TISSUE CULTURE OF WOODY PLANTS AND ITS RELEVANCE TO MOLECULAR BIOLOGY

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Chapter Contents

1. Introduction
2. Organogenesis
3. Somatic embryogenesis
4. Optimization of regeneration
4.1. Biological factors
4.2. Growth regulators
4.3. Macronutrients and micronutrients
4.4. Carbohydrates
4.5. pH
4.6. Physical environment
5. Genetic transformation studies
6. Other applications of tree tissue cultures
6.1. Stress studies
6.2. Secondary metabolite production
6.3. Cryopreservation
6.4. Forest pathology and pest control
6.5. Programmed cell death
6.6. Metabolic and biochemical studies
6.7. Habituation
7. Conclusions and prospectives
8. Acknowledgments
9. References

1. Introduction

The ever increasing demand for forest products and the progressive deterioration of natural forests means that the forest industry cannot continue to rely on the exploitation of natural forests (Jain, 1997; Tzfira et al., 1998). To meet the increasing demand for forest products while more forest land is needed for non-timber uses, the replacement of natural populations of trees with cultivated varieties is absolutely crucial. At present, short-rotation intensive culture of hardwoods appears to augment this need very well. However, there will be continuous pressure on existing forest lands because of limited expansion of arable land, rapid growth of human population, improvement of economic status, environmental degradation, deforestation, global warming, and water shortages (Jain, 1998a). Conventional methods of tree improvement and selection offer limited

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possibilities of meeting the rapidly growing demands of the industry and for reforestation programs. Innovative and cost-effective technologies are needed to develop new hybrids, to select and test desirable genotypes, to rapidly propagate selected genotypes, and to produce genetically modified varieties of commercially important trees (Jain, 1998b).

Methods for large scale monitoring of populations for desirable quantitative traits and the maintenance of clonal germplasm for long-term evaluation of planted material have to be developed. Molecular techniques of gene manipulation (i.e., genetic engineering) and analysis of genetic variation among plant populations (molecular markers) in combination with the techniques of cell and tissue culture, provide us the necessary tools to achieve this. In addition, a better understanding of the regulation of gene activity and plant metabolism should lead to the development of genetically improved varieties that can be clonally propagated to meet the need for plantation stocks.

Research in conventional propagation and breeding has made tree growers more aware of their inability to vegetatively mass propagate many tree species. At the same time, failure to produce a number of desirable inter-specific and inter-generic hybrids due to incompatibility and the long life span of trees has raised interest in the techniques of genetic engineering. Cell and tissue culture techniques have a major role to play in rapid multiplication of elite tree genotypes for large-scale plant production in a short period of time, and are also crucial for genetic engineering of forest and woody plants (Jain, 1998a,b). This technology, although highly labor intensive, is uniquely beneficial to developing countries, where labor is relatively cheap (Govil and Gupta, 1997; Jain, 1998b). In addition to mass propagation of superior genotypes, tissue culture techniques provide the following advantages: (1) isolation of cells, tissues, and organs from the parent organism for cellular and molecular studies as isolated biological units; (2) providing a continuous source for the production of secondary metabolites of economic importance; (3) providing starting material for production of synthetic seeds for low seed yielding species; (4) cloning of hardier varieties which are resistant to environmental and biotic stresses; (5) conservation of germplasm of rare or endangered species; (6) propagation of rare ornamental varieties, such as the highly colored blue spruce; and (7) analysis of gene expression and its role in development

The concept of clonal forestry is increasingly gaining recognition for rapid forest improvement and increased forest productivity (Hasnain et. al., 1986; Smith, 1997). The application of clonal forestry relies on the ability to propagate planting stock of superior quality trees by vegetative methods. The most common vegetative propagation method for woody plants is the rooting of stem cuttings, often referred to as 'macropropagation'. The tissue culture method uses smaller tissue explants (generally from juvenile tissues, e.g. embryos and cotyledons or from mature tissues, such as shoot meristems and needle fascicles) and is referred to as 'micropropagation'. Micropropagation systems based upon the production of multiple shoots directly from explants allow one to maintain clonal material in a juvenile state. A modification of this approach is the production of undifferentiated callus masses from which either roots or shoots can be regenerated *in vitro*. Given the proper stimulus, almost any part of a plant can be induced to form callus, and it is often possible to regenerate roots and shoots from this callus. This method of vegetative propagation is often termed as organogenesis. It is also possible to initiate embryo formation from this callus (somatic embryogenesis) which can lead to production of plantlets. Protoplasts can be produced from callus or intact organs for somatic hybridization and genetic transformation (see Jain et al., 1995a). In addition, haploid and triploid plants can also be produced in tissue culture (Jain et al., 1996a,b,c, 1997b,c).

The techniques of *in vitro* cell and tissue culture of plants form the basis of the above technologies. This chapter is intended to provide an overview of the current status

of cell and tissue culture of forest trees and its relevance to the use of molecular techniques in tree improvement. The chapter is divided into three sections. The first section briefly covers the current status of regeneration (organogenesis and somatic embryogenesis) in cell cultures and its regulation via manipulation of growth media, physical and chemical environment, and the role of intrinsic factors. The second section deals with the use of cell and tissue cultures in genetic transformation studies. The third section outlines various other applications of cell and tissue culture techniques and their role in providing useful knowledge to improve the production of commercial products. The chapter is not meant to be an exhaustive review of any of these areas; each has been extensively reviewed elsewhere (Bajaj, 1995a,c; Jain et al., 1995a,b,c; 1997b,c; 1999a,b). We will only highlight some of the relevant information on this topic.

2. Organogenesis

Organogenesis here refers to the *in vitro* formation and development of organs such as roots and shoots either directly from the explants or via callus differentiation. Trigiano et al., (1992) proposed that organogenesis proceeds *de novo* via organization of meristems from unorganized cell masses through patterned cell divisions. Direct multiple shoot formation or somatic embryo regeneration from an explant is often called micropropagation. Micropropagation is being commercially used for large-scale plant production in agronomic crops (Redenbaugh, 1991), as well as ornamental, fruit and forest trees (Smith and Drew, 1990; Akita et al., 1994). The regeneration of shoots from callus can result in somaclonal variation, which depends on genotype, age and type of the callus, culture medium, type of plant growth regulators used, and number of subcultures (see Jain et al., 1998). Somaclonal variation, while being undesirable for clonal propagation, is ideal for creating genetic variability which can be used for improvement of plants (Karp, 1995). When *in vitro* culture techniques are combined with induced mutagenesis and somaclonal variation, breeding programs can be speeded up (Maluszynski et al., 1995; Jain et al., 1998; Jain, 1998c). However, it is important to identify genetically stable somaclones before their incorporation into breeding programs, otherwise selected somaclones with desirable traits may revert back to wild type due to epigenetic phenomena (seeJain et al., 1998).

