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MATURATION AND CONVERSION OF *LIRIODENDRON TULIPIFERA* SOMATIC EMBRYOS

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SUMMARY

Embryogenic suspension cultures of the hardwood forest tree yellow-poplar (*Liriodendron tulipifera*) have the potential to produce millions of plantlets. However, low conversion frequencies limit the realization of this potential. Using 4 embryogenic yellow-poplar lines, we first tested the ability of somatic embryos, selected for their similarity to mature zygotic embryos, to convert to plantlets, then tested physical and chemical treatments for their effects on promoting maturation of somatic embryos and subsequent plantlet production. Embryos selected based on resemblance to mature zygotic embryos and transferred to a hormone-free basal medium without casein hydrolysate (CH) produced plantlets at a frequency of 63%. Populations of synchronized somatic embryos were obtained by repeated fractionation of liquid medium-cultured proembryogenic masses (PEMs) on stainless steel sieves. These fractionated embryos failed to mature properly when cultured in liquid basal medium, however. Development of embryos cultured in basal medium supplemented with 5×10^{-7} *M* abscisic acid (ABA) was slowed and embryos appeared to mature properly, with separated cotyledons and little precocious germination. However, ABA-treated embryos only rarely converted to plantlets, possibly due to residual effects of the ABA. PEMs fractionated on sieves, transferred to filter paper and placed on solidified basal medium gave a 60-70% synchronous population of mature embryos 10-12 days following plating. Mature embryos transferred to basal medium without CH converted at a frequency of 72%. The percentage of all embryos differentiating from PEMs on filter paper that formed plantlets was 32%.

Key words: Liriodendron tulipifera; yellow-poplar; somatic embryo; conversion; maturation; synchronization.

INTRODUCTION

Somatic embryogenesis has been reported for a number of angiosperm and coniferous forest trees in recent years (25,28). However, in many of these reports, conversion of somatic embryos to plants capable of surviving outside of *in vitro* conditions was not achieved. One review of somatic embryogenesis in woody perennials (25) showed that of 81 species in which somatic embryogenesis had been reported, only 34 had produced plantlets capable of growing in "soil." In addition, in many of the studies reporting conversion, only limited numbers of plantlets were actually produced. The low proportion of species for which large-scale plantlet production has been obtained reflects the fact that in many of these reports somatic embryogenesis has been followed by failure of the somatic embryos to complete the normal stages of embryogeny displayed by zygotic embryos. Among hardwood tree species, developmental failure often results in the production of malformed embryos characterized by fused or multiple cotyledons, double apices, dactyloid clusters, or other abnormal structures (16,21,24,26). However, in other cases, even somatic embryos which appeared to be normal outwardly (i.e. resembling mature zygotic embryos) failed to convert to plantlets (14,23).

Divergence from the normal pattern of development is not limited to somatic embryos of forest trees, and overcoming this problem has been the goal of a number of studies with plant embryogenic systems. Methods employed to induce somatic embryos to mature properly and/or convert to plantlets have included treatment with abscisic acid (ABA) (1,2,3,5,7,27), osmoticum (3,7,), gibberellic acid (GA) (9,18) or benzyladenine (BA) (9), inverting somatic embryos so that the cotyledons are in contact with the medium (2), changing the type of carbohydrate in the medium (22), cold treatment (18,26), and desiccation (8,9,11,15,29). Many of these treatments (e.g. cold, desiccation, GA) were aimed at inducing the somatic embryos to break dormancy or quiescence known to be characteristic of zygotic embryos of the same species, while others (e.g. ABA, osmoticum) were applied to obtain mature embryos that were visually normal and did not germinate precociously.

Not only has the proportion of forest tree embryogenic systems capable of producing plantlets been low, but

even in those studies where plants were obtained, the efficiency of their production was usually not reported. Recently, Becwar et al. (2), provided the first quantitative data on the frequency of maturation, germination and conversion to plantlets of somatic embryos of a coniferous species. Their report showed an overall conversion efficiency of 0.5% for Norway spruce (*Picea abies*) somatic embryos. Similar quantitation has not been reported for any hardwood forest tree species to date.

Since somatic embryogenesis in the hardwood forest tree species yellow-poplar (*Liriodendron tulipifera* L.) was first reported (13), one major obstacle limiting the usefulness of the system for mass propagation has been the low frequency with which somatic embryos have converted to plantlets. Somatic embryos could be produced by the thousands in 4-6 wk by transferring proembryogenic masses (PEMs) from auxin-supplemented induction medium to basal medium. However, total plantlets derived from the somatic embryos only numbered in the hundreds (Merkle, unpublished data). The reason for low plantlet production was the failure of most embryos to complete the normal sequence of embryo maturation. Less than 1% reached a stage resembling zygotic yellow-poplar embryos found in mature seeds, i.e. 1-2 mm long, with 2 distinct cotyledons. Instead, most embryos were malformed, often with fused cotyledons (13). Embryos with fused cotyledons would swell, followed by either radicle elongation (germination), callus formation, or secondary embryo production, usually near the radicle apex. Germination of malformed embryos was only rarely accompanied by apical development and could be described as precocious, in that it began without proper embryo maturation. However, the resulting germinants did not fit the classic symptoms of precocious germination (17), because they were swollen and robust, rather than weak or spindly. The primary characteristic which distinguished them from normal plantlets was their lack of apical development.

