Chapter 6

Germplasm Preservation of *Populus* Through *In Vitro* Culture Systems

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**Introduction**

Demand for germplasm preservation of various plant species has recently increased due to air pollution, climate change, acid rain, natural destruction, illegal collection, and human impact to ecosystem biodiversity. Forested land in the world is rapidly declining at an annual rate of 9.9 million ha (Singh and Janz 1995). As a result, there has been a decrease in the genetic pool of forest tree species, including wild genotypes that could serve as future breeding sources.

Seed storage is common for germplasm preservation of most plant species. For *Populus* species, seed longevity under natural conditions is from 2 weeks to 1 month, depending on the species and/or clone, time of collection, and environmental conditions (Graham et al. 1964; Trappe 1964). The longevity of seed storage can be greater than 2 years by drying the seeds soon after collection, adjusting seed moisture content to 8 percent, using sealed containers in cold storage, or storing vacuum-packed seeds under freezing conditions (Trappe 1964). Even then, seed propagation of selected tree genotypes may not guarantee the preservation of genetic traits such as fast growth and . Because of the vast land area requirement and the difficulty in controlling pests and/or disease, *in situ* and *ex situ* conservation of vegetatively propagated plants is challenging.

In this review we: 1) suggest an alternative approach for germplasm preservation through an *in vitro* culture system; 2) discuss methods of germplasm preservation; 3) discuss the currently available technology; and 4) suggest future strategies.

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**Poplars as a Model System for Germplasm Preservation *In Vitro***

The genus *Populus* includes about 30 species that are widely distributed throughout the North Temperate Zone (between the Arctic Circle and the Tropic of Cancer). This wide distribution represents considerable species’ adaptability and demonstrates its potential over a wide geographic region. Among the woody plants, poplar is one of the most intensively studied species for breeding because of the enormous genetic variation that exists. Thousands of poplar clones are being tested and hundreds of these are commercially propagated throughout the world (Sato 1959). *Populus* spp. are fast growers and have modest nutritional requirements, therefore, they are an economically important source of pulp for paper industries and are considered an energy crop. Also, cryopreservation of cells/tissues requires relatively little space because regeneration of *Populus* plantlets from single-cell/protoplast cultures is possible. Commercial production of selected individuals does not depend on seed reproduction because vegetative macro and micropropagation methods without adulteration or dilution of their genetic potential have been intensively studied (Ahuja 1987; Park and Son 1988). Problems controlling open pollination under natural conditions makes conservation of taxonomically diverse and newly created clones by *in situ* or *ex situ* preservation programs for poplar germplasm difficult.

Development of reliable micropropagation systems is essential for germplasm preservation *in vitro*. Many reports of *in vitro* propagation technology of poplars have further developed this species as a model system for germplasm preservation (Aitken-Christie and Singh 1987; Ahuja 1987; Moon et al. 1987; Park and Son 1988; Son et al. 1991; Stoehr
and Zsuffa 1990). In many clones with a high regeneration capacity in a microenvironment, plantlets from shoot-tip cultures were used in large-scale production and their productivity was found superior to conventionally propagated plants (Chen and Huang 1980; Lester and Berbee 1977; Mehra and Cheema 1980). Most tissues and organs can serve as initial explant materials for in vitro culture of Populus spp. Germplasm preservation through in vitro culture is advantageous because: 1) entire sets of genetic materials can be copied through regeneration schemes; 2) rapid proliferation is achievable; 3) minimal space is required; 4) disease-free plants can be produced and maintained; and 5) risks related to environmental changes are avoided.

Low-Temperature Storage

Low temperature (about 4 °C) has been widely applied for short-term, minimal-growth storage of cultured plant cells, tissues, and organs. To prepare for nursery planting during the optimal growing season, low-temperature storage was used successfully for large-scale synchronization of transplanted propagules (Son et al. 1991). If suitable methods are developed, valuable genotypes can be selected at the in vitro level.

For low-temperature storage in vitro, many techniques can be applied individually or in combination. For example, methods related to maintenance of plant materials under conditions of a mineral oil overlay (Caplin 1959), low-level illumination (Preil and Hoffmann 1985), complete darkness (Marino et al. 1985), reduced temperature (Bhojwani 1981), various osmoticum or plant growth regulators (Henshaw et al. 1978), modified culture atmosphere (Moon and Kim 1987), and modified subculturing schedules (Withers 1985) have been described.

