SHOOT INDUCTION FROM INTERNODES OF ELITE POPULUS DELTOIDES CLONES

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ABSTRACT

Hardwood genetics research at the Institute of Paper Science and Technology (IPST) seeks to produce useful variants of elite Populus deltoides via genetic transformation. Among other prerequisites, transformation requires large numbers of suitable explants and reliable regeneration of plants. Model clones, easily manipulated in culture, are ideal for such research. Elite clones of commercial value, however, often prove recalcitrant. Trials reported here sought to establish shoot cultures of an elite clone, St75. Trial 1 tested varying zeatin (Z) levels. Differences among Z levels were small, but more shoots tended to form at 1 mg/L Z. Few shoots elongated to usable size. Higher Z levels tended to forestall explant deterioration. Trial 2 reexamined promising Z levels, and attempted to reduce deterioration and promote elongation by biweekly or accelerated subculture (ASC). Shoot production was enhanced by ASC on 1 mg/L Z. Explants remained healthy and productive through 161 days. Trial 3 retested this treatment and evaluated gibberellic acid (GA) as a stimulus for elongation. ASC, once again, extended explant life. Explants given GA (1 mg/L) at 115 days produced more usable shoots than in most previous trials.

Keywords: organogenesis, cottonwood, tissue culture, zeatin, gibberellic acid

INTRODUCTION

Hardwood genetics research at IPST centers on producing useful variants of elite eastern cottonwood (Populus deltoides) via genetic transformation. Traits of interest include herbicide tolerance and enhanced auxin synthesis. Herbicide tolerance promises lower plantation establishment costs and increased growth. Enhanced auxin synthesis may influence fiber numbers and/or dimensions (Klee et al. 1987). Increased fiber numbers could raise yields; altered dimensions, e. g., longer fibers with thinner walls, could raise paper quality. Also, ability to alter auxin status at will would permit investigation of mechanisms governing fiber formation.

Developing useful variants requires ready access to large numbers of clean explants, reliable methods for establishing, maintaining, and multiplying cultures, efficient means for effecting transformation and selecting transformed materials, reproducible protocols for regenerating plants, and techniques for confirming genetic change, stability, and utility. Such requirements are readily met by model clones, i. e., those easily manipulated in culture. Models are useful for research, but methods eventually must work with elite clones of commercial value.

Recently, IPST research has focused on methods for establishing cultures from elite clones. Internodes, a readily available and desirable tissue source, are used as explants. Experimental approaches generally have followed

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those of Coleman and Ernst (1989, 1990a, b). Early results showed that internodes from elite clones formed shoots nearly as frequently as those from model clones (Dinus et al. 1992a). However, only small numbers of shoots forming on internodes of elite clones elongated to usable size. As a result, they could not be perpetuated or multiplied in culture.

This report presents findings from three experiments designed to overcome recalcitrance of a valuable elite clone. Each trial was designed to refine or add to treatments giving beneficial results in preceding tests. A model clone, noted for predictable and productive behavior, was used as a "positive" control. Various combinations of growth regulator types, concentrations, and application times as well as subculture frequency were tested in efforts to improve explant health and shoot production.

MATERIALS AND METHODS

Plant Material

Two clones were used throughout the three experiments. Model clone K417 (supplied by Dr. C. S. Prakash of Tuskegee University), was chosen because of ease of manipulation in culture (Prakash and Thielges 1989, Coleman and Ernst 1990a), southerly origin (Fulton Co., KY), and potential utility on sites of interest - bottomlands along the lower Mississippi and Columbia Rivers. Elite clone St75 from Issaquena Co., MS (Mohn et al. 1970) was supplied by Dr. B. *J.* Stanton (James River Corporation, Camas, WA), and was chosen for site adaptability, superior productivity, and high alpha-cellulose content (Olson et al. 1985). Both clones were grown in a greenhouse on a temperature and photoperiodic regime designed to promote year-round growth and ensure continued explant availability. Water was provided as needed, fertilizer was added weekly, and various pesticides were applied alternately across weeks to prevent and/or remedy problems. Ramets were hedged periodically to limit size, maximize explant numbers, and facilitate pesticide application. The same three ramets of each clone were used as explant sources in all trials. Detailed growing conditions are given in Dinus et al. (1992a).