With these problems of maturation and conversion in mind, our objectives in this study were: (1) to test the hypothesis that somatic embryos resembling mature zygotic embryos convert to plantlets at a higher frequency than randomly-picked embryos, (2) to test different chemical and physical treatments for their effect on the percentage of somatic embryos capable of maturing into embryos visually resembling normal zygotic embryos, in both solid and liquid media, and (3) to evaluate the effect of these same factors on conversion rates of somatic embryos into plantlets.

Materials and Methods

Plant culture material. Four embryogenic yellow-poplar lines, initiated as described previously (13), were used for the experiments reported here. Briefly, to initiate cultures, yellow-poplar samaras were disinfested using Clorox (5.25% sodium hypochlorite), and immature zygotic embryos and endosperm were dissected from the samaras and explanted onto a Blaydes' (30) induction medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l BA, 1 g/l casein hydrolysate (CH), 40 g/l sucrose, and 8 g/l Phytagar (GIBCO). Three of the sources for these explants were seeds resulting from controlled pollinations of three unrelated selections at the University of Tennessee's yellow-poplar seed orchard, carried out by Dr. S. E. Schlarbaum (Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN), and one was from an open-pollinated Athens, GA source. Following approximately 6 mo. of growth on induction medium, embryogenic suspension cultures were initiated for each of the lines by inoculating 125 ml flasks containing 40 ml of liquid induction medium with approximately 1 g of proembryogenic masses (PEMs). Suspension cultures were grown in the light at 22° C on a gyratory shaker at 90 rpm, and were maintained by transferring approximately 1 g of PEMs to fresh induction medium at 3 week intervals.

Early selection on solid medium. A preliminary test of our hypothesis that well-formed embryos would convert to plantlets at a level higher than 1% was made using the 4 embryogenic lines described above. For embryo development, approximately 1 g of PEMs from each line growing in induction medium was plated directly on a Blaydes' (30) basal medium, containing no hormones, but with 1 g/l CH, 40 g/l sucrose, and 8 g/l Phytagar, for embryo development. As new embryos differentiated, they were selected under a dissecting microscope based on their physical resemblance to zygotic embryos found in mature yellow-poplar seeds. Mature somatic embryos were placed on one of 4 "secondary media" that had shown promise in preliminary tests in promoting germination and cotyledon expansion. At least 20 embryos were placed on each secondary medium from each clone. Secondary media were (1) basal medium with CH, (2) basal medium without CH, (3) basal medium without CH, and solidified with 0.8 g/l SeaPlaque (FMC Corp.) agarose instead of Phytagar, and (4) basal medium without CH and with only 10 g/l sucrose. After 1 wk, the percentage of embryos with green, expanding cotyledons was computed. Then, those embryos with expanding cotyledons were transferred to test tubes containing 20 ml of plantlet development medium (13), a modified Risser and White's (19) medium with 2% sucrose, for 1 mo. An embryo was considered to have converted to a plantlet when it had germinated and had produced at least one non-cotyledon leaf, since virtually 100% of regenerants reaching this stage survived transfer from *in vitro* conditions (Merkle, unpublished data). Conversion frequency was computed as the total number of plantlets divided by the total number of mature embryos placed on secondary medium. Conversion percentages were transformed using an arcsin transformation (20), and the resulting angles were analyzed using analysis of variance and Duncan's New Multiple Range Test for multiple comparisons of means. The entire experiment was replicated twice, using the same 4 clonal lines.

Embryogeny in liquid medium. In order to test the effects of various treatments on embryo maturation, it was desirable to standardize the developmental stage of the embryos employed in the experiments by synchroniz-

ing them. A protocol previously used to synchronize carrot PEMs and somatic embryos by fractionating them on different screen mesh sizes (10) was modified for use with embryogenic yellow-poplar suspensions. Approximately 1 g of PEMs growing in liquid induction medium was placed in liquid basal medium. PEMs were sieved on a 140 μm stainless steel screen; the fraction that passed through was then sieved on a 38 μm screen. The fraction remaining on the 38 μm screen was cultured for one week in basal medium. Developing embryos derived from the PEMs were sieved on a 230 μm screen; the fraction that passed through was resieved on a 140 μm screen. The fraction remaining on this screen was cultured in liquid basal medium.

In addition to fractionation, treatments were applied to developing embryos during the synchronization in liquid basal medium and tested for their effect on production of mature embryos. These treatments included the following modifications of the liquid basal medium: (1) ABA at 0, 10^{-7}, 10^{-6} or 10^{-5} *M*, (2) sucrose at 1%, 4% or 10% (w/v), and (3) sorbitol or mannitol at 0%, 4%, 6% or 8% (w/v). One week following the second fractionation of developing embryos, mature embryos were plated on hormone-free basal medium without CH for one week. Embryos with green, expanding cotyledons were transferred to test tubes containing 20 ml of plantlet development medium and scored for conversion to plantlets after 1 month.