Although nonfrozen storage systems for germplasm preservation have been reported, such studies focus on herbaceous and/or crop species and may not apply directly to woody plants. During low-temperature poplar storage, the duration, previous subculturing, and various culture media affect the survival rate (Son et al. 1991). With hybrid aspen (Populus alba x P. grandidentata), the previous subculturing period and medium composition are critical variables for the success of germplasm preservation under low-temperature storage (Son et al. 1991). In our experiment, after 9-months of storage at low temperature, hybrid aspen shoots exhibited slow growth, yet continued to possess some healthy, green leaves and stems (figure 1b). After 20-months of cold storage, shoot cultures had nearly stopped incremental height growth and had thin stems and small, chlorotic leaves. According to one report, explant size can greatly influence survival during cold storage (Aitken-Christie and Singh 1987). In our experiment, no significant difference in cold-storage survival was observed among the five shoot sizes (1, 3, 5, 7, and 9 cm) tested; although 3 cm shoots did exhibit a slightly higher survival rate. Under low-temperature storage, survival rates after 2-years and 5-years cold storage were 75 percent and 25 percent, respectively. From 2,000 plantlets transplanted in the greenhouse and/or nursery, less than 1 percent displayed phenotypic variation related to growth performance or pigment accumulation.

Cryopreservation

Cryopreserved plant cells, tissues, and organs stopped their metabolic functions when exposed to ultra-low temperature (-196 °C); yet they retained viability. In this condition, plant cells, tissues, and organs are preserved for a long period of time. Cryopreservation systems can reduce the cost of conventional labor-intensive nursery practices and minimize genetic changes, such as point mutation and/or ploidy level change, which may occur during long-term subculture. However, developing a reliable cryopreservation system involves several complicated steps. In many examples of cryopreservation, only a few species were successfully preserved (Bajaj 1977; Kartha et al. 1979; Kartha et al. 1980; Uemura and Sakai 1980). In other examples, further investigations are needed to optimize treatments at general stages of pregrowth, cryoprotection, freezing, thawing, and recovery (Chen and Kartha 1987). Preconditioning, such as cold treatment during culture or applying osmotically active chemicals to the medium before cryopreservation, was beneficial for viability (Fuchigami et al. 1981). Plant samples require sufficient desiccation before cooling in liquid nitrogen. Without such care, most samples produce intracellular ice crystals that eventually cause cell mortality.

When poplar callus was used for ultra low-temperature storage, optimal results were obtained with the cells from the end of lag phase or exponential growth phase, which occurs approximately 1 week after subculture (data is not shown). This may relate to lower water content and smaller vacuole size of optimal source cells. Pretreatment with cryoprotective chemicals, dimethyl sulfoxide (DMSO), glycerol, polyvinylpyrrolidone (PVP), and dextran were useful separately or in combination. Although its mode of action is not clearly understood, DMSO may form multiple hydrogen bonds that prevent water from crystallizing at intracellular levels. Although cryoprotective chemicals were harmful to plant cells in our experiment, DMSO treatments produced 62 percent survival after long-term preservation; until now, calli were stored for 5 years and survival tests were conducted annually.
Protocols

Species used for low-temperature storage and cryopreservation were *Populus alba* x *P. grandidentata* and *P. glandulosa* (Suwon poplar), respectively. Protocols described here are based on preliminary experiments; supporting data are not included.

**Low-Temperature Storage**

a) Shoot cultures were established by bud cultures from greenhouse-grown stock plants. Shoot multiplication, elongation, and root induction media were Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 1.33 μM 6-benzyladenine (BA), no plant growth regulators, and 0.98 μM indole-3-butyric acid (IBA), respectively. Cultures were maintained under a 16-h light regime (photosynthetic photon flux rate of 40 to 60 μM m⁻² s⁻¹ from cool-white, fluorescent tubes) at 26±1 °C.

b) Shots approximately 3 cm long were used (figure 1a).

c) Shoots were maintained on shoot multiplication medium for 1 month before cold storage.

d) Magenta GA-7 culture vessels (7.6 x 7.6 x 10.2 cm, Magenta Corp., Chicago, IL) containing 50 ml medium and test tubes containing 15 ml of medium were used for cold storage.

e) To prevent drying, culture vessels were double sealed with Nesco film (Bando Chemical Ind., Ltd., Kobe, Japan) and stored within two transparent plastic bags.

f) During cold storage, cultures were maintained at 4 °C without light.

g) After 4-weeks subculture of cold-stored plants on fresh MS medium, each shoot was excised from its root system and transferred to a Polyterra™ plug styrofoam tray (M40045, Techniculture Co., Salinas, CA) and eventually to pots containing artificial soil mix (vermiculite : perlite : peat at 1:1:1) (figure 1f).

