OPTIMIZATION OF THE YELLOW-POPLAR EMBRYOGENIC SYSTEM

S.A. Merkle, R.J. Sotak, A.T. Wiecko and H.E. Sommer[1]

Abstract.--Experiments directed at improving the efficiency of yellow-poplar (Liriodendron tulipifera) somatic embryogenesis have focused on (1) Increasing the percentage of yellow-poplar cultures that become embryogenic and (2) Increasing the percentage of somatic embryos that are well-formed and capable of conversion to plantlets. To raise the percentage of embryogenic cultures, we have concentrated on defining the optimal stage of zygotic embryo explant using morphological and biochemical developmental markers. Yellow-poplar cultures were initiated from developing zygotic embryos from seeds collected every two weeks from 4 weeks post-pollination (June 10) until seed maturity (September 14). Potential of an explant to produce an embryogenic culture peaked during the 8th week following pollination, with over 25 percent of the explants producing embryogenic callus in most full-sib families. Production of cultures with embryogenic callus declined to near zero for mature zygotic embryos. The maximum embryogenic potential for an explanted zygotic embryo corresponded to the globular to early heart stages of embryo development. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of zygotic embryos from 5 sampling datas showed that decline of embryogenic potential appeared to be correlated with an increase in the level of a protein of approximately 55 kD, and some qualitative changes in polypeptides. To promote the production of well-formed somatic embryos capable of conversion to plantlets, we tested physical and chemical treatments on embryogenic suspension cultures. Proembryogenic masses removed from auxin-supplemented medium and placed in hormone-free medium generally produced malformed and clustered somatic embryos that were asynchronous in their development and that germinated precociously. Production of synchronous, mature, well-formed embryos was promoted by fractionating embryogenic suspensions on sieves and culturing in a medium supplemented with 5×10^{-7} M abscisic acid (ABA). However, conversion of the embryos was inhibited once they were transferred to solid medium, possibly due to the effects of residual ABA.

Keywords: Liriodendron tulipifera, somatic embryogenesis, SDS-polyacrylamide gel electrophoresis, abscisic acid.

[1] Assistant Professor, Graduate Research Assistant, Research Technician and Associate Professor, School of Forest Resources, University of Georgia, Athens, GA 30602.

INTRODUCTION

Yellow-poplar (*Liriodendron tulipifera* L.) probably attains the greatest height of any broad-leaved tree in eastern North America and may also reach the largest diameter (Harlow et al. 1979). The species, characterized by straight form, rapid growth and wood of exceptional working quality (Wilcox and Taft 1969) is considered to be one of the most important hardwoods in the United States. Our first report of somatic embryogenesis from yellow-poplar tissue cultures was based on four embryogenic lines derived from immature zygotic embryo explants from seeds of a single mother tree (Merkle and Sommer 1986). Explants produced a fast-growing, pale yellow, nodular callus following 1-2 months culture on a modified Blaydes' (Witham et al. 1971) conditioning medium supplemented with 2 mg/1 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/1 6-benzyladenine (6BA) and 1 g/1 casein hydrolysate (CH). Within 1 month following transfer of embryogenic callus to a hormone-free induction medium, somatic embryos differentiated. A very low percentage of embryos began to convert spontaneously on induction medium and when moved to a Risser and White's (1964) medium (RW), would develop into plantlets. Over the past two years, we have moved hundreds of somatic embryo-derived plantlets from in *vitro* conditions, acclimatized them in a controlled humidity chamber, transferred them to the greenhouse and from the greenhouse to the nursery. The first field planting of yellow-poplar somatic embryo-derived plantlets was made in Spring, 1989. We have also reported on the isolation and culture of protoplasts from yellow-poplar embryogenic suspensions, from which we have been able to regenerate somatic embryos and plantlets of protoplast origin (Merkle and Sommer 1987).

