Chapter 3

Plant Regeneration Through Organogenesis in Poplar[1]

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Introduction

Among woody genera, *Populus* has been extensively studied as a model system for biotechnological research. This genus of the Salicaceae family, widely distributed across the Northern Temperate Zone (FAO 1980), is composed of 5 sections: Leuce (currently termed Populus), Aigerios, Tacamahaca, Turanga, and Leucoides (Dickman and Stuart 1983). Leuce is a large group that includes aspen and white poplars, which have great economic importance. Aigeiros, considered the "true poplars," include the cottonwoods and black poplars. Tacamahaca, containing the balsam poplars, is the largest poplar species group. The remaining 2 sections (Turanga and Leucoides) have minor economic importance.

Populus species and hybrids are ideal for plywood and lumber production, and woody biomass production because of rapid growth, ease of establishment through stem or root cuttings, and relative ease of coppice regeneration (Ahuja 1987; Behrens and Melchior 1978; Hall et al. 1989; Herrmann and Seuthe 1982). Trees of this genus represent wide genetic diversity, exhibit widely ranging site requirements, and respond well to cultural input (Schreiner 1974). Many new, high-quality, rapidly growing poplar clones were developed in various breeding programs from diverse geographic regions (Hall et al. 1989). Vegetatively propagated *Populus* species and hybrid clones have been planted extensively in many countries (Zsuffa 1985). For the difficult-to-root poplar (section Leuce), tissue culture techniques for micro-propagation have become a primary alternative to more conventional propagation procedures. *In vitro* propagation techniques were applied to poplars to overcome limitations such as production cost and availability of specific genotypes for planting material (Ahuja 1987). Furthermore, as a model system for biotechnology programs, poplars have been intensively studied for *in vitro* micropropagation, genetic transformation, and gene expression.

The objectives of this review are to summarize: 1) organogenic regeneration of poplar by adventitious bud induction from various explants; and 2) physiological malformation of *in vitro* plantlets, which is a fundamental problem in poplar micro-propagation.

In Vitro Organogenesis

Since 1980, poplars have been intensively studied for *in vitro* establishment (Chun et al. 1988). Such studies reveal that *Populus* tissue exhibits a high degree of developmental plasticity, similar to tobacco in the herbaceous species. Many *in vitro* cloning techniques were developed that provide an integral basis for biotechnological applications. Published reports on *in vitro* regeneration of poplars are summarized in table 1. Types of shoot regeneration with organs or tissues are ordinarily classified as: 1) adventitious shoot formation or 2) axillary shoot induction. Adventitious shoot formation is amorphous shoots originating from the various explants of leaf, internode, catkin, immature embryo, pollen, vascular or bark cambium, root, etc. Cultures for axillary shoot induction are composed of morphous shoots originating from an apical meristem or axillary bud. Apical meristems containing shoot primordia can produce multiple shoots. For more information on *in vitro* morphogenesis of *Populus* hybrids and species, refer to reviews by Ahuja (1987, 1993), Chun (1993), and Ernst (1993).

Adventitious Shoot Organogenesis Through *In Vitro* Cultured Explants

Adventitious poplar shoots can be induced from *in vitro* cultured leaf, internode, petiole, and root explants that

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Table 1. Published reports on *in vitro* establishment with *Populus* species.

Culture type	Species	Reference
Leaf culture		
immature lamina discs	*P. ciliata*	Mehra and Cheema 1980
leaf, internode, root	*P. alba* x *P. grandidentata*	Chun 1987
leaf discs	*P. maximowiczii* x *P. trichocarpa*	Michler and Bauer 1988
leaf, stem, root	*P. maximowiczii* x *P. trichocarpa*	Kang 1991
Root culture		
bud, stem, leaf, root	*P. tremula*	Ahuja 1983
bud, stem, leaf, root	*P. tremuloides*	Ahuja 1983
root	*P. alba* x *P. grandidentata*	Son and Hall 1990
leaf, stem, root	*P.* x *euramericana*	Kang 1991
Anther culture		
anther	*P. glandulosa*	Kim et al. 1983
anther	*P. deltoides*	Uddin et al. 1988
Embryo culture		
immature embryo	*P. deltoides*	Kouider et al. 1984
immature embryo	*P. deltoides*	Savka et al. 1987
ovary, embryo	*P. alba*	Raquin et al. 1993
ovary, embryo	*P. deltoides*	Raquin et al. 1993
ovary, embryo	*P. trichocarpa*	Raquin et al. 1993
ovary, embryo	*P. lasiocarpa*	Raquin et al. 1993
ovary, embryo	*P. trichocarpa* x *P. deltoides*	Raquin et al. 1993
immature ovary	*P. deltoides*	Kang and Hall 1996
Callus culture		
callus	*P. tremuloides*	Winton 1968
callus	*P. tremuloides*	Wolter 1968
callus	*P. tremuloides*	Winton 1970
callus	*P. tremuloides*	Wolter and Gordon 1975
callus	*P. tremuloides*	Noh and Minocha 1986
callus	*P. maximowiczii* x *P. trichocarpa*	Ostry and Skilling 1988
callus	*P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina*	Ostry and Skilling 1988
callus	*P. nigra* var. *betulifolia* x *P. trichocarpa*	Ostry and Skilling 1988
callus	*P. nigra* var. *charkowiensis* x *P. deltoides*	Ostry and Skilling 1988
callus	*P. nigra* var. *betulifolia* x *P. nigra*	Ostry and Skilling 1988
callus	*P. nigra* x *P. maximowiczii*	Park and Son 1988
cell suspension	*P. ciliata*	Cheema 1989
callus	*P. deltoides*	Coleman and Ernst 1990
callus	*P. alba* x *P. grandidentata*	Michler and Bauer 1991