In plant tissue culture, a variety of explants such as cotyledons, shoot tips, hypocotyls, anthers, internodes, leaf disks, roots, stem and thin cell layers have been used for callus induction on different culture media containing various combinations of plant growth regulators. *In vitro* propagation via organogenesis usually involves four stages including initiation of cultures, multiplication of shoots, rooting of shoots, and acclimatization of plants (Trigiano et al., 1992). Many woody species respond to various modifications of MS medium (Murashige and Skoog, 1962), but some gain from lower salt based on B5 media (Gamborg et al., 1968) or WPM (Lloyd and McCown, 1980). Direct shoot initiation is usually limited to explants obtained from juvenile material; however, several reports have indicated shoot organogenesis from mature tree explants as well (Trigiano et al., 1992). For rooting of regenerated shoots, most species require an auxin treatment or simply growing the shoots under 100% relative humidity in the greenhouse with or without auxin treatment. For recalcitrant species, infection with *Agrobacterium rhizogenes* can also be helpful (Patena et al., 1988; Mariotti et al., 1991; Rugini et al., 1991; Aronen et al., 1996). *In vitro*-grown plant tissues may accumulate large quantities of ethylene in the culture vessels, particularly from rapidly growing non-differentiated callus or suspension cultures. Exogenous ethylene also influences growth

and development in such systems (Biddington, 1992; Gasper et al., 1996). In tissue culture the role of ethylene is quite variable; it may act either as a promoter or inhibitor of regeneration depending on the species (Gasper et al., 1996).

The phenolic exudations into the medium by explants derived from mature trees can often cause difficulties in establishing *in vitro* cultures and inhibit proliferation of axillary buds (Trigiano et al., 1992). The most common approach to prevent this is frequent subculturing of explants. Davis and Keathley (1989) made transfers of *Robinia pseudoacacia* mature explants every 4-7 days to minimize accumulation of inhibitory compounds in the medium. Similar approaches were taken in tea (Jain et al., 1993) and rose tissue culture (Jain, 1978). *In vitro* regenerated tea shoots did not leach phenolic compounds into medium probably because of their juvenile nature (Jain et al., 1993). Antioxidants, e.g. polyvinylpyrrolidone (PVP) or ascorbic acid have also been used to prevent browning of the medium (Trigiano et al., 1992; Khattar and Mohan Ram, 1982). However, antioxidants did not prove advantageous in *Prosopis tamarugo* cultures (Jordan, 1987).

Cost-effective micropropagation systems for woody plants are essential for commercial production of plantlets. A widespread commercial use of this technique for tree species is still restricted due to the high cost of production of plants. Some of the reasons for high cost of micropropagation include:, (1) long periods required for each culture stage; (2) low multiplication rates; (3) loss of plantlets due to contamination, and physiological and morphological disorders during the multiplication stage; (4) relatively high percentage of death of plantlets as a result of environmental stresses during acclimatization; (5) significant costs of infrastructure, chemicals and energy; (6) large space requirement; and (7) over- or under-production of plantlets due to unpredictable circumstances (Kozai, 1991). Sugar in the medium is essential as a carbon source, and is partially responsible for increasing the cost of plant production because extra precautions have to be taken for preventing contamination. Chlorophyllous shoot explants and plantlets produced *in vitro* may possess high photosynthetic ability, but their photosynthetic activity is restricted, largely by low carbon dioxide concentration in the vessel during the photoperiod, and in part by the presence of sugar in the medium (Kozai, 1988). Kozai (1991) has described some of the advantages of photo-autotrophic micropropagation which include: (1) the growth and development of plantlets are promoted under high carbon dioxide and high photosynthetic photon flux (PPF) conditions; (2) physiological and morphological disorders can be reduced and relatively uniform growth and development can occur; (3) biological contamination can be minimized thus reducing the loss of plantlets; (4) larger culture vessels can be used leading to robotization and automation of micropropagation; (5) rooting and acclimatization *in vitro* and *ex vitro* or both can be achieved easily; (6) environmental control in the culture vessels is possible; and (7) the use of growth regulators and other organic addenda can be reduced.

3. Somatic Embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells without going through a sexual cycle. The process is developmentally similar to zygotic embryogenesis, except that it occurs in the artificial environment of a culture vessel. The process was first described in *carrot (Daucus carota)*. Sandal wood *(Santalum album)* was the first angiospermic tree species to show somatic embryogenesis while the first gymnosperm species to show this developmental pattern was *Norway spruce (Picea abies)*. Cloning of trees through somatic embryogenesis is an important technology for commercial mass

propagation of improved genotypes (Gupta et al., 1993; Sutton and Polonenko, 1999). The topic of somatic embryogenesis in conifers as well as angiosperms has been extensively reviewed in recent years (Bajaj, 1995a; Klimazewska et al., 1997; Rival et al., 1997; Jain, 1998a,b; Jain et al., 1995a,b,c; 1999a,b). The formation of somatic embryos from cultured cells is a prime example of the process of controlled development and differentiation in plants. Cultures capable of producing somatic embryos with high frequency provide an ideal experimental system where hormonal regulation of development can be studied and its mechanism of action can be elucidated both at the molecular and biochemical levels. In addition to providing uniform stocks of selected genotypes for direct use in planting (synthetic seed technology), these cultures are valuable tools for testing: (a) the resistance of clonal material to pathogens and environmental stress; (b) virus elimination; (c) metabolite production; and d) *in vitro* mycorrhizal initiation (Vicient and Martinez, 1998).