Plating fractionated PEMs. Due to problems with conversion of somatic embryos following prolonged culture in liquid medium (see Results), a third protocol for maturation and conversion of somatic embryos was adopted, which was a combination of the two protocols described above (fractionation in liquid medium and early selection on solid medium). The objective was to obtain large numbers of roughly synchronous embryos which developed from PEMs on solid medium rather than in liquid. The protocol was tested using the same 4 clones used with the other methods. Approximately 1 g of PEMs growing in induction medium was placed in liquid basal medium. PEMs were sieved on a 140 μm stainless steel screen and the fraction passing through was resieved on a 38 μm screen. The fraction remaining on the 38 μm screen was backwashed from the screen onto black filter paper. The excess liquid medium was allowed to drain into 2 layers of gel dryer filter paper backing (Bio-Rad Co.) placed below. Then the filter paper with PEMs was placed directly on a 60 mm petri dish containing solidified basal medium and incubated at 30° C under a 14 h photoperiod with cool white fluorescent light for 12 days. Total embryos (globular stage or later) and mature embryos on each plate were counted and the percentage of mature embryos was calculated. The white embryos were easily visible against the black filter paper, facilitating counting.

Samples of mature embryos from each of the four clones were used to test the same 4 secondary media as were tested with early-selected embryos for their effect on conversion frequency. Conversion frequency was computed as the percentage of mature embryos that formed plantlets. Also, an overall conversion rate was computed by multiplying the percentage of embryos on the filter paper that were mature by the percentage of mature embryos that formed plantlets. Conversion rates were analyzed using the procedures described for early-selected embryos. The experiment was replicated twice.

RESULTS

Early selection on solid medium. Fig. 1 *A* shows mature yellow-poplar somatic embryos developing from a mass of asynchronous PEMs prior to selection for transfer to a secondary medium for further development and germination. Effects of the 4 secondary media on conversion variables of early-selected embryos are shown in Table 1. Cotyledon expansion percentage, scored while the early-selected embryos were still on secondary medium, was highest for basal medium without CH (92.3%), which was significantly higher than that for basal medium with CH (40.9%), but not significantly higher than on the other two secondary media. Conversion frequency for embryos germinated on basal medium without CH was 63.2%, which was significantly higher than for any other secondary medium. Basal medium with CH resulted in a conversion rate of only 16.2%. However, even this relatively low conversion frequency supported our hypothesis that selected well-formed mature embryos were capable of converting at rates higher than the rates (<1%) typical of non-selected embryos. Averaged over all secondary media and clones, the selected mature embryos converted at a rate of 36.6%.

Embryogeny in liquid medium. PEMs of any of the 4 embryogenic lines transferred from liquid induction medium to liquid basal medium without fractionation developed asynchronously and often formed clusters of embryos at various stages of embryogeny (Fig. 1 *B*). Fractionation of PEMs had two beneficial effects on the production of somatic embryos. First, fractionation effectively synchronized development, at least in the outward appearance of the embryos. Second, fractionation accelerated the differentiation of somatic embryos from PEMs almost 3-fold over non-fractionated cultures allowed to differentiate in liquid or on solid medium. Fig. 1 *C* shows the selected fraction of somatic embryos 3 days after their second sieving (10 days following transfer to hormone-free medium and first sieving). Typically at this point, we could obtain at least 90% pure globular stage embryos, with very few clusters of embryos or undifferentiated cells. However, over the next week, embryos maintained in liquid medium failed to mature properly. Fig. 1 *D* shows the same culture of fractionated embryos as Fig. 1 *C*, 1 wk later (10 days following second sieving). As demonstrated in Fig. 1 *D* few embryos would proceed through the classic heart or torpedo stages of embryo development. Instead, most appeared to skip these final stages of maturation and begin elongation of both the radicle and the cotyledons, with no distinct separation of the cotyledons. A few of these embryos would also produce secondary embryos.

Of the treatments applied to the fractionated embryos while in liquid culture, ABA had the most evident impact on embryo development, although response to a given ABA level varied somewhat with clone. At 10^{-7} *M*, ABA