normally never reproduce vegetatively *in vivo* (Ahuja 1993; Chun 1993; Ernst 1993). Most aspen, cottonwood, and their hybrids produce adventitious shoots in Murashige and Skoog (MS) (Murashige and Skoog 1962) media or Woody Plant Media (WPM) (Lloyd and McCown 1980) supplemented with 0.2 to 0.5 mg/l, 6-benzyladenine (BA) or 2.0 mg/l zeatin as a cytokinin source. Many *Populus* species were successfully regenerated through adventitious shoot induction from cultured explants including node, internode, leaf, and root segment (Agrawal and Gupta 1991; Chun 1990; Coleman and Ernst 1990; Douglas 1984; Kim et al. 1994a, 1994b; Nadel et al. 1992; Rutledge and Douglas 1988).

Adventitious regeneration *in vitro* may generate a much higher rate of shoot production than proliferation of axillary shoots. Commercial-scale regeneration systems were

established from leaf and root cultures of European aspen (*P. tremula*), quaking aspen (*P. tremuloides*), and their hybrids (Ahuja 1984). Plant regeneration from stem internodes was accomplished in *P. trichocarpa* x *P. tacamahaca* (Douglas 1984). Park and Son (1988) demonstrated that an average of 178 shoots was directly produced from a punctured leaf of *P. nigra* x *P. maximowiczii* after 6-weeks culture.

Morphogenetic responses of explants from *in vitro* cultured poplar plantlets depend on the explant source and the combination of exogenously applied plant growth regulators. Chun (1990) demonstrated that abaxial side culture of whole-leaf explants was best to induce adventitious shoot buds from a hybrid aspen (*P. alba* x *P. grandidentata*) plantlet. Kim et al. (1994a) showed that the form and concentration of nitrogen sources (ammonium and nitrate) and combination of BA with naphthaleneacetic acid (NAA) greatly affect adventitious shoot induction from leaf-explant cultures of *P. davidiana*.

Kang and Hall (1996b) successfully established a nodal culture system to maximize shoot production of a cottonwood hybrid (*P.* x *euramericana*). They used 4 explants: node, internode, node with the axillary bud excised, and reutilized stem. The shoot proliferation capacity of nodal explants and modified explants was compared, and the origin of multiple shoots was investigated. For these explants, most shoots were derived from the axillary meristems. Shoots also originated from the vascular cambium and occasionally from the lenticels (figures 1-4 to 1-6). A conventional method of directly using nodal explants produced a mean (± standard error) of 5.7 ± 0.6 shoots by treatment with 2.0 mg/l zeatin. However, shoot production from internodes and modified nodes was better using 0.5 mg/l BA with 2.0 mg/l zeatin, which produced averages of 21.7 ± 1.1 and 29.0 ± 0.9 shoots, respectively (figures 1-1 to 1-3). Shoot formation from lenticels was examined in thin sections using bright-field microscopy, and intact specimens were observed with a stereo microscope (figures 1-8 and 1-9).

For adventitious shoot production from *in vitro* cultured leaves of hybrid aspen clones (*P. alba* x *P. grandidentata* cv. 'Crandon' and cv. 'Hansen'), thidiazuron (TDZ) and NAA test combinations were more effective than BA and NAA test combinations or TDZ test concentrations without NAA (Kim et al. 1994b). Among 40 combinations of TDZ and NAA tested, 0.05 μM TDZ with 1.0 μM NAA produced the most adventitious shoots; 18 shoots per leaf explant for 'Crandon' and 15 shoots per leaf explant for 'Hansen.' Increasing TDZ concentrations (0.1, 0.2, 0.5, 1.0, and 2.0 μM) had varying effects on the frequency of regeneration and vitrification of the 2 hybrid aspen clones (Kim et al. 1994b). Significant clonal differences were observed for the number of adventitious shoot regenerated. After 2 to 3 weeks on TDZ/NAA regeneration media, adventitious shoots initiated on the proximal cut surface of the petiole and near punctured areas on the leaf surface. On BA/NAA regeneration media, most adventitious shoots formed at the proximal cut surface of the petiole, while only a few formed on the cultured leaf surface.