Whereas zygotic embryos develop under a highly restrictive environment of the maternal tissue, somatic embryo development can be affected dramatically by changes in the physical and chemical environment of the medium (Goldberg et al., 1989; Carman, 1990). Unlike the embryogenic cultures of herbaceous angiosperms (e.g. carrot, celery), where it is difficult to visually recognize the embryogenic cells, embryogenic tissues in conifers are characterized by the presence of long suspensor-like proliferative cell masses. These cell masses continue to produce early-stage somatic embryos that must undergo 'maturation' under a different set of chemical and physical environment. Somatic embryo development in conifers often has to be stimulated by exogenous abscisic acid (ABA) (Hakman and Fowke 1987a,b; Nagmani et al., 1987; Hakman and von Arnold, 1988; Boulay et al., 1988; Harry and Thorpe, 1991). This is generally not the situation with angiosperm somatic embryo cultures (Bajaj, 1995a; Jain et al., 1995b).

Zygotic embryos undergo dessication during the development of seeds (Senaratna et al., 1990). For *in vitro*-produced conifer somatic embryos, ABA treatment is often used during dessication. In addition, heat shock, cold stress, and osmotic stress have also been used to achieve similar results (Tautorus et al., 1991). Increased osmolarity can affect the efficiency of induction as well as maturation of somatic embryos. Addition of various sugars and polyethyleneglycol (PEG) has been shown to promote somatic embryo maturation in both angiosperms and gymnosperms (Brown et al., 1989; Attree et al., 1991; Tautorus et al., 1991).

Bioreactors are being tested to scale-up the production of somatic embryos in a cost effective and efficient manner. Although shake-flask cultures are useful to establish growth kinetics, nutritional requirements for growth, embryo yield, and to study developmental regulation, bioreactors provide an added advantage by increasing the working volume while maintaining homogeneous cultures under controlled environment for optimum growth. These cultures can be used either for secondary metabolite production or for mass propagation via somatic embryos. Substrate requirements and utilization, pH of the growth media etc., have been studied for several tree species grown in bioreactors (Goodchild and Givan, 1990; Tautorus et al., 1992; 1994; Tautorus and Dunstan, 1995). Even though liquid cultures produce large numbers of somatic embryos, the continuous submersion of somatic embryos in the liquid medium results in vitrification. This exposure to excessive liquid may cause damage to somatic embryos at early stages of development. The damaged somatic embryos may leach undesirable chemicals in the medium that may lead to a reduction in somatic embryo production and germination. Etienne et al., (1997) suggested that the problem of vitrification and excessive exposure of somatic embryos to liquid could be solved in part by growing the cultures on solid media and adding only small amounts of liquid media on top of solid medium, or by culturing in fermentors with bubbled aeration, or by placing the explants

on partially-submerged inert supports. An alternate approach is to submerge the growing somatic embryos in liquid medium intermittently rather than continuously (Teisson et al., 1995; Teisson and Alvard, 1995). *Citrus* (Cabasson et al., 1997) improved and *Hevea brasiliensis* (Etienne et al., 1997) somatic embryo development was improved significantly by intermittent immersion in liquid medium. To date, however, application of this technology for large-scale plantlet production in woody species has not been demonstrated.

The high efficiency production of somatic embryos in bioreactors will lead to major changes in the way crops are planted, e.g. through the use of synthetic seed technology (Litz and Gray, 1995). Synthetic or somatic seeds are somatic embryos encapsulated in a protective coating that can be used in commercial plant production in a way analogous to zygotic seeds (Attree and Fowke, 1993; Carlson and Hartle, 1995; Gray et al., 1995). There is an increasing interest in creating synthetic seeds that possess protective outer coatings which provide the somatic embryos with the storage and handling qualities of natural seeds. Both somatic embryo quality and manufactured seed design must be improved before the implementation of this technology on commercial scale. Although somatic embryos are morphologically similar to zygotic embryos, the former lack many of the stored reserves that the latter have available for their growth following germination (Gray et al., 1995). Somatic embryos produced in tissue culture do not have endosperm and testa around the embryo. These embryos must be encapsulated in an artificial endosperm-testa matrix that will allow respiration, contain sufficient quantity of nutrients and protectants, and will be tough enough for handling but allow the embryo to germinate (Sorvari et al., 1997). Duplicating the chemical and physical attributes of natural seeds which are responsible for rapid emergence of normal germlings under field conditions will be the most effective way to use synthetic seed technology (Carlson and Hartle, 1995). Several encapsulation methods have been tested some of which involve the addition of nutrients (Redenbaugh, 1991; Fujii et al., 1992; Sanada et al., 1993; Mukunthakumar and Mathur, 1992; Carlson and Hartle, 1995). Carson et al., (1992) even included oxygen as part of encapsulation process; this improved the performance of the synthetic seeds. In general, inclusion of nutrients in the capsule improves the efficiency of germination. Sorvari et al., (1997) demonstrated that encapsulation with combinations of galactomannans and polysaccharides as well as calcium alginate works well for the germination of carrot somatic embryos. They further showed that starch may be used in the capsule as artificial endosperm for the nourishment of embryo. Synthetic seed coats of two types have been manufactured and tried so far: hydrophobic coat (Friend, 1993) and wax impregnated paper seed coat (Carson et al., 1992). The latter has an advantage over the hydrophobic coatings since in this case there is no danger of cytotoxic effects to the gametophyte/endosperm from organic solvents. Piccioni and Standardi (1995) encapsulated apical and sub-apical buds of six different woody plants using sodium alginate, with or without nutrient enrichment. Rates of regrowth after encapsulation were different for different species and growth was observed only on nutrient enriched medium. For more details on applications of synthetic seeds see Attree andFowke (1993), Carlson andHartle (1995), and Gray et al. (1995).

4. Optimization of Regeneration

4.1. BIOLOGICAL FACTORS

Among the factors that determine the fate of a particular tissue in terms of regeneration in culture, the most important are the intrinsic factors, i.e. the type of tissue explant (leaf,

stem, embryo, etc.) and developmental stage of the tissue (juvenile vs. mature). One needs to optimize regeneration response in a tissue culture environment by choosing the right type of tissue and developmental stage of the explant. For example, in the case of radiata pine, embryogenic cultures can be initiated only from embryos excised from immature seeds (Smith, 1997), whereas in spruce, mature seeds can be used for this purpose (Tremblay and Tremblay, 1995; von Arnold et al., 1995; Minocha et al., 1993; 1995). Similarly, one can induce somatic embryogenesis from leaf tissue in angiosperms but in most conifers one has to use zygotic embryos. Similarly, physiological age of the tissue may also determine its regeneration potential, e.g. in most cases juvenile shoots sprouting from the base of a tree are better suited for micropropagation than are tissues taken from young branches in the crown .