**Cryopreservation**

a) Callus was induced from cambial tissues isolated from *in vitro*, greenhouse-, and field-grown donor plants.

b) Callus proliferation was conducted on MS medium with 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D).

c) Cold hardening was performed at 10 °C for 1 week before placing the callus into cryogenic plastic vessels and adding DMSO (callus : DMSO at 1:5 weight/volume) as a cryoprotectant solution.

d) Further cooling was initiated by incubating the callus on ice for 1 to 2 h.

e) A freezing temperature ranging from -25 to -45 °C was obtained using a programmable freezer controller to drop the cooling temperature by 0.5 to 1 °C per minute, followed by liquid nitrogen immersion.

f) For regrowth of stored callus, temperature was increased in reverse of the freezing procedures but quicker. Before culturing, calli were rinsed several times with MS liquid medium containing no plant growth regulators.

g) After subculturing on MS basal medium supplemented with auxin at the appropriate levels, newly grown callus was collected for use as shoot induction material.

h) For regeneration, selected callus was subdivided into small pieces (5 to 10 mm in diameter) and cultured on a shoot induction medium supplemented with optimal levels of zeatin (figure 2c).

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**Limitations**

Plant cell and tissue culture techniques have developed rapidly and the knowledge associated with culturing systems has increased significantly in comparison to other plant science fields. Based on the theory of totipotency, any cells, tissues, and organs should be regenerable; however, successful establishment of reliable regeneration systems is limited, especially for woody plants. This restriction frequently confines the application of tissue culture methods and restricts plant biotechnological applications for many valuable species.

A basic premise of *in vitro* conservation techniques for plant germplasm is that genetic materials must be preserved without genetic alteration at any level. Most tissue culture work is based on adventitious shoot regeneration systems. These systems are typically associated with a prolonged undifferentiated phase before regeneration. However, such culture systems have generated phenotypic, genotypic, and/or biochemical variation (Bebeli and Kaltsikes 1990; D’Amato 1978; Whelan 1990). Although this variation frequently occurs at negligible levels, the potential for genetic variation should be addressed.

There is also potential for genetic alteration associated with low and ultra-low temperature storage methods. In a nonfrozen storage system, for example, phenotypic variation was reported for traits such as color accumulation in leaves (Son et al. 1991), organ shape, seed yield, and photoperiodic response (Grout 1990). During cryopreservation at -196 °C, most cellular chemical reactions cannot occur because the energy levels are too low to allow sufficient molecular motion to complete enzymatic reactions. Nevertheless, certain chemical reactions that affect genetic alteration by damaging nucleic acids via ionizing radiation can accrue at unacceptable levels. Although this kind of chromosome damage is observed in plants and microorganisms under storage conditions, DNA repair mechanisms to overcome damage from freezing or low temperature conditions are not fully understood.

Most storage systems have not provided 100 percent survival and are not adaptable for diverse species. Intensive studies are needed to develop...
methodology and accumulate relevant knowledge for successful germplasm storage of target plant materials.

Conclusions and Prospects

Due to rapid environmental destruction and associated declines in biological diversity, genetic resource conservation has become critical. Recently, major approaches to genetic resource conservation such as ex situ and in situ preservation programs were established for forestry. Although these approaches have merit, a successful genetic conservation program will require modification and/or a combination of existing methods with new technology to meet current needs.

Advances in plant tissue culture systems are widely applicable throughout plant science. This technology is also a valuable tool for tree germplasm conservation through low- or ultra-low temperature storage. Recent progress in somatic embryogenesis may provide other opportunities for storage material. In particular, somatic embryos exhibit high genetic stability and are easily manipulated. Above all, relatively simple desiccation methods that reflect the physiology and development of zygotic embryos offer additional potential for extending the longevity of germplasm storage in vitro.

Because poplar has demonstrated totipotency at diverse cell levels, it is a model species for mass clonal propagation, genetic transformation, and other biological research of woody plants. For the conservation and preservation of tree germplasm, intensive studies are needed to refine associated methods and to provide reliable and reproducible results.

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Literature Cited