Explants

Stems were cut just below the eleventh node. Leaves were removed and stems rinsed with tap water. Earlier research (Douglas 1984, Coleman and Ernst 1989, Dinus 1992a) showed that response varied with internode position or age. To increase response and predictability, the present work used only internodes six through nine. After cutting into individual stem segments, all materials were surface-sterilized according to the procedures of Coleman and Ernst (1989). Segments were then aseptically dissected into 5 mm internodal explants. Nodes were discarded. Explants were placed in liquid WNA medium (Coleman and Ernst 1989), supplemented with antibiotics (500 mg/L carbenicillin, 50 mg/L tetracycline, and 15 mg/L rifampicin), and shaken in the dark for 24 hours to minimize systemic bacterial contamination (Coleman, personal communication). After rinsing three times in sterile deionized water, explants were placed horizontally on culture media.

Media and Experimental Design

Explants were cultured on WNA medium as modified by Coleman and Ernst (1989). WNA was supplemented with 0.5 mg/L 2,4-D to induce callus formation (CIM) (Coleman and Ernst, 1990b), and with Z to promote shoot formation and

elongation (Coleman and Ernst, 1990b; Dinus et al. 1992a). Growth regulators used in each experiment are described below. WNA was used at pH 5.8, solidified with 0.25% Phytagel (Sigma Chemical Co., St. Louis, MO), and autoclaved at 121°C and 1.4kg/cm^2 for 20 minutes. For the first 28 days of culture, carbenicillin (500 mg/L) was included in all media, regardless of growth regulator content, to control systemic bacterial contamination. Carbenicillin, Z, and GA were filter sterilized and added after autoclaving. Media (20 ml) were dispensed into 100 x 20 mm sterile polystyrene Petri plates.

To the extent possible, individual replications were filled with explants from one stem of a particular ramet from each clone. When one stem did not yield sufficient explants for a replication, explants from the next harvested stem were used. All work on a replication was completed on a given day by the same operators. This proved logistically efficient and potentially reduced experimental error by associating any variability caused by ramet condition or explant processing with replications. Each combination of replication, clone, and treatment (one petri plate) with seven explants constituted an experimental unit, and observations were based thereon.

Culture was done in darkness at 22°C for 10 days, and then under a 16 hour/day photoperiod at 22°C with illumination from 40W preheat- rapid start agro-lite lamps (15 µmoles M^{-2} s^{-1} of photosynthetically active light). Cultures were moved to fresh media every 28 days unless indicated otherwise. Observations of contamination, callus production, shoot formation and/or elongation , and explant health were recorded every seven days until deterioration or death dictated termination. Usable shoots (5 mm or longer) were counted, and collected for other research every two weeks beginning at day 63. Harvested shoots were rooted as per Dinus et al. 1992a.

Trial 1 sought to examine effects of elevated Z levels, and to confirm findings from previous work. St75 explants were arrayed in a randomized block design with six treatments and eight replications. Treatments consisted of 4 days on CIM (CIM4) followed by transfer to WNA supplemented with 0.00, 0.25, 0.50, 1.00, 2.00, and 4.00 mg/L Z. After exposure to CIM4, K417 material was placed only on the 0.5 mg/L Z level, a treatment found to induce shoots in earlier work (Coleman and Ernst 1990b; Dinus et al. 1992a). Comparisons between clones were based on eight replications of this treatment.

Trial 2 reexamined promising Z levels, and attempted to improve explant health and shoot elongation via modified subculture regimes. For St75, the experiment initially was organized as a randomized block design with 4 treatments and 12 replications. Treatments included WNA without Z, and CIM4 followed by transfer to WNA with 0.50, 1.00, or 2.00 mg/L Z. To offset explant deterioration and stimulate shoot elongation, the standard subculture regime was altered at days 49 and 56. The 12 replications for each Z level were distributed equally and randomly among subculture treatments: accelerated subculture (i.e., biweekly rather than monthly transfer to fresh medium) beginning on day 49 (ASC49); accelerated subculture beginning on day 56 (ASC56); standard or monthly subculture to medium without growth regulators (NOGR); and the standard subculture regime (CONTROL). Thus, each combination of Z level and subculture regime (16 treatments) was replicated three times. For analyses from day 70 through day 154, this arrangement was considered a completely random design. K417 was employed to track agreement with earlier trials. Eight replications were pretreated with CIM4, placed only on 0.5 mg/L Z, and given the standard subculture regime.