4.2. GROWTH REGULATORS

Auxins are usually required for the induction of callus from a variety of tissue explants; 2-4, D being the most effective and the most frequently used auxin. The other essential and commonly used hormone is a cytokinin such as BA or kinetin. Cytokinins are generally used in combination with auxins for initiation and maintenance of callus cultures. Cytokinins also induce multiple shoot formation in the case of micropropagation from embryos, shoot apices, or axillary buds. When applied in an appropriate concentration along with auxins, cytokinins can induce shoot regeneration from callus. Even though cytokinins play critical roles in the regeneration process, their biosynthetic pathways are still not fully elucidated to facilitate *in situ* manipulation of their concentrations in the cells (Prinsen et al., 1997).

Abscisic acid is known to affect growth of cultures and induce embryo development in most tree species (See reviews in Bonga and Durzan, 1987; Jain et al., 1995a; Bajaj, 1995a; Capuana and Debergh, 1997; Astarita and Guerra, 1998). The role of ABA in conifer embryo development has been well studied and pertains largely to the process of embryo maturation. As with auxins and cytokinins, *in situ* manipulations of ABA levels are not feasible at present, so exogenous supplies are used to affect development. Ethylene, which has not been extensively studied under cell and tissue culture conditions, is known to inhibit cell division and promote cell senescence (Mattoo and Suttle, 1991; Magdalita et al., 1997; Ma et al., 1998). It plays a dual role of inhibitor as well as promoter of morphogenesis. A competitive interaction between the biosynthesis of polyamines and ethylene has been suggested to play an important role in modulating somatic embryogenesis in carrot cell cultures (Suttle, 1991; Minocha and Minocha, 1995; Bastola and Minocha, 1995). The existence of such an interaction has not been established in conifer somatic embryo development.

It is not easy to formulate specific recommendations on the use of growth regulators in woody plant tissue culture since various studies have used a wide range of concentrations in numerous combinations accompanied by little or no quantitative data (Minocha, 1987a). Another complicating factor is that the relationship of exogenous levels of these growth regulators with their endogenous concentrations at the time of induction of morphogenesis is not clear. Besides directly affecting cell division and morphogenesis through modulation of gene activity, growth regulators are also known to indirectly affect the uptake of various inorganic and organic ions from the medium (Saftner and Wyse, 1984). Recent advances in the field of hormone receptors and hormone induced gene regulation should lead to a better understanding of the mechanisms of their action and thus optimization of growth media for plant cell cultures. An extensive review on the role of plant growth regulators in cell and tissue culture of forest trees was

published by Minocha (1987a). Since then most of the progress in understanding their role in plant development has been with herbaceous plants, particularly *Arabidopsis thaliana*.

4.3. MACRONUTRIENTS AND MICRONUTRIENTS

There are about a dozen formulations of various media available for woody plant growth in culture (McCown and Sellmer, 1987). While these media vary significantly in their ionic strength, they all contain the same essential Macronutrients. Although many plant tissues can grow well on most of these media, some species/genotypes show considerable differences in their growth rates as well as morphogenetic potential on different media. A few possible explanations for these differences may be: inhibition by high ionic strength, effects of total N levels, Ca^{++} deficiencies, and chloride sensitivity (McCown and Sellmer, 1987). Bon et al., (1998) showed that on media lacking growth regulators, growth and development varied significantly in response to varying macronutrient composition. Morphogenetic responses were also species specific. Morard and Henry (1998) showed that in *Catharanthus roseus* cell cultures changes in mineral composition of the medium caused changes in the production of biomass as well as solamargine, a secondary metabolite.

For the growth and development of plants in culture, nitrogen is of singular importance. Both the form and the amount of nitrogen in the medium have significant effects on the rate of cell growth, cell morphology and regeneration potential (Kirby et al., 1987; Wink, 1997). In some tissues the importance of reduced nitrogen (ammonia and/or amino acids) has been demonstrated as a specific requirement for embryo development and improvement of morphogenesis (Halperin and Wetherell, 1965; Kirby et al., 1987). In carrot cultures, for example, ammonium nitrate increased the rate of embryogenesis as compared to potassium nitrate (Halperin and Wetherell, 1965). Most plant tissue culture media, including those used for the culture of cells and tissues of forest trees, incorporate both nitrate and ammonium salts as nitrogen source for growth (Gamborg and Shyluk, 1981). An organic nitrogen source (e.g. amino acids and casein hydrolysate) is sometimes added to stimulate the growth of plant cells and tissues in culture. Organic nitrogen represents a readily assimilated and energetically inexpensive nitrogen source that may be significant in sustaining the growth of cells in culture at times when their nitrate and ammonium assimilative mechanisms are not fully operative (Kirby et al., 1987).

It has been shown that inappropriate levels of micronutrients can also adversely affect development and growth. The common practice of treating all of the essential micronutrients as a group may be simplistic since chemical speciation phenomena combined with nutrient interactive effects on uptake could affect the availability of micronutrients. This is complicated even further by the fact that tissues in different developmental stages may vary in their requirements for particular micronutrients (Teasdale, 1987). Commonly used additives such as activated charcoal, which presumably causes promotion of growth in tissue cultures by adsorption of toxic compounds from the culture medium, would affect the availability of macro- and micronutrients in an unpredictable manner (Herman, 1991).

4.4. CARBOHYDRATES

Cultured cells always require an exogenous supply of carbohydrates as a carbon and energy source, sucrose being the best for this purpose. However, different developmental

responses sometimes display differential requirements of the type and the level of specific carbohydrates other than sucrose (Thompson and Thopre, 1987; Tautorus et al., 1991 and references therein;Jain et al., 1997b;Karsai andBedo, 1997; Travert et al., 1997).

