OPTIMIZATION OF A CRYOPRESERVATION PROTOCOL FOR EMBRYOGENIC CULTURES OF YELLOW-POPLAR *(LIRIODENDRON TULIPIFERA)*

Wagner A. Vendrame[1], Christopher P. Holliday[1], Dale R. Smith[2], and Scott A. Merkle[1]

Somatic embryogenesis has the potential to be used for mass propagation of superior and genetically engineered forest tree genotypes. The long time needed for evaluation of regenerated and/or transgenic material, however, makes it imperative that the source cultures be maintained for long periods of time. Cryopreservation has been used as a suitable and efficient means for long term storage of plant tissue and cell cultures. Applications in forest species include white spruce (Kartha et al., 1988), radiata pine (Hargreaves and Smith, 1992), and silver birch (Ryynanen, 1996), among others. Embryogenic cultures in particular have been proven amenable to cryostorage. Cryopreservation promotes reduction in labor and supply costs, reduces chances for contamination, provides retention of embryogenic capacity, limits somaclonal variation, maintains transgene stability, and reduces culture deterioration. Thus, cryopreservation is an especially attractive tool for forest tree embryogenic cultures.

Three embryogenic yellow-poplar culture lines (one initiated in 1992 and two initiated in 1997) were selected for this study. In phase I, suspension culture lines were proliferated for one week liquid induction/maintenance (IM) medium (Merkle and Sommer 1986) to induce an optimum stage for cryopreservation. The cultures were then submitted to osmotic pretreatment using IM medium supplemented with sorbitol, aiming to minimize the formation of microscopic ice crystals within the cells. Two sorbitol levels were tested, 0.2 M and 0.4 M. The cultures were placed on an orbital shaker (100 rpm), in the dark, for 24 hours. In order to moderate dehydration injury, cultures were subjected to pre-chilled DMSO (dimethylsulfoxide), pretreatment, which acts as a cryoprotectant. However, DMSO is toxic to the cells. Therefore, three levels of DMSO, 5%, 10%, and 15%, were tested in order to find the lowest level to minimize toxicity while still providing cryoprotection.

Half of the cultures were submitted to phase II, while the other half were used as controls. Control cultures were collected after phase I on filter paper using mild vacuum, and placed on a square of 30-um nylon mesh, which was placed onto semi-solid IM medium in Petri dishes. They were then transferred to fresh IM medium at 1 hr, 24 hr, and 7 days, for removal of residual DMSO. Plates were then stored in a dark incubator at 24 °C. Recovery was evaluated after 2 weeks.

Cultures subjected to phase II were cryopreserved using a modified protocol by Hargreaves and Smith (1992). Aliquots of 1 ml from each pretreatment were pipetted into pre-chilled 2-ml cryovials and placed into a pre-chilled Nalgene freezing container (Mr. Frosty). The container was transferred to the ultra-cold freezer (-70°C) for 1.5 hr. This allows a freezing rate of approximately 1°C per minute to -70°C. Samples were then placed into a Nalgene cryobox and submerged in liquid nitrogen (-196°C) in a Cryosafe freezer for 24 hr. Following removal of the material from liquid nitrogen, cryovials were transferred from the cryobox to the floating tray component of the Mr. Frosty container and placed into a water bath at 40°C for 20 minutes to allow gradual thawing. The cultures were then collected on filter paper under mild vacuum and plated on nylon mesh as in phase I, and recovery was evaluated after 2 weeks.

Rates of recovery were variable among the three lines and all six treatments. Survival rates from 25% to 50% were observed for the combinations of 0.2 M sorbitol with either 10% or 15% DMSO. The variable survival rates were probably due the toxic effect of DMSO to the cells. However, one particular 1997

[1]D.B. Warnell School of Forest Resources, University of Georgia, Athens, GA, 30602-2152, USA
[2] Metagenetics, 93 State Highway 30, RD4, Rotorua, New Zealand

line showed recovery of 100% for the same treatments. Combinations of 0.2 M sorbitol with 5% DMSO, and 0.4 M sorbitol with 15% DMSO achieved survival rates varying from 50 to 75%, for all 3 lines. The best treatments however were the combinations of 0.4 M sorbitol with 5% DMSO and 0.4 M sorbitol with 10% DMSO. In both cases, 100% recovery was obtained for all 3 lines.

In this initial experiment, contamination was observed two weeks following recovery of cultures from cryopreservation. We found that the contamination source was the sterilization protocol for the nylon mesh used to collect the cultures after thawing. However, despite the contamination, cultures showed continued growth until they were discarded to avoid contamination spread. The whole experiment has recently been replicated with a new sterilization protocol for the nylon mesh. Cultures have been thawed and are in the process of recovery evaluation. Our preliminary observations confirm the results obtained during the first experiment. For more accurate evaluation of the results, cultures will be weighed on a weekly basis for growth analysis. No contamination has been observed in this replication to date.

Our results indicate that cryopreservation offers the potential to become a routine procedure in tissue culture laboratories, where cultures will be able to be stored for long periods of time and retrieved according to the researcher's needs. Challenges include further improvements in the protocol, comparisons of cryopreservation of cultures in liquid suspensions vs. semi-solid media, and most importantly, the recovery analysis of cultures following longer periods of cryostorage, such as one year or more.

Keywords: cryopreservation, somatic embryogenesis, yellow-poplar, *Liriodendron tulipifera*

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