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Stable ploidy levels in long-term callus cultures of loblolly pine *

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ABSTRACT

Ploidy levels were calculated for callus cultures of loblolly pine (*Pinus taeda* L.), based on nuclear DNA content measured by Feulgen cytophotometry. The nuclear DNA content of initial stem explants showed a predominant 2C condition with less 3C and 4C, in ratios approximating those expected from diploid cells as they replicate DNA in the mitotic cell cycle. Cells with higher ploidy were produced during callus initiation, as indicated by a sharp reduction in the 2C population and a concomitant increase in higher DNA levels up to 8C. A gradual decrease in the higher ploidy levels occurred in subsequent subculture intervals, so that by 18 weeks the diploid nuclear DNA distribution was again observed, with complete elimination of DNA levels greater than 4C. Established callus cultures derived from stem or embryo explants and cultured on three different nutrient media for 48–76 weeks also showed the diploid nuclear DNA distribution with no indication of polyploid cells.

ABBREVIATIONS

BAP, benzylaminopurine; NAA, α-naphthaleneacetic acid; BL, Brown and Lawrence's medium; BLG, modified BL medium; LM, Litvay's medium

INTRODUCTION

Genetic stability of cells in culture is advantageous if clonal propagation of plants through *in vitro* techniques is desired, but genetic instability in the form of higher ploidy levels is common in the cell cultures of many species (Bayliss 1980, Berlyn et al. 1986). Retention of normal diploid levels in long term culture is not common, but has been reported in the callus cultures of both herbaceous and woody plants, e.g. wheat (Shimada 1971) and Norway spruce (Hakman et al. 1984). Though DNA content in cultured cells of some gymnosperms is stable, polyploidy has been reported in cultures of Coulter pine (Patel and Berlyn 1982) and loblolly pine (Renfroe and Berlyn 1985). We have monitored cellular DNA content during loblolly pine callus initiation and long-term culture, as a screen for nuclear aberrations in the form of increased ploidy levels.

MATERIALS AND METHODS

General callus culture:

Media used in this study were BL (Brown and Lawrence 1968), BL with the entire nitrogen complement replaced by 10 mM filter sterilized glutamine (BLG), and LM (Litvay et al. 1981). All media for callus initiation and subsequent subculture contained 2 μM BAP, 2 μM NAA and 0.5% (w/v) sucrose, and were solidified with 1% (w/v) agar. All cultures were grown under constant flourescent light (36 $\mu Es^{-1}m^{-2}$ [PAR]) at 23±2°C. Callus initiation occurred during the first 6 week interval. The cultures were then transferred to fresh media of the same composition, and were subsequently subcultured at 3 week intervals.

Callus initiation from stem explants:

Succulent stem pieces 5–6 cm in length, selected from the flush growth of 3-year-old greenhouse-grown loblolly pine trees (open-pollinated family 7–56 from NCSU-Industry Tree Improvement Co-operative), were used for callus initiation. After removing the fascicles and scale leaves, the stem pieces were washed with liquid detergent, rinsed several times in tap water, surface sterilized in 0.5% calcium hypochlorite solution for 15 min, and washed several times in sterile distilled water. Stem pieces were cut into discs 0.5 mm in thickness which were placed flat on BLG medium for the callus initiation study, or on BLG, BL, and LM media for generating long-term callus stocks.

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Callus initiation from embryos:

Loblolly pine seeds (family 7-56) were scarified by cutting the seed coat at the micropylar end, and were germinated at 30°C in hydrogen peroxide (1% solution changed daily for 3 days and 0.03% solution for the fourth day). Seed coats were removed from the germinated seeds, and the embryos along with the gametophytic tissue were surface sterilized in 0.5% calcium hypochlorite solution for 5 minutes. Each embryo was aseptically removed from the gametophytic tissue, minced into small pieces and transferred to BLG medium as a clump.

Sampling procedure:

At the end of culture intervals, samples were fixed in Carnoy's #2 fixative (Berlyn and Miksche 1976) for one hour at room temperature, and then transferred to 70% ethanol and stored at 4°C until further processed for Feulgen cytophotometry. As a test against potential sampling bias, some treatments were sampled twice. One sample was taken from the outer 2-3 mm of the callus, and another from the inner core.

Nuclear DNA content was measured in three sets of cultures:

1. Stem explants, callus at the time of initiation from stem explants, and the callus up to 18 weeks in culture, on BLG medium.
2. Three separate sets of long-term callus cultures, derived from stems and subcultured for about one year or more on BLG, BL and LM media, respectively.
3. Embryo derived long-term callus cultures subcultured for more than one year on BLG medium.