4.5. pH

Plant cells maintain the cytoplasmic pH within a tightly regulated range, even when exposed to a wide range of external pH (Minocha, 1987b). The pH of the growth medium may, however, have significant effects on growth and differentiation of cells in some cases, whereas in others growth rates are not affected over a wide range of initial pH of the medium except at extreme (<3.5 or > 8.0) values (Seabrook, 1980). Although most tissue culture media are adjusted to a pH of 5.2-5.6, the cells in culture can significantly alter the pH of the external medium through differential uptake of nutrients such as ammonium and nitrate and/or through H^+ efflux. This may partly account for the lack of visible effects of initial pH of the culture medium on growth of cells. The pH of the medium presumably has profound effects on uptake of nutrients (Minocha, 1987b).

4.6. PHYSICAL ENVIRONMENT

Only a few detailed studies have involved variation in physical parameter for maintenance of cell/tissue cultures. An interaction between temperature and other chemical and physical parameters may indirectly affect growth rates. Most laboratories seem to follow temperature and light regimes that are empirically set depending upon the sophistication of instrumentation available. Generally, plant cultures are maintained within a narrow range of temperatures (22-28 °C) and different light/dark cycles. No specific recommendations have been made to optimize these parameters. The light and temperature requirements for conifers have been reveiwed by Ellis and Web (1993). An important concern in recent years has been the type of gelling agent used to solidify the medium. The effect of different gelling agents on micropropagation and somatic embryo development of various woody plants has been analyzed by several workers (Pochet et al., 1991; Tremblay and Tremblay, 1991; Huang and Gbur Jr., 1998). It is also recommended that vessels which permit adequate air exchange should be used. The use of liquid versus solid media has also been examined in some cases. The roles of dissolved gases such as oxygen and carbon dioxide in somatic embryogenesis have been emphasized by Kvaalen and von Arnold (1991).

5. Genetic Transformation Studies

Genetic improvements through conventional breeding in most tree species have been rather limited due to a combination of two factors: the long period before flowering and a very slow selection process. While the conventional methods of breeding through hybridization and selection will continue to play a significant role in the improvement of forest trees, one must also incorporate the techniques of somatic cell hybridization and gene manipulation through genetic engineering in order to maximize gains over a short period.

The availability of established cell and tissue culture techniques and protocols for regeneration of whole plants from these cultures is generally a prerequisite for the application of genetic transformation technologies for plant improvement. In comparison

with herbaceous species, the variety of tissues from which whole plants can be regenerated is rather limited for woody plants. Embryogenic cell masses capable of regeneration can be initiated from juvenile material or zygotic embryos in most conifers. In hardwood species such as *Populus*, *Eucalyptus*, and a few others, plants can be regenerated either via somatic embryogenesis or organogenesis (for more details see reviews in Ahuja, 1993; Bajaj, 1995a). The available literature on genetic transformation of woody plants includes several conifers and a few angiosperms (Klopfenstein et al., 1993; Machado et al., 1997; Mullins, 1997; Moralejo et al., 1998; Minocha and Minocha, 1999).

Plant protoplasts, suspension and callus cultures, somatic embryos, leaves, and roots are all useful starting materials for genetic transformation. For using the techniques of direct DNA uptake, micro injection, electroporation and liposome fusion, a healthy protoplast culture capable of regeneration, is required. Only a few reports on transformation using protoplasts of woody plants are available (Bekkaoui et al., 1988, 1995; Gupta et al., 1988; Tautorus et al., 1989; Wilson et al., 1989; Wilde and Merkle, 1994). No stable expression and regeneration from protoplasts was obtained in any of these studies (Minocha andMinocha, 1999).

Agrobacterium tumefaciens has been widely used for transformation of numerous plants and is quite suitable for woody plants as well (Ellis, 1995). Regeneration from selected tissues is achieved either by direct shoot formation or via callus production. A list of species that have been transformed by *Agrobacterium tumefaciens* is presented in Minocha and Minocha (1999). Biolistic bombardment technique, which has also been used successfully for transformations of conifers as well as angiosperms, depends upon regeneration of somatic embryos from embryogenic cell masses or formation of shoots from callus (Ellis et al. 1993; Walter et al., 1994; Charest et al. 1996; McCown et al., 1991).

While most published studies on transformation have employed only the selectable marker or reporter genes, some of the commercially important phenotypes that have been targeted for transformation of forest trees include insect resistance, herbicide tolerance, early flower development, lignin type, and biomass production. Transgenic *Populus* plants have been produced that were tolerant to glyphosate (Karnosky et al., 1997) and to damage by Coleopteran larvae (Heuchelin et al., 1997). There have also been a few reports on genetic manipulations of lignin biosynthesis (Sederoff et al., 1994; Atanassova et al., 1995; Kajita et al., 1997). Attempts are also being made to increase the harvest index of a species (Robson et al., 1996) and to produce male sterile plants through genetic engineering (Walter et al., 1998). There is a need to extend transformation effort at genetic manipulation of enzymes involved in primary or secondary metabolic pathways in order to increase our understanding of basic processes of growth and development. For example, it may be possible to increase the embryogenic potential of woody plant cell cultures through manipulation of their metabolism, as was seen in carrot cell cultures where genetic manipulation of polyamine biosynthesis promoted somatic embryogenesis (Bastola andMinocha, 1995).

A major impediment in the production of transgenic plants in most conifers is the inability of transformed callus to regenerate into a whole plant (Ellis, 1995). Initial work on optimization of transformation techniques and on analysis of basic processes of growth and development may still be done with cultures that are incapable of regenerating whole plants; however, in order to fully understand the dynamics of the effects of an introduced gene on other interrelated pathways, one needs to work with whole plants regenerated from these cultures.

6. Other Applications of Tree Tissue Cultures

6.1. STRESS STUDIES

Trees, by their perennial nature and immobility, are constantly exposed to a variety of biotic and abiotic stress factors. Stress induces a variety of physiological and developmental changes that can be long-term or short-term in nature. Whereas some of these changes are quantitative, i.e. increase or decrease in cell size or the cellular content of one or more metabolites, others are qualitative, e.g. the presence or absence of certain metabolites or proteins. A low-stress event may induce tissue responses that result in acclimation and induction of repair mechanisms but a high-stress event often causes significant damage that can result in cell and plant death (Lichtenthaler, 1998).