The third trial was part of a larger test designed to contrast effects of Z with those of another growth regulator, thidiazuron. Only 1 mg/L Z plus ASC56 and subsequent manipulations are described here. Six replications of St75 and three of K417 explants were placed on CIM4 and transferred as usual to WNA plus 1 mg/L Z. ASC56 was implemented at day 56 and continued thereafter. Additional measures to stimulate elongation were taken on day 115. At that time, replications for each clone and treatment were distributed randomly between two supplemental treatments. Three replications of St75 continued on Z and ASC56, while three were transferred to WNA supplemented with 1 mg/L GA (Welander et al. 1989) and subcultured biweekly. This arrangement was considered a completely random design with two treatments (GA and no GA) and three replications. As a check, two K417 replications were given GA; the third remained on the original treatment. Data and usable shoots were collected through day 203.

Data Collection and Ana ysis

Observations for each experimental unit were summarized as percentages of explants forming callus, forming shoots (FS), deteriorating, and elongating shoots (ES) on days 63, 91, 112, and later where noted. Observations of some treatments were extended to allow greater opportunity for St75 shoot elongation and to maximize numbers of harvested shoots. Data were subjected to analyses of variance (Steel and Torrie 1960) for the designs described above, using the general linear models procedure of SAS Institute (1985). When main effects were significant, means were compared using Duncan's New Multiple Range Test. All tests of significance were made at P = 0.05.

RESULTS AND DISCUSSION

In the first trial, all K417 explants formed callus and shoots; ES averaged 80% or more over the course of the experiment. Such findings typify those from earlier trials (Dinus et al. 1992a, b). Time course analyses of development (Figure 1, K417) showed that K417 callus formation began between 7 and 14 days after start of culture. Parallel increases in FS and ES occurred roughly 14 and 35 days thereafter, respectively. Explants remained healthy, with usable shoots harvested at 14-day intervals through or beyond day 112.

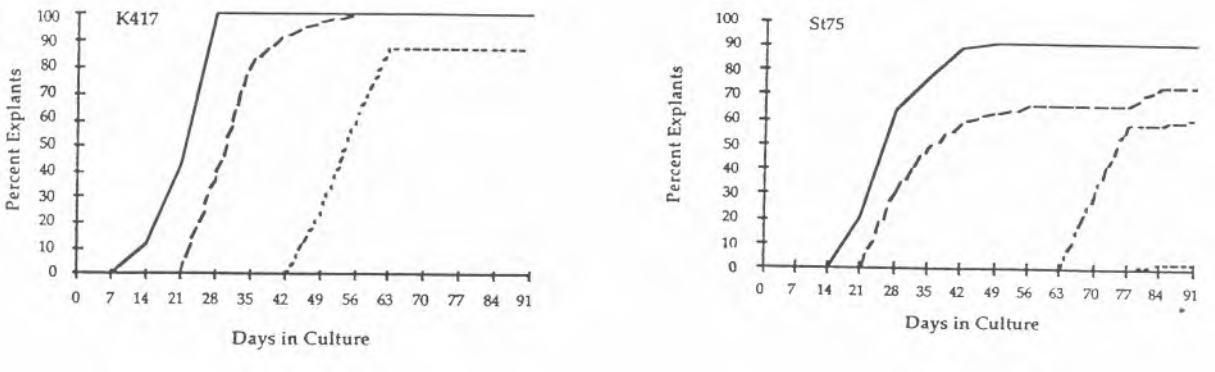

Figure 1: Time course of development for K417 and St75 on 0.5 mg/L zeatin over 91 days in culture. Callus formation _____, Shoot formation ___, Shoot elongation ------- , Deterioration -------