Shoot proliferation occurred on cultured root segments of quaking aspen, hybrid aspen (*P. alba* x *P. grandidentata*), and hybrid cottonwood (*P.* x *euramericana*) (Ahuja 1983; Chun 1990; Kang 1991; Son and Hall 1990). Using 'Crandon' and 'Hansen' clones of *P. alba* x *P. grandidentata*, Son and Hall (1990) tested root segments of various ages for shoot regeneration. They reported that root tip containing explants from 60-day-old roots produced the most shoots (an average of 111 shoots per root segment for 'Crandon' and 98 for 'Hansen') on WPM supplemented with 4.8 mg/l or 3.0 mg/l zeatin, respectively. Clonal propagation was used with root segments of hybrid cottonwood (*P.* x *euramericana*) clones (Kang 1991). Among 4 clones ('Canada Blanc,' 'Eugenei,' 'I-45/51,' and 'Wisconsin #5'), the most shoots (17.60 ± 8.96) were induced from 'Canada Blanc' explants on WPM with either a low concentration (0.2 mg/l) of BA or a high concentration (5.0 mg/l) of zeatin.

Generally, morphogenetic responses of *in vitro* cultured explants depend on the species, explant source, and combination of exogenously applied plant growth regulators. Research efforts to develop more generalized organogenesis techniques for explant culture of poplar species and hybrids include: 1) applications of physical treatment, such as the leaf-surface puncturing technique of Park and Son (1988); 2) the effects of various plant growth regulator combinations, such as 2,4-dichlorophenoxyacetic acid (2,4-D) vs. NAA; and 3) optimization of culture conditions for explant source material.

Adventitious Shoot Induction From Anther, Ovule, Embryo, and Catkin Culture

Haploid plantlets were produced from anther cultures of *P. glandulosa*, *P. deltoides*, and *P. maximowiczii* (Kim et al. 1983; Stoehr and Zsuffa 1990; Uddin et al. 1988). In a breeding program, embryo cultures can potentially rescue aborting embryos of interspecific hybrids. Several cultures were reported with ovules or embryos of *Populus* species (Kouider et al. 1984; Raquin et al. 1993; Savka et al. 1987). Recently, Kang and Hall (1996a) reported shoot formation