Cell suspension cultures are valuable model systems for gaining insights into the effects of stress inducing factor(s) under controlled conditions. This is especially true for tree species, for which it is extremely difficult and cost prohibitive to create a controlled environment for studies with whole plants. High osmoticum, salinity, heavy metals, nutrients, herbicides, and biological elicitors are some examples of the types of stress factors that have been studied using plant tissue cultures in order to elucidate their effects on various organelles, nucleic acids, proteins, amino acids, carbohydrates, secondary metabolites, inorganic ion uptake, cell ultrastructure, and gene activity (Berglund and Ohlsson, 1995; Serrano, 1996; Tabaeizadeh, 1997; Bressan et al., 1998; Lichtenthaler, 1998). Cell/tissue cultures can also be exploited for selecting mutant cell lines of potential economic importance (Dirks et al., 1996; Vollmann et al., 1997; Mejia et al., 1998). However, such studies with tree species have been rather limited.

The most critical step in alleviating a particular stress in plants is to detect and characterize the stress response at an early stage. The need to assess a set of early biochemical markers of stress in trees is obvious because, in most cases, the visual symptoms of stress appear too late to reverse the damage through management practices and/or treatments. Thus the availability of markers that can assess the current status of stress in visually healthy appearing trees in a forest is crucial for planning a potential treatment or management practice for alleviating the adverse effects of the stress or removing its cause. Models using cell cultures can identify biochemical and molecular processes that are early indicators for a particular stress, e.g. Al exposure (Minocha et al., 1992, 1996). Results obtained using a model system can be tested further for their validity and applicability to mature trees growing in nature. Following is one such example where this was accomplished by using suspension cultures and mature trees of red spruce (*Picea rubens*) to study the effects of Al on cellular processes.

Among the physiological and molecular responses of cells to Al treatment are the changes in cellular content of polyamines, metabolism of malate, oxalate, and phenylpropanoids and induction of novel gene expression (Delhaize and Ryan, 1995; Zhou et al., 1995; Ma and Miyasaka, 1998; Yamamoto et al., 1998). The similarity of results obtained by Minocha et al. (1996) using cell suspension cultures of red spruce with those obtained by others using seedlings of red spruce (Cronan and Grigal, 1995) indicates that *in vitro* grown cell cultures are suitable for investigating the effects of Al on various biochemical pathways. Minocha et al. (1997) observed that the conclusions drawn from field studies conducted on physiological and biological effects of exposure to various concentrations of Al on the foliar tissue of mature red spruce trees were similar to

those drawn from their controlled experimental study with cell cultures (Minocha et al., 1996). They also demonstrated that an increase in foliar putrescine and/or spermidine concentrations in response to direct or indirect stress imposed on red spruce trees by Al exposure may possibly be used as an early warning tool for assessing and predicting tree health before the appearance of visual symptoms.

Another application of studies with cell cultures is to investigate the effects of genetic transformation on stress tolerance of a cell line as compared to untransformed control. Kishor et al. (1995) and Kiyosue et al. (1997) have suggested that genetically engineered plants that overproduce proline might acquire osmotolerance, i.e. ability to tolerate water as well as salinity stress. Shen et al, (1997) demonstrated that targeting mannitol biosynthesis to chloroplasts in tobacco increased the resistance of these cultures to oxidative stress. Suspension cultures of hybrid poplar have been transformed with ornithine decarboxylase gene for overproduction of putrescine using biolistic bombardment are being used to investigate the role of polyamines in stress (unpublished data from R. Minocha and S.C. Minocha's laboratories). The topic of genetic application of stress tolerance in plants has been recently reviewed (Holmberg and Bülow, 1999).

6.2. SECONDARY METABOLITE PRODUCTION

Plants are not only the primary source for food and fiber, but also a source of pharmaceutical and industrial products such as drugs, dyes, scents, etc. However only a small number of the compounds sold commercially are produced in cell cultures, primarily because yields are low (Alfermann and Petersen, 1995). The topic of plant tissue cultures for the production of industrial biochemicals has been thoroughly reviewed (Staba, 1980; Fujita, 1990; Bajaj, 1995a, Cosson, 1997; Yeoman and Yeoman, 1997). The major advantages of producing secondary metabolites in cell/tissue cultures are: (1) year round availability of cultures; (2) only specific cells/organs can be grown *in vitro*; (3) secretory products can be easily purified; (4) production of a single compound can be enhanced through the use of chemical elicitors, biotransformation or by transgene expression; and (5) the production of recombinant biopharmaceuticals such as mammalian hormones, enzymes, vaccines and monoclonal antibodies. Taxol, an anticancer drug, was initially discovered in extract from the bark of *Taxus brevifolia* (Nutt). The most promising long-term solution to taxol production seems to be from cell suspension cultures using modern biotechnology approaches (Shortle and Minocha, 1999). Ciddi et al. (1995) showed that the addition of several fungal cell extracts and culture filtrates resulted in an increase in the production of taxol in cell cultures of *Taxus*. Yukimune et al., (1996) were able to achieve increased production of taxol and its precursor baccatin III in taxus cell cultures by adding methyl jasmonate. In another study, production of several secondary metabolites such as alkaloids and phenolics was shown to increase by the addition of acetylsalicylic acid to *Catharanthus roseus* cell cultures (Godoy-Hernandez and Loyola-Vargas, 1997). Auxins have also been shown to affect alkaloid production in transgenic cell lines of this species (Whitmer et al., 1998).

Manipulation of natural product biosynthesis in plants through genetic engineering is very promising but technically a very challenging goal. The potential contribution of genetic engineering and plant biotechnology to the production of secondary metabolites has been reviewed recently (Cosson, 1997; Yeoman and Yeoman, 1997). Grotewold et al., (1998) have demonstrated that different secondary metabolites can be produced in cultured maize cells by ectopic expression of the appropriate regulatory genes. The use of plants as bioreactors for the production of recombinant biopharmaceuticals has been reviewed by Miele (1997).

The biosynthesis of secondary metabolites may not be as stringently regulated as that of primary metabolites because the former may not be essential for the survival or viability of the cell or plant. Thus manipulation of the biosynthetic pathway of a secondary metabolite through genetic engineering may be less complicated when compared to a primary metabolic pathway (Taylor, 1998). On the other hand, the fact that the secondary metabolic pathways are complex and generally require several enzymatic steps to produce the desired end product, may require the transfer of multiple genes (Oksman-Caldentey and Hiltunen, 1996). Flavonoid biosynthesis (Grotewold et al., 1998) and phenylpropanoid pathways are likely the first targets for genetic manipulation for secondary metabolite production because they have been studied in great detail.