Development for St75 followed a similar pattern, with some critical exceptions (Figure 1, St75). Callus and shoot formation occurred several or more days later than for K417, and neither attained levels as high as those for K417. Both callus and shoot formation frequencies varied somewhat with Z level, though differences were not significant through day 77 (Figure 2). Nonetheless, explants not receiving Z were least responsive, and those given 1 mg/L Z tended to form shoots more often. Later in the experiment, FS for explants receiving Z proved significantly greater than that for explants not given Z. Also noteworthy were the observations that St75 shoot elongation lagged behind that for K417 by roughly 35 days (Figure 1), and that it never exceeded 7 percent regardless of Z level (Figure 2). In addition, St75 explants began deteriorating on or shortly after day 63; browning, callusing-over, and death became increasingly frequent with time (Figure 1, St75).

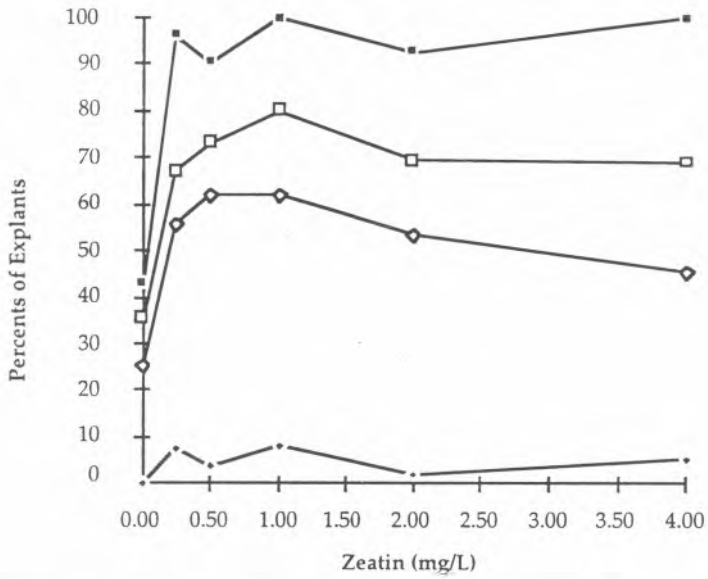

Figure 2: Responses of St75 explants to varying zeatin levels through 77 days in culture. Differences among zeatin levels were not significant. Callus formation —•—, Shoot formation —□—, Shoot elongation —◇—, Deterioration —·—·—

The 35 day lag in St75 shoot elongation and rapid decline of St75 explants suggested the need for some additional measure(s) to maintain explant health and/or stimulate elongation. The fact that 1 mg/L Z tended to raise FS, while higher Z levels tended to lessen deterioration (Figure 2) further suggested that moderate but more unifoim Z levels might improve health and/or promote elongation. Though FS was significantly rower for St75 that for K417 (Figure 1), 60 percent or more of St75 explants had shoots available for elongation by day 49. Such findings infer that shoot elongation requirements differ from those for formation. Accordingly, subsequent experiments sought to reevaluate the more promising Z levels, and to intervene in the window of opportunity between peak shoot formation (day 49) and onset of deterioration (day 63).

Results from the second trial largely confirmed those from the first. K417 FS and ES matched earlier levels, and exceeded those of St75 by the usual margins. Performance of St75 through day 63 was also in agreement, but later results differed markedly. On average, St75 explants produced more usable shoots, and shoot elongation occurred over a longer period of time than in earlier trials. Proportionately larger numbers of explants remained healthy and productive through day 112, an average over all treatments of 13.2 percent relative to a maximum value of 7.0 percent for Trial 1.

One notable similarity occurred in that the 1 mg/L Z treatment, when averaged across all subculture treatments, gave significantly greater shoot elongation than other treatments. By day 112, roughly 33 percent of explants given 1 mg/L produced usable shoots, a frequency almost three times greater that the next highest (12 percent). Significant differences also occurred among subculture regimes, when averaged over all Z levels. Though similar to most regimes, response to ASC56 significantly exceeded that to NOGR (19.8 versus 1.7 percent). All regimes except NOGR produced more usable shoots than observed in the first trial and in earlier work (Dinus et al. 1992a and b).