Although the embryogenic cultures which we initiated in 1984 produced thousands of embryos, there remained problem areas which limited the usefulness of the system for large scale production of plantlets. One of these was our ignorance regarding the optimum explant for use in initiating embryogenic cultures. Although we determined that only immature zygotic embryos had the potential to intiate embryogenic callus, we did not know if one particular developmental stage had greater potential than other stages. Neither did we know if the ability to produce embryogenic callus was genotype-specific, since all cultures in our first report originated from a single mother tree. There were also problems at the other end of the process, in the area of conversion of somatic embryos to plantlets. Although thousands of somatic embryos were produced, over 99% were malformed, with fused cotyledons. These malformed embryos, almost without exception, failed to convert. A number of these embryos germinated, if germination is defined as radicle elongation. However, the shoot apices failed to develop. Malformation and low conversion rate were even worse problems when somatic embryos were differentiated in liquid media shake cultures. Until the study described here, we had never obtained a plantlet from an embryo raised in liquid medium.

The research reported here was undertaken with the goal of overcoming the culture initiation and conversion problems described above, with the following objectives: (1) to find the optimal developmental stage of zygotic embryo for explanting to obtain embryogenic cultures, and to look

for biochemical markers to help identify that stage, and (2) to discover nethods for the high-frequency production of well-formed somatic embryos with a high conversion percentage.

MATERIALS AND METHODS

Explant optimization

Our approach to determining the optimal zygotic embryo developmental stage for explanting was to sample developing fruit from a number of controlled crosses made at the University of Tennessee's yellow-poplar 'reeding orchard in 1987 and 1988 by Dr. S.E. Schlarbaum and Mr. R.A. Cox. fruit were sampled at 2-week intervals throughout the summer and early fall of each year. In 1987, samples were collected at 10, 12, 14, 16 and 18 weeks post-pollination. In 1988, samples were collected at 4, 6, 8, 10, 12, 14, 16 and 18 weeks post-pollination. As each sample was received, the samaras of each cross were divided into 2 subsamples. In subsample 1, samaras were surface-sterilized and dissected, and the embryos and endosperm were placed on solid conditioning medium as described in Merkle and Sommer (1986). Explants and callus growing from them were transferred monthly to fresh conditioning medium and at the end of 3 months, the cultures were scored for production of embryogenic callus. In subsample 2, samaras were dissected and embryos were measured for total length, cotyledon length and hypocotyl thickness. Then, the embryos were placed in 2DMH sample buffer (Mayer et al. 1987) and stored at -70 C until all samples had been received. Stored embryos were assayed for protein developmental markers by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a Bio-Rad Mini-Gel (8 cm wide by 7 cm long) apparatus, using 7 or 10% polyacrylamide, run at 150 V (constant voltage) for approximately 45 min. Proteins were visualized in the gels either by staining with Coomassie Brilliant Blue R-250 or by silver staining using a protocol supplied by R.P. Feirer (Institute of Paper Chemistry, Appleton WI, personal communication). Buffer, gel and stain recipes used can be found in Sotak (1989). We then tried to relate sampling date, embryo measurements and quantitative and qualitative changes in embryo proteins to the potential of an explant to initiate an embryogenic culture.

Improving embryo form and conversion rates

Our first step in raising the numbers of plantlets we could obtain from each culture was to use embryos which differentiated on solid induction medium. Our standard procedure with our first cultures was to wait until embryos began to green-up and elongate before picking individual well-formed embryos off of the plates and moving them to RW medium to complete conversion. Since the embryos were at least 5 mm long at this point, it meant we were actually waiting for conversion to begin before transferring the embryos. Using this method, a typical yield of 10-20 plantlets per gram of embryogenic callus moved to a plate of induction medium was achieved. Given that some clonal lines produced thousands of embryos per gram of callus, the conversion rate was 1% or less. Our first attempt to improve the conversion rate was to make early selections of well-formed embryos at the 1.5-2 mm torpedo stage, prior to greening. This size corresponds to

that of a mature zygotic embryo, and selection required the use of a dissecting microscope. The primary criterion for selecting an embryo was that it have 2-3 separated cotyledons. Usually 20-30 such embryos could be picked off of a plate every 2 weeks for approximately 2 months. Individual embryos were transferred to one of four "secondary" media (induction medium, induction medium minus CH, induction medium minus CH and solidified with agarose instead of agar, and induction medium minus CH and with only 1% sucrose instead of 4% sucrose) for 7-10 days, scored for the presence of expanded and greening cotyledons, transferred to RW medium and scored for conversion after 1 month.