6.3. CRYOPRESERVATION

Cryopreservation is the suspension of the metabolic functions of a living cell through temperature reduction to -196°C (temperature of liquid N_2). Cryopreservation allows the cells to be stored for long periods of time without loss of embryogenic potential or aging (Hargreaves and Smith, 1994; Sakai, 1995). To maintain viability of cultured cells and meristems, the freezing and thawing must take place in a manner that the water molecules within the cells do not crystalize and damage the integrity of the cell membranes and other structures, thus killing the cell. Research has shown that a combination of freezing in stages, thawing quickly, and using protective dehydrating chemicals called "cryoprotectants", prevents damaging crystal formation during cryopreservation (Sakai, 1995).

The irreversible loss of naturally occurring diverse germplasm is a major concern since this germplasm is critical for future use in tree improvement programs (Chen and Kartha, 1987; Day and McLellan, 1995). Traditionally, seed storage at low temperature and low humidity has been the main avenue for maintaining germplasm. However, in cases where seeds cannot be dried without immediate injury and loss of viability or trees that produce seeds only once in several years or plants which do not produce seeds, alternate ways, such as cryopreservation of cultured embryos, cells, or shoot apices can be adopted for the storage of germplasm.

Among the various uses for cryopreservation are: conservation of existing natural variability in the germplasm; the preservation of germplasm of endangered species and commercially useful breeding lines; and conservation of the variability introduced, *in vitro*, through genetic manipulation. The problem of poor plant regeneration from explants of mature trees can be augmented by cryogenic storage of juvenile tissues. The phenomenon of physiological aging imposes limitations on clonal forestry. The ability to store reference clonal material, such as embryogenic cultures should allow the juvenile state to be maintained while field tests of the clonal material are carried out. Embryogenic lines of *Pinus radiata* are known to lose their embryogenic potential within 3-4 months of subculture. Through cryopreservation, however, the embryogenic potential of these lines has been maintained for several years (Hargreaves and Smith, 1994).

Another major advantage of cryopreservation is to bypass the need for large quantities of hybrid seeds, since such breeding is very costly. Instead, cryogenic storage of some of these hybrid clones either as zygotic embryos, seeds, or *in vitro* induced buds or meristems, could provide a continuous supply of superior clones of desired genotypes (Chen and Kartha 1987). Cryopreservation is a helpful tool for increasing the storage period of even synthetic seeds, and for international exchange of germplasm (Bajaj, 1995b). Embryogenic conifer cultures (Kartha et al., 1988; Gupta et al., 1987; Klimazewska et al., 1992; Hargreaves and Smith, 1994; Sakai, 1995 Find-Jens et al.,

1998; Park et al., 1998). (Pence, 1996), seeds or isolated embryonic axes of *Azadirachta indica* (Berjak and Dumet, 1996), *in vitro*-grown shoot tips of cherry and sweet cherry (Niino et al., 1997) and shoot tips of silver birch (Ryyananen, 1998) have all been cryopreserved successfully.

6.4. FOREST PATHOLOGY AND PEST CONTROL

Tissue cultures have been used successfully for studying a variety of host pathogen interactions, including the mechanisms of disease resistance and apoptosis, at the cellular and molecular level (Able et al., 1998; Furukawa and Kunihiko, 1998; Herbers and Sonnewald, 1998; Ragazzi et al., 1998). Most of the work published thus far pertains to herbaceous plants. The progress in this area of research helps to demonstrate the usefulness of these studies to woody plants in the near future. *In vivo* studies to analyze host pathogen interactions and resistance mechanisms are confounded by site and microclimate variability, physiological age of the tissues at the inoculation site, and infections by other pathogens besides the one under study, along with abiotic stresses. *In vitro* studies, on the other hand, offer ready access to selected cells, tissues, and organs under controlled and aseptic growth conditions, permitting one to accurately investigate the inoculation events and response mechanisms in these cells/tissues. The synthesis, regulation, and activities of pathogen induced compounds such as phytoalexins, phytotoxins, polyphenols, and pathogenesis related proteins (PRP's) can be studied in response to exposure of cultured explants and tissues to pathogens. However, one must bear in mind the possibility that the behavior of the host or the pathogen can prove to be artifactual under *in-vitro* conditions. The information generated using *in vitro* systems must, therefore, be verified with whole plant studies (Diner and Kharnosky, 1987).

6.5. PROGRAMMED CELL DEATH

The death of a cell may be accidental resulting from a physical injury or it may occur in a very predictable pattern (e.g. during development) by a programmed pathway for cell suicide (Greenberg, 1996; Mittler et al., 1997). The latter is known as programmed cell death (PCD). In plants, PCD is often activated by recognition by the cell of an invading pathogen or abiotic factor. According to Greenberg (1996), plants, like animals, also have two types of PCD. One form of PCD which is very common resembles apoptosis in animals and the other represents a morphologically distinct form of cell death. Apoptosis is a well studied topic in mammals and exhibits morphologically identifiable steps toward programmed cell death. These steps are regulated by genes that have homologues in other animal phyla (Heath 1998). Although plants and fungal plant pathogens have shown forms of developmentally regulated cell death, only a few steps involved in this process have been delineated, e.g. cleavage of DNA into oligonucleosomal fragments (Ryerson and Heath 1996; O'Brien et al. 1997). Parallels between plant and animal apoptosis have also been discussed by Havel and Durzan (1996). The hypersensitive response (HR) is a form of programmed cell death which helps to restrict the spread of pathogens from the infection site. Using suspension cultures of tobacco, Bonner et al., (1996) suggested that a particular ratio of glutamine to other amino acids established *in vivo* might influence the susceptibility of cells to apoptosis. The role of Ca in this process has also been suggested by Levine et al. (1996). The generation of reactive oxygen species in response to the bacterial elicitor harpin in cell cultures of *Arabidopsis* triggered a cell death program (Desikan et al., 1998). While no studies with woody plants have been reported, cell cultures provide a unique material for such studies with trees, where response of the

whole organism is difficult to manipulate.