Considering all combinations of Z levels and subculture regimes, response to 1 mg/L Z and ASC56 (74.6 percent) significantly exceeded that of most other combinations. Response to 1 mg/L Z and ASC49 (41.9 percent), although not differing significantly from many other combinations, was roughly twice as large as any other response except that to 1 mg/L Z and ASC56 (74.6 percent), and 2 mg/L Z and CONTROL (38.1 percent).

The generally better performance of ASC and higher Z levels suggests that a relatively high but uniform Z level is important to continued explant health and supportive of shoot elongation. Poor response to NOGR combinations, regardless of initial Z level, supports such an argument. That is, more frequent subculture and/or higher initial Z levels seem to favor shoot production, whereas elimination of Z generally has an opposite effect. Absence of clear trends across Z levels within individual subculture regimes, possibly resulting from having only three replications, precluded drawing firm conclusions. As a result, another experiment was undertaken to confirm utility of ASC and evaluate need for additional measures to stimulate elongation.

In the third trial, K417 explants performed predictably, with callus and shoots forming soon after start of culture. Once again, K417 shoots elongated in large numbers throughout the test, regardless of GA treatment. GA was observed to increase shoot length, but effects on usable shoot numbers remained unclear. Both GA treatments produced more shoots than needed for other research, and definitive counts were not maintained. Observations also indicated that K417 shoots given GA were long and spindly. Harvest and transfer to rooting media were difficult. Further work is needed to gauge the nature of GA effects on such clones; lower GA concentrations deserve testing.

St75 shoots, as expected, began to elongate well after those of K417. Performance of ST75 nevertheless differed to some extent from that in the second trial; i.e., elongation occurred on smaller numbers of explants and somewhat sporadically across time, even though ASC56 had been implemented. Results agreed with earlier findings, however, in that ASC56 maintained explant health for a longer time. Indeed, explant condition was such that GA application to promote elongation was delayed beyond the time originally though appropriate for intervention (day 115 versus day 49 to 60). Within 21 days of application, shoots on explants given GA began to elongate, and usable shoots became available shortly thereafter. Explants given GA produced usable shoots

through day 203, a productive lifetime up to two times longer than those observed earlier (Table 1). In addition, numbers of usable shoots harvested across that time period were comparable to, or greater, than those produced by best treatments in most preceding trials.

Table 1. Summary of results for St75 from Trials 1-3 and a previous trial by Dinus et al. 1992a, b).

Trial	Treatments	Productive Explant Life (Days)	Usable Shoots/Explant (No.)
Previous	0.0 Z	91	0.00
	CIM4 ⇒ 0.5 Z	"	0.00
1	CIM4 ⇒ 0.5 Z	105	0.04
	CIM4 ⇒ 1.0 Z	119	0.14
2	CIM4 ⇒ 1.0 Z	126	0.00
	CIM4 ⇒ 1.0 Z + ASC56	161	1.85
3	CIM4 ⇒ 1.0 Z + ASC56	161	0.10
	CIM4 ⇒ 1.0 Z + ASC56 + GA	203	1.79

SUMMARY AND CONCLUSIONS

As apparent from Trials 1 and 2, moderate and relatively uniform Z levels seem more effective than other levels and monthly subculture at stimulating shoot production from internode explants of recalcitrant clones such as St75. Results from Trials 2 and 3 further indicate that ASC56 and 1 mg/L Z are beneficial to health of explants. The fact that the protocol fostered elongation only in Trial 2 infers that improved explant health may not be adequate for elongation and that some additional stimulus is required to obtain consistent results. GA application seems to be one approach to ensuring elongation, and more definitive research toward clarifying the contributions of ASC regimes and GA levels is underway. Shoots harvested from the present research were rooted at frequencies approaching 100 percent; regenerated plants are being maintained in culture and used as a source of explants for research on genetic transformation.

ACKNOWLEDGMENTS

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