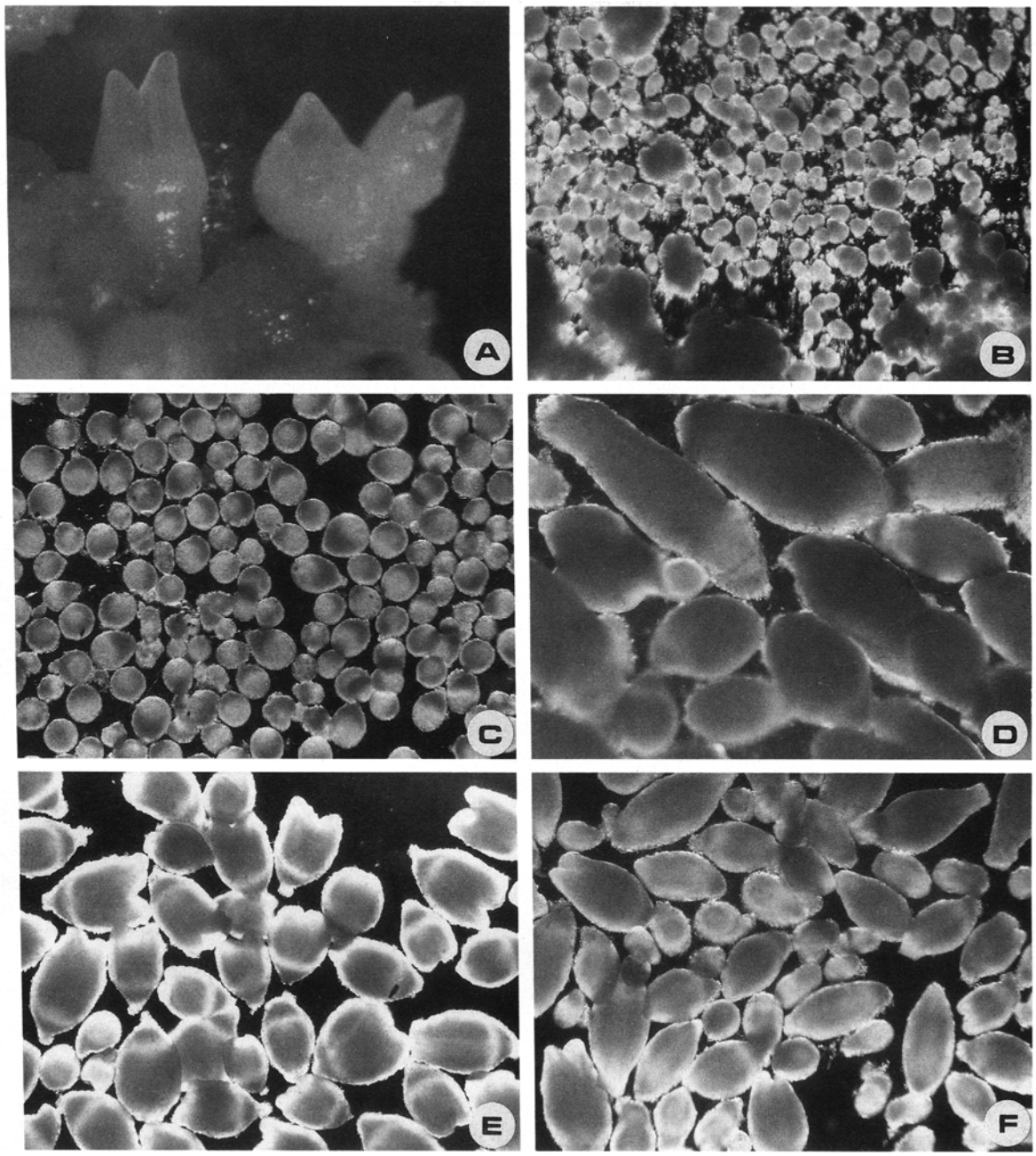

FIG. 1. Effects of fractionation, ABA and sucrose on maturation of yellow-poplar somatic embryos. *A*, Mature yellow-poplar somatic embryos just prior to transfer to secondary medium (67×). *B*, Non-fractionated yellow-poplar proembryogenic masses (PEMs) in liquid basal medium one week following transfer from induction medium (21×). *C*, Globular stage somatic embryos derived from fractionated PEMs, 3 days following second sieving, in liquid basal medium (21×). *D*, Elongating somatic embryos derived from fractionated PEMs, 10 days following second sieving, in liquid basal medium (21×). *E*, Heart-stage somatic embryos derived from fractionated PEMs, 7 days following second sieving, in liquid basal medium with 5×10^{-7} *M* ABA. Note distinct cotyledons and proliferation of cells around periphery of embryos (21×). *F*, Torpedo stage somatic embryos derived from fractionated PEMs, 7 days following second sieving, in liquid basal medium with 10% sucrose (21×).

had little effect on embryo maturation, and embryos continued to elongate prematurely. At 10^{-5} *M* ABA, embryos appeared to be blocked at the globular stage, with no further maturation or elongation. In most cases free cells proliferated around the periphery of embryos cultured in 10^{-5} *M* ABA. ABA at 10^{-6} *M* (later modified to 5×10^{-7} *M*) appeared to provide a balance between the two responses, allowing the embryos to complete maturation while preventing premature elongation. In some cultures, up to 50% of the embryos continued development to the heart or early torpedo stages about 1 wk following their second sieving (Fig. 1 *E*). Embryo clusters and the production of secondary embryos were virtually eliminated.

TABLE 1

EFFECT OF SECONDARY MEDIUM ON COTYLEDON EXPANSION AND CONVERSION OF SOMATIC EMBRYOS SELECTED FOR MATURITY FROM ASYNCHRONOUS CULTURES ON SOLID BASAL MEDIUM

Secondary Medium	% Embryos With Expanded Cotyledons (S.E.)[a]	% Conversion of Mature Embryos (S.E.)
Basal without CH[b]	92.3 (7.5) a[c]	63.2 (9.2) a
Basal without CH, with agarose	78.0 (9.4) a b	38.6 (5.2) b
Basal without CH, with 1% sucrose	68.2 (10.8) a b	28.7 (5.2) b
Basal	40.9 (15.0) b	16.2 (6.4) b

[a] Values represent the means of 4 clones, with 2 determinations per clone.

[b] CH = 1 g/l casein hydrolysate.

[c] Means followed by the same letter were not found to be significantly different at the $\alpha = .05$ level using Duncan's Multiple Range Test. Percentages were transformed to angles (arcsin transformation) prior to analysis.