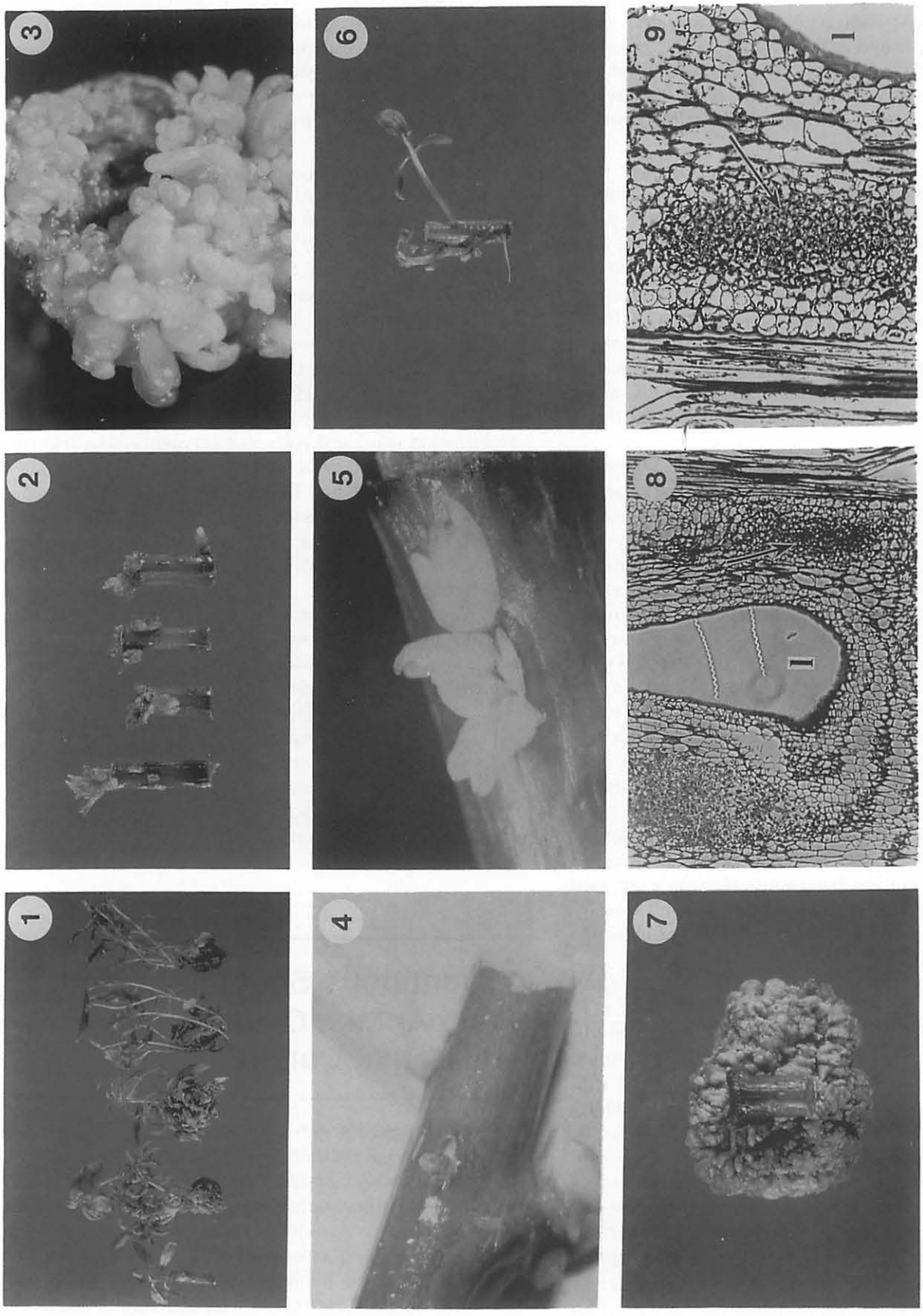

Figure 1. Shoot proliferation from different explants of the 'Ogy' clone (*Populus* x *euramericana*). 1-1) Multiple shoots from the explants of a control node, modified node with the axillary bud excised (2.0 mg/l zeatin), reutilized stem (0.5 mg/l BA), internode (0.5 mg/l BA), and node (2.0 mg/l zeatin). 1-2) Shoot formation from the internodal explants; 1-3) Shoot initiation from the vascular cambium of a stem internode. 1-4 to 1-6) Shoot initiation and elongation from lenticel. 1-7) Shoot induction with a high concentration of zeatin (5.0 mg/l) from a modified node axillary bud. 1-8 and 1-9) Longitudinal section of a partial lenticel with a shoot. l=lenticel (Kang and Hall 1996a).

