in field populations. Unfortunately, despite extensive efforts, the initiation of SE in tissues excised from mature conifer trees has so far, with a few exceptions (29), failed. This failure includes attempts at inducing SE in cultures derived from nucellar tissue from ovules, a procedure that has been effective with nucellar tissues of several angiosperm tree species. Cultures derived from conifer nucellar tissue are

fast growing, but do not respond to treatments that normally induce SE. Rejuvenation does seem to occur when micrografting techniques are applied (30,31), and perhaps an integrated approach of micrografting and induction could be exploited.

Haploid Culture and Somatic Hybridization

For some agricultural species, especially cereal crops, haploid culture, derived from pollen, followed by diploidization and regeneration of plants, has been a powerful tool in genetic improvement. The plants thus generated are homozygous diploid, ideal material for controlled hybridization and, potentially, capture of hybrid vigor. Obtaining haploid tissue from conifer pollen has been proved to be very difficult. However, regeneration of adventitious embryos from tissues derived from haploid megagametophytes, rather than from pollen, has been accomplished with *Larix*. Another process that could lead to genetically improved planting stock is by fusion of haploid protoplasts obtained from two different parents. This process is called *somatic hybridization* and has been used to create hybrids between parents that cannot be sexually crossed. Sexual barriers are thus bypassed and novel genotypes are created. This has been effective for some nonconiferous tree species, most notably *Citrus* spp. The first step in the process, regeneration of conifer embryos or plants from haploid and diploid protoplasts, has been achieved (32,33).

Genetic Engineering

Genetic engineering has become common practice in agricultural crops. In conifers, however, the technology is still mostly experimental. Conifers present a number of daunting problems (34–37). Because of the longevity of trees, foreign genes that have been introduced at conception *in vitro* have to remain active for many years to be of value. Foreign gene expression has been achieved for conifers (35) and is being field tested for long-term stability (37–39). A concern with transgenic forest tree species is that, even if they belong to a population that has been bred for several generations, they will still be close to their wild-type relatives, with which they can easily breed. Containing the transgenic genes within the transformed population is possible only if the trees are male and female sterile, if a buffer zone free of trees with which they can interbreed is created, or if the transgenic trees are removed before they reach sexual maturity. Most satisfactory would be transgenic trees that are sexually sterile. Therefore, the development of trees with novel transgenic traits that are at the same time sexually sterile is currently a main focus of genetic transformation research in several tree species, including conifers (36,37). A potential additional benefit of creating sexually sterile trees is that this may divert energy from seed and pollen formation into vegetative development, thus accelerating tree growth (34). Other major areas of genetic transformation research of trees are the introduction of genes that will modify lignin content and composition, reduce the juvenile growth period, change growth habit, improve the rooting capacity, remove toxins from the environment (phytoremediation), and provide resistance to herbicides, insects, or diseases (35–37). Much work has been carried out in recent years on genetic transformation of poplar, in which many physiological traits have been studied. Included in these traits are, among others, glutamine metabolism, lignin, and cellulose biosynthesis; plant growth regulator signaling; wood development; flowering; and plant–pathogen and plant–pest interactions (40,41). Together with large-scale genomics projects on poplar and spruce, the knowledge base of genetic control in transgenic trees is rapidly developing, affecting future directions of tree biotechnology.

Because unrestricted use of transgenic trees could cause severe environmental problems, many countries have instituted strict rules for commercial deployment of transgenic trees. In North America, it is mandatory that promising transgenic material is evaluated in field trials conducted under conditions that will confine the test plants and limit any environmental impact before it will be approved for commercial use, again under strictly controlled conditions (42). In Europe, transgenic trees may only be released into the environment for research purposes or commercial use in conformity with Directive 2001/18/EC of the European Parliament. This Directive provides detailed guidance on the objectives, elements, general principles and methodology of environmental risk assessment, and details of appropriate safety and emergency response measures before approval is granted (43). The first significant results of the confined field trial in Canada of 5-year-old transgenic white spruce expressing the synthetic version of *Bacillus thuringensis* endotoxin gene against spruce budworm suggested that certain trees were lethal to the feeding larvae (44).

CONCLUSION

Breeding, followed by SE, cryopreservation of SE clones, and subsequent mass propagation of selected cryopreserved clones, either by SE or by SE followed by rooting of cuttings, is an attractive means of achieving genetic improvement of conifer planting stock. Unfortunately, the use of SE as a mass production device has so far been limited because it is labor intensive. However, somatic embryo production in bioreactors, and the mechanical sowing of the desiccated, mature embryos thus produced, has recently been achieved. This promises that mass cloning of conifers by SE on an industrial scale will soon be a reality (3,45). By combining improved SE methods with the rapid progress that is being made in genetic transformation, we can look forward to interesting new genotypes becoming available for future large-scale planting.

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