Osmoticum treatments (mannitol, sorbitol, sucrose) tested for their effects on embryo maturation affected embryo development only at the highest levels tested, i.e. 8% for sorbitol and mannitol, 10% for sucrose. At these levels, embryo growth was slowed or completely inhibited. However, embryo maturation was not outwardly improved as was observed with ABA. Sucrose at 10% allowed a few of embryos to develop distinct cotyledons prior to elongation (Fig. 1 *F*).

Well-formed, mature somatic embryos completing development to the torpedo stage in liquid culture were expected to convert at a high frequency following transfer to solid basal medium minus CH, as had mature embryos selected from solid medium (*see* Early Selection on Solid Medium results above). However, the majority of embryos treated with ABA failed to even germinate (data not shown), and except for a few plantlets, those that did germinate showed no apical development, even though cotyledons greened and expanded slightly. It should be noted here that a number of treatments were applied to these mature-appearing embryos to induce them to convert following their transfer from liquid medium. These treatments included: (1) culture on solid basal medium without CH (control), and the following treatments followed by culture on solid basal medium minus CH: (2) culture on agar-water for 1 week, (3) one-hour shake-rinse in distilled water, or 10^{-4}, 10^{-5} or 10^{-6} *M* GA, (4) cold treatment (stratification) on basal medium at 6° C for 1, 2 or 3 months. We also tested the following modifications of the basal medium: (a) addition of 2 g/l activated charcoal, (b) addition of 10^{-5} or 10^{-6} *M* GA, (c) addition of 10^{-5} or 10^{-6} *M* 6BA. None of these treatments resulted in an improvement in conversion frequency (data not shown).

Plating fractionated PEMs. Fractionation of PEMs growing in liquid medium immediately followed by plating on basal medium with filter paper produced roughly (60–70%) synchronous populations of somatic embryos (Fig. 2 *A*). As with fractionated embryos grown in liquid medium, differentiation to the globular stage was rapid, averaging only 8 days. Also similar to fractionated liquid-differentiated embryos, embryos occurred singly and there was little secondary embryo production. Thus each PEM appeared to produce a single embryo (Fig. 2 *B*). Embryos matured rapidly, with the majority of torpedo stage embryos appearing on each plate within 12 days after plating of PEMs (Fig. 2 *C*). For the 4 clones, total numbers of embryos differentiated per gram of sieved PEMs averaged approximately 750, while the number of these embryos that appeared mature after 12 days averaged 410 (data not shown). Mature embryos were harvested from the primary medium plates and germinated (Fig. 2 *D*) on 4 types of secondary medium prior to transfer to plantlet development medium. Results of combining this fractionation/plating method with 4 different secondary media are presented in Table 2. For mature embryos, basal medium without CH gave a significantly higher conversion frequency (71.9%) than basal medium with CH (53.4%) or basal medium without CH with only 1% sucrose (44.6%). The relative performance of embryos germinated on basal medium with CH versus those germinated on basal medium without CH is illustrated in Fig. 2 *E*, which shows the embryos just prior to transfer to plantlet development medium. Without CH, the embryos germinated more vigorously and cotyledons expanded more rapidly. Replacing agar with agarose in the CH-free secondary media did not significantly alter conversion of mature embryos (64.2%). As shown in Table 2, conversion frequency of total embryos formed on primary medium (overall conversion frequency) was also highest for embryos germinated on basal medium without CH (32.5%), although not significantly higher than for those germinated on regular medium (25.3%).

DISCUSSION

The primary aim of the early selection experiment was to test our hypothesis that selection of yellow-poplar somatic embryos for maturity resulted in a higher frequency of conversion to plantlets. In this experiment, however, embryos were selected and isolated from masses of asynchronously-developing embryos and PEMs. Thus, the higher conversion rates obtained in this study may have been due to microenvironmental changes resulting from removal of embryos from the hormonal or osmotic effects of surrounding embryos and callus. It is possible that embryos allowed to develop from PEMs to maturity and germinated free of the influence of other embryos would mature properly and convert to plantlets. However, as shown by the impact of the different secondary media, other factors were involved in conversion. The presence or absence of CH in the secondary medium on which embryos were germinated was responsible for an average of 50% difference in the subsequent conversion of the