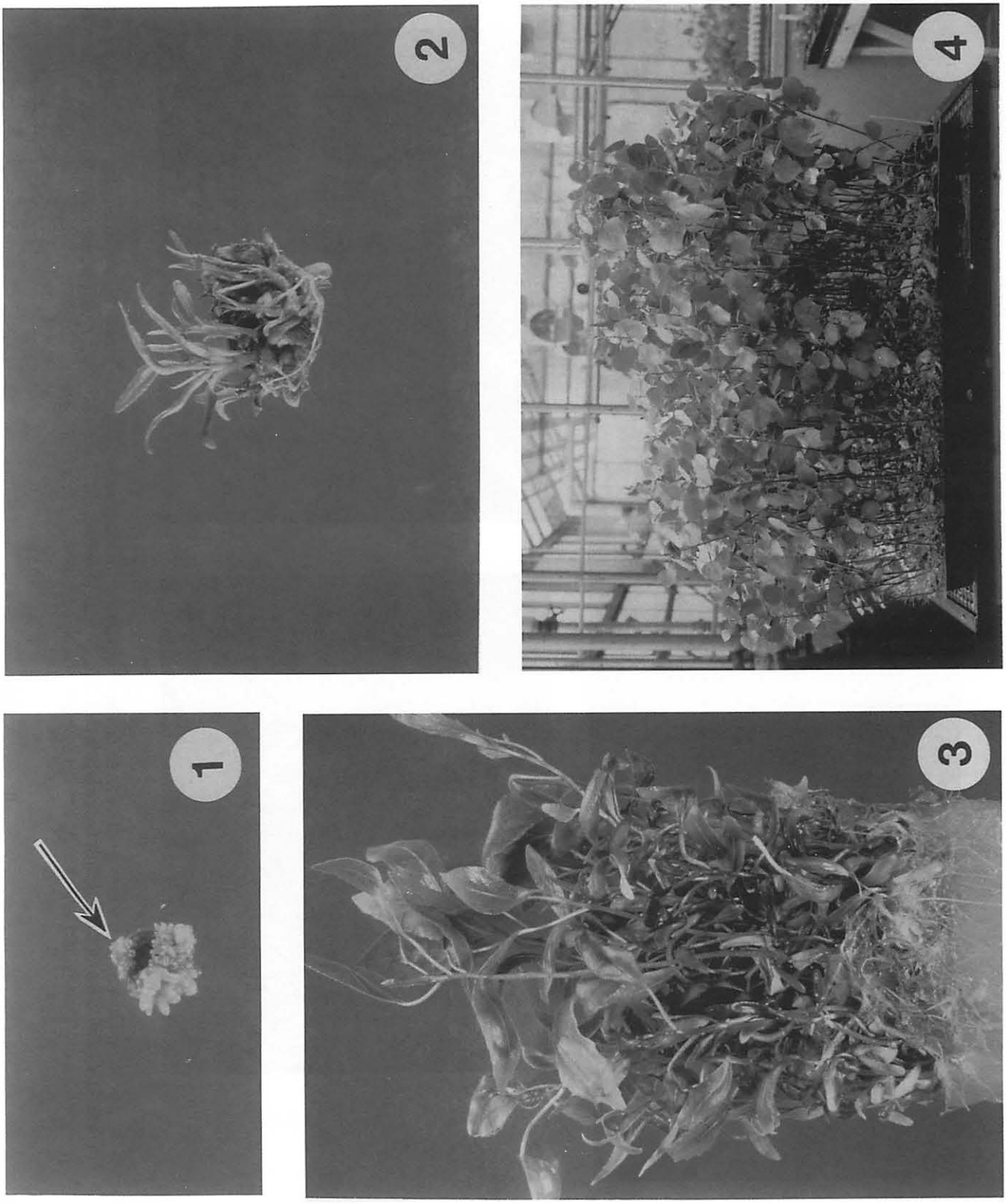

Figure 2. Shoot formation from immature ovules of *Populus deltoides*. 2-1) Shoot initiation from immature embryo and expanded cotyledon; arrow indicates the multiple shoots from the cotyledon. 2-2 and 2-3) Shoot elongation of multiple shoots in half-strength medium containing 0.02 mg/l IBA. 2-4) Plants after transplanting to soil mix and 6-weeks growth in a greenhouse (Kang and Hall 1996b)