Additional experiments to improve embryo form and conversion percentage were conducted using embryogenic suspension cultures as the starting point. Suspension cultures were used for a number of reasons. First, suspension cultures have the potential to be "scaled-up" into systems such as bioreactors, where very large numbers of propagules may be produced with small amounts of labor, compared to cultures grown on solid media. Secondly, using suspension cultures as the starting point had the advantage of allowing synchronization of the developing embryos by fractionation of the proembryogenic masses (PEMs) and embryos on sieves. Such a synchronization would also enhance any scale-up for mass propagation since a bioreactor-type system would only be useful if it produced large numbers of mature embryos at the same time. However, our main reason for fractionating the embryos was that we felt that only by observing our treatment effects on synchronized embryos would we be able to determine if our treatments aimed at producing well-formed embryos were effective.

Our fractionation/synchronization procedure was based on that used by Giuliano et al. (1983) with carrot somatic embryos. Briefly, approximately 1 g of PEMs growing in liquid conditioning medium was placed in liquid induction medium. PEMs were sieved on a 140 pm stainless steel screen and the fraction that passed through was sieved once more on a 38 pm screen. The fraction remaining on the 38 pm screen was saved and cultured for one week in induction medium. Then the developing embryos derived from the PEMs were sieved on a 230 pm screen, and the fraction that passed through was sieved on a 140 pm screen. The fraction remaining on this screen was saved and cultured for 1 week, producing embryos that were, at least in gross a^{p}pearance, synchronized at the early torpedo stage. Then, embryos were moved to solid induction medium for conversion.

Treatments aimed at improving embryo form and conversion percentage were of two types: (1) those imposed during synchronization in liquid induction medium, and (2) those imposed post-synchronization, following transfer of embryos from liquid induction medium. Treatments imposed during synchronization were primarily aimed at producing well-formed embryos, i.e. with 2-3 distinct cotyedons, not fused in a multiple-embryo cluster, and with no premature radicle elongation. We assumed that well-formed embryos would have a high probability of converting. Treatments consisted of the supplementation of liquid induction medium with varying levels of abscisic acid (ABA), mannitol, sorbitol or sucrose. ABA was of particular interest, since Ammirato (1974) showed that ABA at the proper concentration in liquid medium produced well-formed caraway somatic embryos with distinct cotyledons

that of a mature zygotic embryo, and selection required the use of a dissecting microscope. The primary criterion for selecting an embryo was that it have 2-3 separated cotyledons. Usually 20-30 such embryos could be picked off of a plate every 2 weeks for approximately 2 months. Individual embryos were transferred to one of four "secondary" media (induction medium, induction medium minus CH, induction medium minus CH and solidified with agarose instead of agar, and induction medium minus CH and with only 1% sucrose instead of 4% sucrose) for 7-10 days, scored for the presence of expanded and greening cotyledons, transferred to RW medium and scored for conversion after 1 month.

Additional experiments to improve embryo form and conversion percentage were conducted using embryogenic suspension cultures as the starting point. Suspension cultures were used for a number of reasons. First, suspension cultures have the potential to be "scaled-up" into systems such as bioreactors, where very large numbers of propagules may be produced with small amounts of labor, compared to cultures grown on solid media. Secondly, using suspension cultures as the starting point had the advantage of allowing synchronization of the developing embryos by fractionation of the proembryogenic masses (PEMs) and embryos on sieves. Such a synchronization would also enhance any scale-up for mass propagation since a bioreactor-type system would only be useful if it produced large numbers of mature embryos at the same time. However, our main reason for fractionating the embryos was that we felt that only by observing our treatment effects on synchronized embryos would we be able to determine if our treatments aimed at producing well-formed embryos were effective.