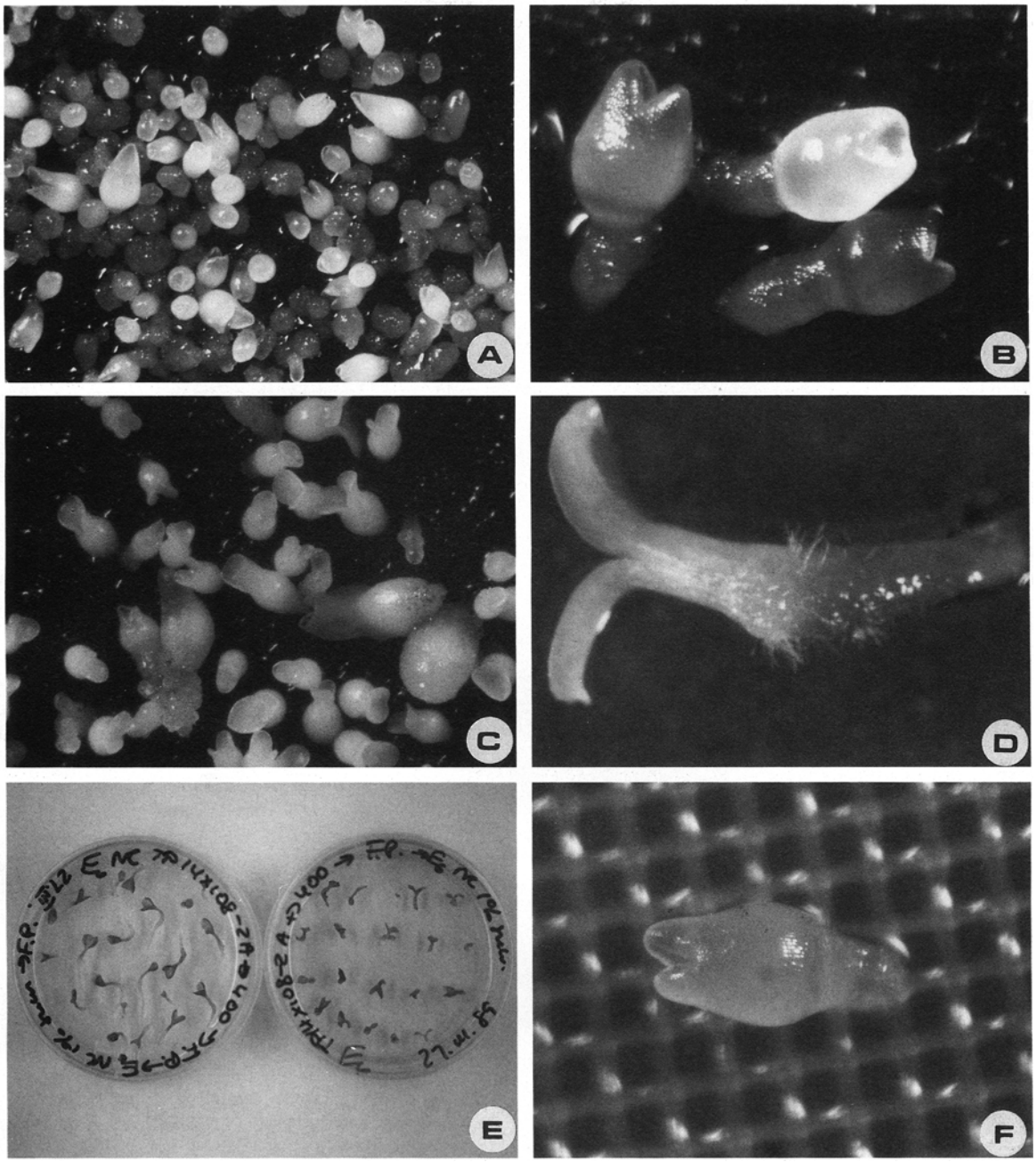

FIG. 2. Effects of fractionation and filter paper/nylon mesh plating of yellow-poplar PEMs on somatic embryo maturation and germination. *A*, Population of roughly synchronous somatic embryos, derived from fractionated PEMs, 10 days following plating on filter paper on top of solid basal medium (16×). *B*, Early torpedo-stage somatic embryos developing independently on filter paper on solid basal medium, each apparently derived from a single PEM (67×). *C*, Torpedo stage somatic embryos, 12 days following plating of fractionated PEMs on filter paper on top of solid basal medium (16×). *D*, Germination of selected mature embryo on basal medium without CH, 6 days following transfer from filter paper (16×). *E*, Effects of 2 secondary media on cotyledon expansion and germination of somatic embryos derived from fractionated PEMs cultured on filter paper. Embryos in the left plate were cultured on basal medium without CH, while those in the right plate were cultured on basal medium with 0.1% CH. *F*, Early torpedo stage somatic embryo derived from fractionated PEM plated on nylon mesh on top of solid basal medium (67×).

germinants to plantlets on plantlet development medium. Although one explanation of this impact may be that the metabolism of the developing embryos was altered by the loss of the constituent amino acids of CH, we believe that a simpler explanation is that the lower osmotic potential of the medium lacking CH promoted germination of mature embryos placed on it. This conclusion is supported by experiments which established that 1% CH prevented precocious germination of barley zygotic embryos by generating high osmotic potential in the

TABLE 2

EFFECT OF SECONDARY MEDIUM ON CONVERSION OF MATURE EMBRYOS AND OVERALL CONVERSION OF EMBRYOS OBTAINED FROM FRACTIONATED PEMs PLATED ON FILTER PAPER

Secondary Medium	% Conversion of Mature Embryos (S.E.)[a]	% Conversion Overall (S.E.)[b]
Basal without CH[c]	71.9 (4.7) a[d]	32.5 (3.6) a
Basal without CH, with agarose	64.2 (3.8) a b	30.0 (3.6) a b
Basal	53.3 (5.2) b c	25.3 (3.9) a b
Basal without CH, with 1% sucrose	44.6 (5.0) c	20.7 (3.7) b

[a]Values represent the means of 4 clones, with 2 determinations per clone.

[b]Overall conversion percentage was computed by multiplying the percentage of embryos on the filter paper that were mature by the conversion percentage of mature embryos.

[c]CH = 1 g/l casein hydrolysate.