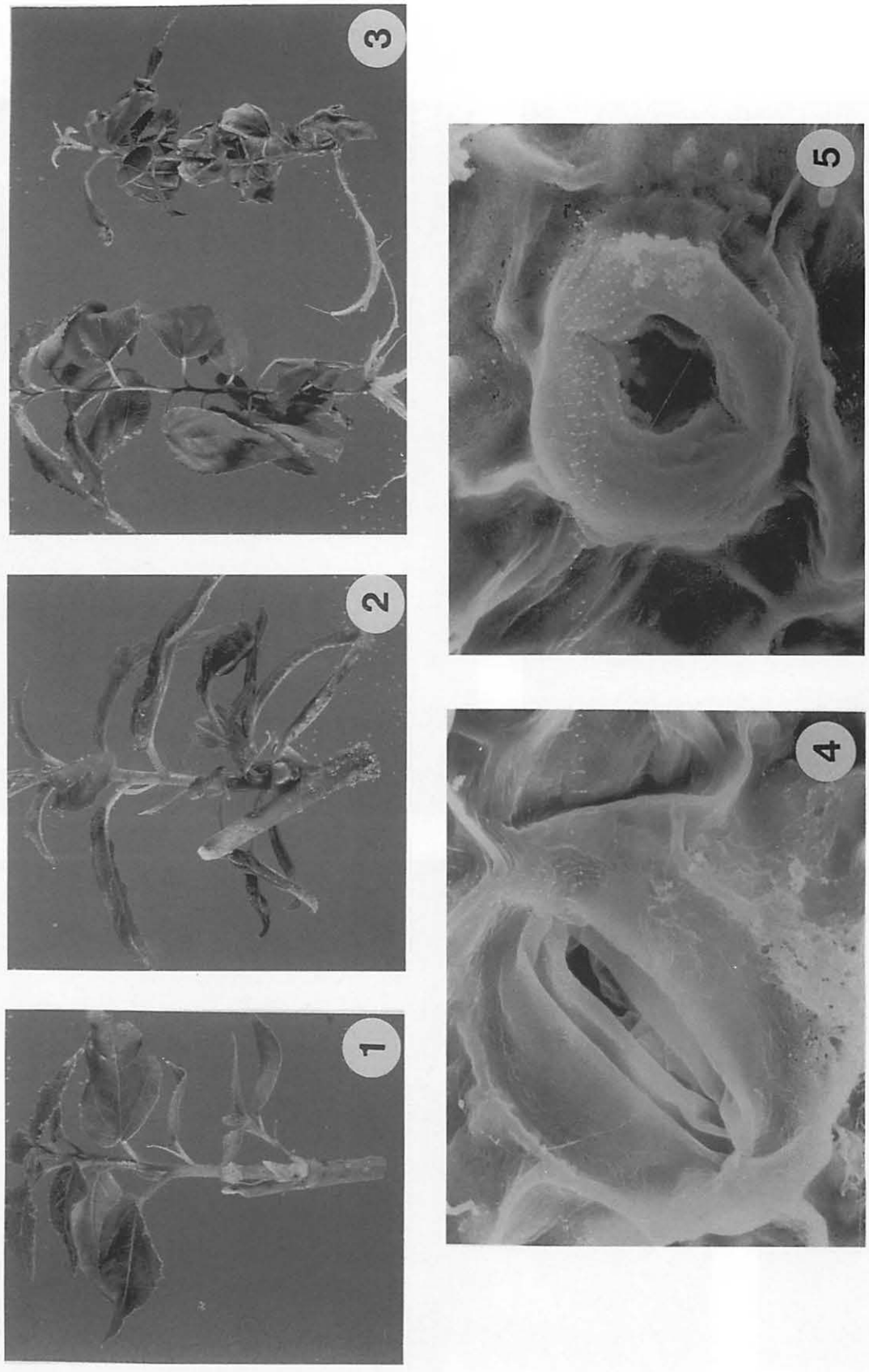

Figure 3. Shoot vitrification from stem nodal cultures of *Populus alba* x *P. grandidentata*, 'Crandon' clone. 3-1) Normal shoot induced from an axillary bud. 3-2) Vitrified shoots induced from an axillary bud. 3-3) Shoot elongation on the half-strength Murashige and Skoog (MS) medium. 3-4) Normal stomate of nonvitrified leaf. 3-5) Abnormal stomate of vitrified leaf (Kang and Hall 1996c).

from developing ovules cultured on vitamin-supplemented WPM containing BA and zeatin as cytokinins. Multiple shoots were obtained from immature ovules collected 20 days after pollination. Generally, zeatin was better than BA for inducing multiple shoots. The highest mean number (56.1 ± 7.4) of shoots was from immature ovules on WPM supplemented with 5.0 mg/l zeatin (figures 2-1 and 2-2). Roots formed after small clumps of shoots were transferred to half-strength WPM containing 0.02 mg/l indole-3-butyric acid (IBA) (figure 2-3). A survival rate of 97 percent was achieved by transferring plantlets to a greenhouse environment (figure 2-4).

Development of shoots from excised flower buds is extremely rare in tree species. Bawa and Stettler (1972) cultured female catkin primordia of *P. trichocarpa*. Although unsuccessful in developing whole plants, they found a general tendency for callus formation with increasing culture age, occasionally followed by a reversal to vegetative growth. Chung et al. (1993) successfully established a flower-bud culture system to maximize shoot regeneration of *P. deltoides* and *P. maximowiczii*. They used small slices (5 to 6 mm thick) of catkin prepared from well-developed (3 to 4 cm long) flower buds that were cultured on MS medium. An average of 3.8 shoots from *P. deltoides* and 4.1 shoots from *P. maximowiczii* were regenerated from catkin after culturing on MS medium containing 1.0 mg/l BA with 0.05 mg/l NAA or 0.5 mg/l BA with 0.05 mg/l NAA, respectively. More than 20 shoots can be regenerated from a single mature catkin of poplar such as *P. deltoides*, which is typically recalcitrant to shoot regeneration. Adventitious shoots were initiated from 3 locations on the explant; the outer and the cut surface of stalks, and the marginal area of small bracts.

Callus Culture

Calli, groups of undifferentiated cells, are induced from various explants, such as stem, leaf, root, embryo, pollen, or protoplast. Plants regenerate in 2 ways from calli manipulation: 1) organogenesis in which shoots form directly from calli; and 2) somatic embryogenesis from embryogenic development. Pioneering studies for *in vitro* shoot regeneration were conducted on callus cultures of triploid quaking aspen (Winton 1968; Wolter 1968) and quaking aspen (Winton 1970). Wolter (1968) suggested that shoots were induced on callus explants of *P. tremuloides* by supplementing the basal medium with 0.2 to 0.5 mg/l BA, and that fresh shoots were rooted on the same medium supplemented with 0.04 mg/l 2,4-D and 1.0 mg/l kinetin. *In vitro* plantlets were regenerated through organogenesis in *Populus* species (Coleman and Ernst 1990; Noh and Minocha 1986; Park and Son 1988). Cytokinin treatments of 0.2 to 1.0 mg/l BA or 2.0 to 5.0 mg/l zeatin were used for shoot regeneration.