6.6. METABOLIC AND BIOCHEMICAL STUDIES

Cell cultures are ideally suited for investigations aimed at achieving a greater understanding of the primary and secondary metabolic pathways. In addition, they can be used for biotransformation of synthetic precursors into commercially useful products. Several metabolic pathways such as flavanoids, polyamines, and antioxidants have been studied in details using cell cultures of various plants. Pathways for the uptake and metabolism of various hormones have also been elucidated.

Numerous studies have focused on understanding the physiological, biochemical and molecular aspects of regeneration of roots, shoots, and embryos in cell and tissue cultures. The importance of the extracellular matrix and its role in development and differentiation of carrot somatic embryos has been studied (Knox, 1990; 1993). Extracellular glycoproteins such as arabinogalactans and chitinases have also been implicated in somatic embryogenesis (LoSchiavo et al., 1990; De Jong et al., 1995; Kruger and van Holst, 1996; Toonen et al., 1997). The levels of specific arabinogalactan proteins, peroxidases and chitinases in Norway spruce cultures were shown to be related to their embryogenic potential (Egertsdolter and Von Arnold, 1995; Mo et al., 1996). The genes for some of these glycoproteins have recently been cloned (Dong and Dunstan, 1997b). The expression of several genes belonging to various gene families such as heat shock genes, LEA protein genes, and ABA responsive genes has been shown to be linked with embryo development in spruce. The expression of these genes can be modulated by changes in concentration of ABA (Dong and Dunstan, 1996a,b; 1997a; for more details, see chapter by Dong and Dunstan in this volume). Detailed studies on changes in endogenous hormone levels in developing seeds and zygotic embryos of *Picea glauca* and during growth of somatic embryogenic masses in hybrid larch have been reported (Kong et al., 1997; Jourdain et al., 1997) . Precociously germinating somatic embryos of *Vitis vinifera* were shown to have lower ABA and IAA levels than their germinating zygotic counterparts (Faure et al., 1998). These results suggest that the role of ABA and IAA needs to be further studied during normal germination in order to achieve a better control over this process. A basic understanding of the biochemical and molecular processes that underlie formation of somatic and zygotic embryos is essential for the control of somatic embryogenesis, and production of superior quality somatic embryos (Misra, 1995). Most of these studies are fragmentary in nature and more research needs to be conducted before this information can be used to fully understand the emergence and development of organized structures *in vitro*. The success in this field is further hampered by the low proportion of cells that participate in the regeneration process (Dekerk et al., 1997).

Several studies comparing developing zygotic and somatic embryo have revealed that the two processes are biochemically quite similar. Identification of stage-specific molecular and biochemical markers should aid in optimizing somatic embryogenesis protocols (Feirer, 1995; Misra, 1995; Minocha et al., 1999). For example, recent reports on polyamine ratios as indicators of somatic embryo development suggest that manipulating polyamines through media supplementation or genetic manipulation may improve somatic embryogenesis (Bastola and Minocha, 1995; Berros et al., 1997; Santanen and Simola, 1997; Yadav and Rajam, 1998; Minocha et al., 1999). Misra (1995) has suggested the use of seed storage proteins and their cDNAs as potential markers to gauge the maturity of developing somatic embryos in conifers. Dodeman et al. (1998) have reported that protein patterns of carrot somatic embryos at torpedo stage were different from those of zygotic embryos. They concluded, however, that these

differences in protein patterns may be due to culture conditions, since most of the differences disappeared upon conversion of these somatic embryos to plants that were growing on carbon free medium. The identification of the expression of specific genes during development would lead to cloning of their promoters which can then be used for the expression oftransgene in a developmental stage specific manner.

6.7. HABITUATION

Habituation is a physiological and developmental change in the behavior of cells that occurs spontaneously in cell culture. It is characterized by hormone independence, loss of morphogenetic potential, and the occurrence of fast-growing undifferentiated cells (Hagege, 1995, 1996). It has been proposed that habituated cells are more efficient than normal cells in scavenging deleterious free radicals that are produced in cell cultures (Hagege,1996). Habituated cells have various cytological and ultrastructural features that resemble those of animal tumor cells. These cells have altered biochemical processes such as ethylene production, cytoskeleton organization, elaboration of cell walls, and photosynthesis. Habituated cells have been reported to contain low ethylene and high polyamine levels (Hagege, 1995; Bisbis et al., 1998). While habituation is generally presumed to be an epigenetic phenomenon, several biochemical and cytological changes underlie this process. An understanding of the molecular basis of habituation should lead to the establishment of genetically stable cell cultures while maintaining their morphogenic potential.

7. Conclusions and prospectives

Establishment of reliable protocols for regeneration of plants from tissue explants and callus cultures has been instrumental in the success of genetic engineering techniques with crop plants. Although tremendous progress has been made in this regard with woody species, including forest trees, the step of large scale multiplication, especially via somatic embryogenesis from morphogenetically stable cultures, is still a major impediment for widespread use of transformation techniques with trees. The difficulty of regeneration from protoplasts, and the fact that only juvenile material can be used for tissue culture in most tree species, further compounds the situation. Thus it is imperative that we focus our attention on increasing our understanding of the physiological and molecular basis for the loss of regeneration potential in plant cell cultures. A better understanding of the biochemical and molecular basis of habituation would help prevent this phenomenon and achieve continued regeneration of shoots or somatic embryos in cell cultures. There is a need for the development of methods for recognizing cell populations with high potency for regeneration within mixed cultures. The relatively long lag periods needed for evaluation of improved phenotypes necessitates the development of biochemical and molecular markers that can aid in an early selection of the desired genotypes. Likewise, molecular markers to evaluate genetic diversity in regenerated plants must be developed.

Tissue culture techniques are being used commercially for large-scale plant production; however, the protocols are highly labor intensive. As a result it is often not cost-effective to use these protocols for commercial mass propagation. Combined use of bioreactors for large scale somatic embryo production and robotics for handling of cultures and plantlets could be extremely valuable in reducing this cost and increasing productivity. The technology of encapsulation of somatic embryos (i.e. the production of synthetic seeds) also has to be optimized for commercial use. Since trees have a long life

cycle, it is important that somatic seedlings, particularly those that are transformed, must maintain their genetic fidelity. In the long run, genetic instability among somatic plantlets may lead to economic losses. The techniques of cell and tissue culture form the basis for all these future developments. It is the synergistic application of molecular biology techniques and the tissue culture techniques that will set the stage for desired gains in the genetic improvement of trees.

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