[d]Means followed by the same letter were not found to be significantly different at the $\alpha = .05$ level using Duncan's Multiple Range Test. Percentages were transformed to angles (arcsin transformation) prior to analysis.

medium (31). In our case, basal medium with 0.1% CH had an average osmotic potential of −393 kPa, which was significantly more negative than that of basal medium lacking CH (−350 kPa), as measured using a Wescor PR-55 psychrometer microvoltmeter and C-52 sample chambers (data not shown). Thus even at 0.1%, CH could have some impact on germination by its effect on the osmotic potential of the medium. However, lowering the sucrose concentration of CH-free medium from 4% to 1%, one effect of which was to further lower the water potential, actually decreased conversion frequency. Since our design did not test the interactive effects of CH and sucrose, their roles in promoting or inhibiting conversion remain unclear.

Fractionated PEMs developing into embryos in liquid medium appeared to follow a normal developmental pattern, at least up to the globular stage. However, as with embryos allowed to remain on solid basal medium, they subsequently seemed to proceed into a germination phase without reaching full maturity. Thus synchronization in itself did not improve maturation. The failure of these embryos to complete maturation could be modified, at least outwardly, by the application of ABA and to a lesser extent by raising the sucrose level. These treatments seemed to slow embryo development. Premature elongation was also inhibited, at least to the point where distinct cotyledons appeared prior to elongation. ABA at its optimal level (5×10^{-7} *M*) had the additional effect of suppressing the formation of multiple embryos from individual PEMs and the production of secondary embryos. As was reported by Ammirato (1), the effect of ABA was highly dependent on the stage at which the developing embryos were exposed to it. For example, when the fraction of PEMs that failed to pass the 140 μm screen was cultured in basal medium with 5×10^{-7} *M* ABA, precocious germination was much less inhibited than with the fraction that passed the same screen. Thus PEMs exposed to ABA at an early stage (i.e. while they are smaller) were more likely to have their development altered by the exogenous ABA.

As evidenced by our failure to obtain reliable conversion of synchronized embryos matured in liquid medium, the production of embryos that appear to be mature and well-formed does not guarantee that they will convert. Although ABA- and osmoticum-treated embryos which differentiated in suspension culture appeared similar to embryos allowed to mature on solid medium, they did not behave similarly when placed under conditions designed to promote conversion. The recalcitrance of these embryos to convert or even germinate may be related to the fact that in many cases, these embryos were exposed to ABA or high levels of osmoticum for long periods of time. These treatments were applied precisely because they were known to prevent precocious germination (4,6). It is possible that prolonged exposure to these chemicals induced embryo dormancy or quiescence, which none of the treatments we subsequently applied (see Results) was able to break.

Sieved PEMs were cultured on filter paper to facilitate retrieving PEMs of the desired fraction for plating on solid medium. Our approach was similar to a previously reported procedure (12) in which embryogenic alfalfa cell clusters were fractionated on nylon mesh and the desired fraction was spread into a thin layer on the mesh, which was then placed directly onto solid hormone-free medium. However, in our protocol, we backwashed the desired PEM fraction from the screen and onto a support through which the liquid medium could be drained before placing it on solid medium. Besides filter paper, we also tried Miracloth, nylon mesh (120 μm pore size, Fig. 2 *F*), Millipore filter membranes and Celgard (Celanese Corp.) membranes. Of these materials, filter paper and nylon mesh gave adequate drainage so that PEMs would not flow off the edge with the liquid medium. Both materials appeared to produce large numbers of roughly synchronous, mature embryos, but filter paper performed more consistently, especially when care was taken to thoroughly wick the liquid medium using 2 layers of gel dryer filter paper backing beneath the filter. Similar to results reported by McKersie et al. (12), sieving served to enrich the culture for embryogenic cells, and may have almost completely eliminated nonembryogenic cells, since unlike the results with alfalfa, yellow-poplar embryos appeared to develop from PEMs directly on the filter paper, with no underlying bed of callus (Figs. 2 *B-C*).

The high frequency of embryos able to complete maturation following fractionation and filter paper plating suggested that this technique may have altered the environment of the developing embryos in a number of ways. First, similar to the early selection experiment, fractionation and plating of PEMs in a single layer removed the developing embryos from the influence of embryos at other developmental stages. The array of

hormonal and other chemical gradients presumably present in asynchronous cultures was probably replaced by more uniform conditions. Second, the filter paper was a physical barrier between the medium and the developing embryos, so that the embryos were not in direct contact with the medium matrix. Measurements of the water potential of the filter paper and the medium beneath it suggested that using the filter paper kept the water potential experienced by the embryos during their development more negative (Merkle, unpublished data). Thus, it is possible that modifying the environment of developing somatic embryos by altering matric potential, rather than the osmotic potential of the medium, may be useful in controlling later stages of development.