Our fractionation/synchronization procedure was based on that used by Giuliano et al. (1983) with carrot somatic embryos. Briefly, approximately 1 g of PEMs growing in liquid conditioning medium was placed in liquid induction medium. PEMs were sieved on a 140 pm stainless steel screen and the fraction that passed through was sieved once more on a 38 pm screen. The fraction remaining on the 38 pm screen was saved and cultured for one week in induction medium. Then the developing embryos derived from the PEMs were sieved on a 230 pm screen, and the fraction that passed through was sieved on a 140 pm screen. The fraction remaining on this screen was saved and cultured for 1 week, producing embryos that were, at least in gross appearance, synchronized at the early torpedo stage. Then, embryos were moved to solid induction medium for conversion.

Treatments aimed at improving embryo form and conversion percentage were of two types: (1) those imposed during synchronization in liquid induction medium, and (2) those imposed post-synchronization, following transfer of embryos from liquid induction medium. Treatments imposed during synchronization were primarily aimed at producing well-formed embryos, i.e. with 2-3 distinct cotyedons, not fused in a multiple-embryo cluster, and with no premature radicle elongation. We assumed that well-formed embryos would have a high probability of converting. Treatments consisted of the supplementation of liquid induction medium with varying levels of abscisic acid (ABA), mannitol, sorbitol or sucrose. ABA was of particular interest, since Ammirato (1974) showed that ABA at the proper concentration in liquid medium produced well-formed caraway somatic embryos with distinct cotyledons

Prior to 10 weeks post-pollination, zygotic embryos were too small to isolate for use in protein analysis, so SDS-PAGE could only be used to visualize proteins from embryos from 10, 12, 14, 16 and 18 weeks postpollination. However, since the 10-weeks postpollination embryos showed the second highest embryogenic potential (Fig. 2), we believed comparisons among these stages would still be useful. The most conspicuous marker was a protein of approximately 55 kD, which showed a large increase in abundance from the first through the fifth sampling dates, correlating with an increase in embryo size and a decrease in embryogenic potential. This polypeptide was barely visible 10 weeks postpollination, but became easily visible at 12 weeks and was the most abundant protein for weeks 14-18. A protein with apparent molecular weight of 62 kD was present at 10 and 12 weeks postpollination, but disappeared in embryos from weeks 14-18, when embryogenic potential dropped to essentially O. The identity of these proteins is unknown, but it is possible that the 55 kD polypeptide is a seed storage protein.

Improving embryo form and conversion rates

Early selection of well-formed somatic embryos at the torpedo stage of development for movement to a secondary medium resulted in an average (over 3 clonal lines and 4 media) of 68% embryos with expanded and greening cotyledons and 37.3% conversion to plantlets one month following transfer to RW medium. Of the secondary media used, induction medium minus CH gave significantly (a = .05) higher percentages of embryos with expanded and greening cotyledons (84.6%) and significantly higher conversion percentages (63.3%) than did regular induction medium (40.9% and 16.2% respectively). Lowering the sucrose concentration to 1% or substituting agarose for agar in the CH-free induction medium did not significantly change the conversion percentage from that of regular CH-free induction medium.

For embryogenic suspension cultures, we found that fractionation on stainless steel screens worked quite well in gross synchronization of developing embryos. However, fractionation did not completely eliminate the production of clusters of multiple embryos from the PEMs. It also failed to raise the frequency of well-formed embryos in liquid media. Embryos continued to enlarge and elongate without developing distinct cotyledons.

The addition of ABA to the liquid induction medium had an observable impact on the development of fractionated somatic embryos. Depending on the clonal line used, ABA at a concentration between 10^{-7} M and 10^{-5} M prevented the production of multiple embryos, inhibited elongation of embryos and raised the frequency of embryos with 2-3 distinct, separated cotyledons. In general, concentrations lower than 10^{-7} M had little apparent effect on somatic embryo development, while concentrations higher than 10^{-5} M completely inhibited development of embryos. In the latter case, embryos would rarely develop past the globular stage and loose cells would proliferate around the periphery of the embryos. For most lines tested, the ABA concentration producing the highest proportion of well-formed embryos (approximately 20-30%) was 5×10^{-7} M. When the fractions that did not pass the 140 pm mesh in the first fractionation or the 230 pm mesh in the second fractionation were cultured in the same concentration of ABA, embryo

development was less affected, resulting mainly in the production of larger, malformed embryos. Thus it appears that for ABA to have an effect on yellow-poplar somatic embryo development, the embryos must be exposed to it at an early stage, which we selected for by culturing the smaller fractions.