McCown et al. (1988) defined nodules from cell suspension culture as independent, spherical, dense cell clusters that form a cohesive unit and display a consistent internal cell/tissue differentiation. They demonstrated that nodule culture has a high potential for organogenesis. Chung and Chun (1991) obtained fine nodules from cell suspension cultures of *P.* x *euramericana* and *P. nigra* x *P. maximowiczii* on MS medium with an optimal combination of BA and NAA. When these fine nodules were transferred to liquid regeneration medium with BA and NAA, an average of 27 shoots per nodule was induced after 8-weeks culture.

Overall, plant morphogenesis through *in vitro* culture depends on species, explant, type and concentration of plant growth regulators, culture medium composition, and culture environment. For further application, systems for successful plant regeneration need careful study to optimize these parameters.

Physiological Changes of *In Vitro* Cultured Plantlets

Vitrification is a physiological disorder affecting *in vitro* plantlets (Kevers et al. 1984). Although most vitrification studies have focused on herbaceous species, some woody species have been studied (Letouze and Daguin 1987; Marin et al. 1988; McLaughlin and Karnosky 1989). Numerous studies have reported on the abnormalities caused by factors such as medium type (Earle and Langhans 1975), gelling agents (Debergh 1983), organics (Zimmerman and Cobb 1989; Ziv et al. 1983), inorganics (Vieitez et al. 1987), and plant growth regulators (Leshem et al. 1988; Paques and Boxus 1987). In comparison with normal leaves of *in vitro* plantlets, vitrified plantlets have malfunctional stomata (Brainerd et al. 1981; Marin et al. 1988; Miguens et al. 1993; Ziv et al. 1987); unorganized vacuolated, spongy mesophyll cells (Earle and Langhans 1975; Vieitez et al. 1987); large intercellular spaces (Brainerd et al. 1981); reduced epicuticular waxes (Sutter 1985); and low chlorophyll content (Phan and Letouze 1983; Ziv et al. 1983). Vitrified *in vitro* plantlets also poorly acclimatize after transfer to soil, a problem for commercial production (Debergh et al. 1981). Vitrified plantlets demonstrated an increased cellular volume (Paques and Boxus 1987; Vieitez et al. 1985; von Arnold and Ericksson 1984), and vascular bundles were less developed in vitrified leaves (Leshem 1983; Ziv et al. 1983). Several rescue methods using modifications of media