REFERENCES

1. Ammirato, P. V. The effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). Bot. Gaz. 135(4):328–337; 1974.
2. Becwar, M. R.; Noland, T. L.; Wyckoff, J. L. Maturation, germination, and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants. In Vitro Cell. Dev. Biol. 25:575–580; 1989.
3. Carman, J. G.; The *in ovulo* environment and its relevance to cloning wheat via somatic embryogenesis. In Vitro Cell. Dev. Biol. 25:1155–1162; 1989.
4. Crouch, M. L.; Sussex, I. M. Development and storage protein synthesis in *Brassica napus* L. embryos in vivo and in vitro. Planta 153:64–74; 1981.
5. Dunstan, D. I.; Bekkaoui, F.; Pilon, M., et al. Effects of abscisic acid and analogues in the maturation of white spruce (*Picea glauca*) somatic embryos. Plant Sci. 58:77–84; 1988.
6. Finkelstein, R.; Crouch, M. L. Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol. 81:907–912; 1986.
7. Fujii, J. A.; Slade, D.; Redenbaugh, K. Maturation and greenhouse planting of alfalfa artificial seeds. In Vitro Cell. Dev. Biol. 25:1179–1182; 1989.
8. Gray, D. J. Quiescence in monocotyledonous and dicotyledonous somatic embryos induced by dehydration. HortScience 22:810–814; 1987.
9. Gray, D. J. Effects of dehydration and exogenous growth regulators on dormancy, quiescence and germination of grape somatic embryos. In Vitro Cell. Dev. Biol. 25:1173–1178; 1989.
10. Giuliano, G.; Rosellini, D.; Terzi, M. A new method for purification of the different stages of carrot embryoids. Plant Cell Rep. 2:216–218; 1983.
11. Hammatt, N.; Davey, M. R. Somatic embryogenesis and plant regeneration from cultured zygotic embryos of soybean (*Glycine max* L. Merr.). J. Plant Physiol. 128:219–226; 1987.
12. McKersie, B. D.; Seneratna, T.; Bowley, S. R., et al. Application of artificial seed technology in the production of hybrid alfalfa (*Medicago sativa* L.). In Vitro Cell. Dev. Biol. 25:1183–1188; 1989.
13. Merkle, S. A.; Sommer, H. E. Somatic embryogenesis in tissue cultures of *Liriodendron tulipifera*. Can. J. For. Res. 16:420–422; 1986.
14. Merkle, S. A.; Wetzstein, H. Y.; Sommer, H. E. Somatic embryogenesis in tissue cultures of pecan. HortScience 22:128–130; 1987.
15. Parrott, W. A.; Dryden, G.; Vogt, S., et al. Optimization of somatic embryogenesis and embryo germination in soybean. In Vitro Cell. Dev. Biol. 24:817–820; 1988.
16. Preece, J. E.; Zhao, J.; Kung, F. Callus production and somatic embryogenesis from white ash. HortScience 24:377–380; 1989.
17. Raghavan, V. Embryogenesis in Angiosperms, A Developmental and Experimental Study. Cambridge: Cambridge University Press; 1986:303.
18. Rajasekaran, K.; Mullins, M. G. Embryos and plantlets from cultured anthers of hybrid grapevines. J. Exp. Bot. 30:399–407; 1979.
19. Risser, P. G.; White, P. R. Nutritional requirements of spruce tumor cells *in vitro*. Physiol. Plant. 15:620–635; 1964.
20. Snedecor, G. W.; Cochran, W. G. Statistical Methods, Sixth Edition. Ames: Iowa State University Press; 1967:593.
21. Sommer, H. E.; Brown, C. L. Embryogenesis in tissue cultures of sweetgum. For. Sci. 26:257–260; 1980.
22. Strickland, S. G.; Nichol, J. W.; McCall, C. M., et al. Effect of carbohydrate source on alfalfa somatic embryogenesis. Plant Sci. 48:113–121; 1987.
23. Trigiano, R. N.; Beaty, R. M.; Graham, E. T. Somatic embryogenesis from immature embryos of redbud (*Cercis canadensis*). Plant Cell Rep. 7:148–150; 1988.
24. Trigiano, R. N.; Beaty, R. M.; Dietrich, J. T. Somatic embryogenesis and plantlet regeneration in *Cornus florida*. Plant Cell Rep. 8:270–273; 1989.
25. Tulecke, W. Somatic embryogenesis in woody perennials. In: Bonga, J. M.; Durzan, D. J., eds. Cell and Tissue Culture in Forestry, Vol. 2. Dordrecht: Martinus Nijhoff Publishers; 1987:61–91.
26. Tulecke, W.; McGranahan, G. Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci. 40:57–63; 1985.
27. von Arnold, S.; Hakman, I. Regulation of somatic embryo development in *Picea abies*, with emphasis on ABA effects. J. Plant Physiol. 132:164–169; 1988.
28. Wann, S. R. Somatic embryogenesis in woody species. In: Janick, J., ed. Horticultural Reviews, Vol. 10. Portland: Timber Press; 1989:153–181.
29. Wetzstein, H. Y.; Ault, J. R.; Merkle, S. A. Further characterization of somatic embryogenesis and plantlet regeneration in pecan (*Carya illinoensis*). Plant Sci. 64:193–201; 1989.
30. Witham, F. H.; Blaydes, D. F.; Devlin, R. M. Experiments in Plant Physiology. New York: Van Nostrand-Reinhold Co.; 1971:245.
31. Zieber, N. K.; Brink, R. A.; Graf, L. H., et al. The effect of casein hydrolysate on the growth *in vitro* of immature *Hordeum* embryos. Am. J. Bot. 37:144–148; 1950.

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