Mannitol or sorbitol at 8% had similar effects to ABA in that the elongation of embryos was inhibited, but in general, cotyledon development was not enhanced by addition of these osmotica at any concentration we tried. However, raising the sucrose concentration of the induction medium from 4 to 10% had very similar effects to that of ABA in that many embryos formed distinct cotyledons, although we did not quantify the percentage of well-formed embryos for this treatment.

Although both ABA and sucrose appeared to have a positive effect on the production of well-formed embryos, once these embryos were transferred to solid medium, they failed to convert to plantlets at a rate higher than embryos not selected for distinct cotyledons. Thus our assumption that well-formed embryos would convert at a high frequency, based on our experience with embryos differentiated on solid medium, did not apply to embryos differentiated in liquid medium. Furthermore, none of the post-synchronization treatments listed above succeeded in raising the frequency of conversion of liquid-differentiated embryos above the few plantlets obtained by simply transferring embryos to regular induction medium. Of the thousands of well-formed embryos differentiated in liquid medium to date, we have obtained less than 100 total plantlets.

We believe that the long period of time somatic embryos spend differentiating in shake culture may be responsible for their poor performance once transferred to solid medium, since well-formed embryos differentiated on solid medium which appear the same outwardly as liquid-raised embryos have a very high conversion percentage. It is also possible that residual ABA from the liquid medium prevents conversion. Currently, we are attempting to combine the high frequency production of synchronous somatic embryos in liquid medium with the higher conversion rates of embryos differentiated on solid medium by fractionating embryogenic suspension cultures at the PEM stage and immediately plating the selected fractions on solid induction medium. Experiments are underway to determine which modifications of the solid induction medium will give the most efficient plant let production.

LITERATURE CITED

Ammirato, P.V. 1974. The effects of abscisic acid on the development of somatic embryos from cells of caraway (Carum carvi L.). Bot. Gaz. 135(4):328-337.

Giuliano, G., D. Rosellini, and M. Terzi. 1983. A new method for the purification of the different stages of carrot embryoids. Plant Cell Rep. 2:216-218.

Harlow, W.M., E.S. Harrar, and F.M. White. 1979. Textbook of Dendrology, Sixth Edition. McGraw-Hill, New York. 510 p.

Mayer, J.E., G. Hahne, K. Palme, and J. Schell. 1987. A simple and general plant tissue extraction procedure for two-dimensional gel electrophoresis. Plant Cell Rep. 6:77-81.

Merkle, S.A., and H. E. Sommer. 1986. Somatic embryogenesis in tissue cultures of Liriodendron tulipifera. Can. J. For. Res. 16:420-422.

Merkle, S.A., and H.E. Sommer. 1987. Regeneration of Liriodendron tulipifera (family Magnoliaceae) from protoplast culture. Amer. J. Bot. 74(8):1317-1321.

Risser, P.G., and P.R. White. 1964. Nutritional requirements for spruce tumor cells in vitro. Physiol. Plant. 15:620-635.

Sotak, R.J. 1989. Optimization of explant choice for initiation of embryogenic yellow-poplar cultures. M.S. Thesis, School of Forest Resources, University of Georgia, Athens, GA. 152 p.

Wilcox, J.R., and K.A. Taft. 1969. Genetics of yellow-poplar. USDA Forest Service Res. Pap. WO-6. 12 p.

Witham, F.H., D.F. Blaydes, and R.M. Devlin. 1971. Experiments in Plant Physiology. Van Nostrand-Reinhold Co., New York. 245 p.