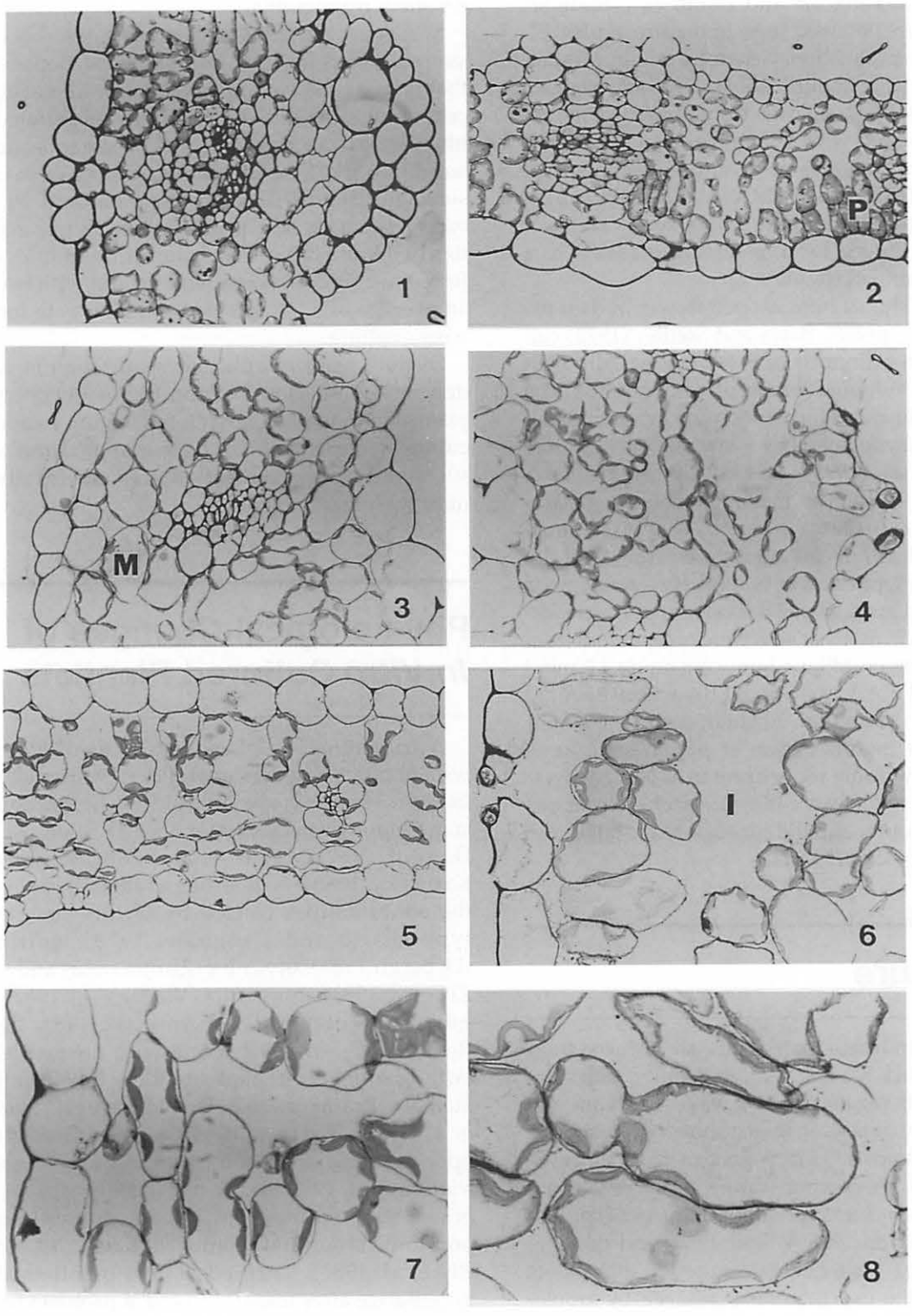

Figure 4. Morphological characteristics of leaf vitrification in the culture of *Populus alba* x *P. grandidentata*. 4-1) Normal leaf of 'Crandon' clone. 4-2) Adaxial orientation of normal 'Crandon' clone, P=palisade parenchyma. 4-3 and 4-4) Abnormal leaf of 'Crandon' clone; M=spongy mesophyll. 4-5) Normal leaf of 'Hansen' clone. 4-6) Abnormal leaf of 'Hansen' clone; I=enlarged intercellular space. 4-7) Normal leaf of 'Hansen' clone. 4-8) Abnormal leaf of 'Hansen' clone (Kang and Hall 1996c).

components have been reported (McLaughlin and Karnosky 1989; Sato et al. 1993).

In liquid culture, vitrified plantlets produce more ethylene, suggesting that increased 1-aminocyclo-propane-1-carboxylic acid (ACC) and ethylene production is a nonwound response to stress (Kevers et al. 1984). Subsequently, ACC and ethylene cause metabolic changes that activate peroxidases such as indole-3-acetic acid (IAA) and ACC oxidases. Increased peroxidase activities were detected in vitrified tissue of herbaceous and woody plantlets (Kervers et al. 1984).

Kang and Hall (1996c) studied *in vitro* morphological abnormalities in hybrid aspen (*P. alba* x *P. grandidentata* cv. 'Crandon' and cv. 'Hansen'). Morphological differences in stomata and other cell types were investigated through scanning electron microscopy. Phenotypic differences are shown in figures 3-1 to 3-3. In vitrified leaves of 'Crandon' and 'Hansen,' stomatal shape was circular and inflated with larger stomatal apertures (figures 3-4 and 3-5); however, stomatal densities were similar between normal and vitrified leaves. Vitrified leaves were thicker than those of normal leaves (figure 4). Mesophyll cells were arranged irregularly and palisade parenchyma was less developed in vitrified leaves (figures 4-1 to 4-4). Chloroplasts of vitrified leaves were arranged sparsely within the larger mesophyll cells, whereas they were highly condensed in normal leaves (figures 4-5 to 4-8).

Although many reports relate to vitrification studies in herbaceous horticultural species, only a few papers have been published for woody species. The morphological study of Kang and Hall (1996c) is the only report on the physiological variations related to vitrification in *Populus* species. Studies on such physiological phenomena provide a basis for developing successful *in vitro* culture systems.

Summary

Organogenesis has become a basic technique for advancing studies such as genetic manipulation and can provide a primary plant regeneration system to obtain "true-to-type" plantlets. Fundamental studies on *in vitro* physiology are prerequisites for developing and optimizing a successful tissue culture system.

Biotechnology can support future industrial applications to produce synchronized plantlets for tree-breeding programs and secondary metabolites for pharmaceutical programs. Further applications of biotechnology require more studies on: 1) *in vitro* physiological phenomena; 2) development of generalized regeneration systems for poplar species and hybrids; 3) variation among plantlets from different explants; and 4) transplanting for greenhouse growth.

Acknowledgments

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