Feulgen cytophotometry:

Samples were hydrated to water and squashed on glass slides. Each slide contained smears of chicken erythrocytes fixed in Carnoy's #2 fixative, for use as an internal standard (Dhillon et al. 1977, 1980). Feulgen cytophotometric techniques described by Miksche and Dhillon (1984) were followed, including the acid hydrolysis protocol of 5N HCl for 30 min. at 25°C (see also Renfroe and Berlyn 1984). Five callus samples were examined per time period and one hundred cells per sample were screened for their nuclear DNA content using a Vickers M86 scanning microdensitometer.

Ploidy determination:

The absolute DNA values for different 'C' levels were determined based on Feulgen cytophotometric measurements of DNA in 40 telophase and 70 metaphase figures from the root apical cells of germinating loblolly pine seeds. Pooled values of metaphase and telophase figures showed the 2C DNA value for loblolly pine to be 27 picograms $\pm$5 pg standard deviation (cf. 26 pg for loblolly pine previously reported by Renfroe and Berlyn 1984). Based on this 2C value and its coefficient of variation, expected DNA contents for other C levels were estimated as follows in pg DNA per nucleus: 1C=13.5$\pm$2.5 3C=41$\pm$7.5, 4C=54$\pm$10, 6C=81$\pm$15 and 8C=108$\pm$20. Nuclear DNA contents higher than 8C were not observed in this study.

The measured nuclear DNA content of each cell was transformed into a normal standard deviate of the DNA content for each ploidy level, and the probability that the observation belonged within that distribution was calculated. Each observation was assigned to the ploidy level showing the highest probability, and the percentage of total nuclei in each ploidy level was calculated for each callus treatment.

RESULTS

Our method of assigning a continuum of observed nuclear DNA quantities into discrete bins representing ploidy levels ('C' levels, Fig. 1A) compares favorably to the pattern obtained when these values are sorted into smaller arbitrary bins of 3 pg DNA increments (Fig. 1B). For this reason, figures used in this paper contain bin widths presented according to ploidy levels, since this method of presentation does not misrepresent the data and it simplifies visualization of results. No difference was seen in nuclear DNA contents of samples taken from the outer vs. inner zones of those calli sampled in this manner. Data presented represent pooled values from both zones, where applicable.

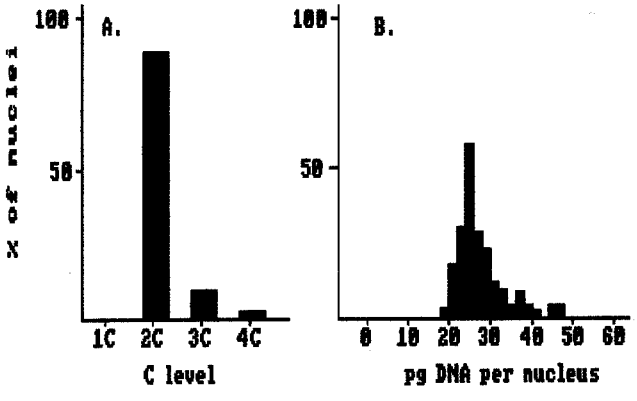

Figure 1. Distribution of nuclear DNA content in loblolly pine embryo-derived callus cultures grown on BLG medium for 60 weeks, with subcultures at 3 week intervals. A. Nuclear DNA content distributed in bins corresponding to C-levels, as explained in the text. B. The same data as in A, but distributed in bins arbitrarily chosen to have widths of 2 pg DNA.

The distribution of nuclear DNA content for initial stem explants (Fig. 2A) shows a predominant 2C condition (62%). Thirty four percent of cells were found in the 3C level and only 4% in the 4C condition. Ploidy levels higher than the 4C condition were not observed in the stem explants. However, by 3 weeks in culture, the nuclear DNA content of the emerging callus showed a reduced 2C population (12%) and elevated 3C and 4C levels (Fig. 2B). Ploidy levels as high as 6C and 8C were also seen at low percentages in these cultures.

The higher ploidy levels seen during callus initiation were gradually eliminated over time. By six weeks (end of callus initiation period), the 8C population was no longer observed and a return to the predominantly 2C condition was seen (Fig. 2C). From this time point onwards a gradual decrease in the higher ploidy levels was observed and by 18 weeks (fifth culture interval) the complete elimination of nuclear DNA content higher than 4C was observed (Fig. 2E). A second trial also showed increased ploidy levels during the callus initiation period in contrast to normal nuclear DNA contents in older cultures, on LM medium (data not shown).

Another set of cultures was examined which was also derived from stem, but subcultured at 3 week intervals on three different media for 48-76 weeks. Similar DNA distributions were seen among these cultures, with DNA levels predominantly at 2C and not exceeding 4C. Long term (60 weeks) embryo-derived callus also showed a similar DNA distribution (Fig. 1A).

DISCUSSION

Feulgen cytophotometric measurement of nuclear DNA content is a quick and easy method to estimate cell ploidy levels compared with conventional techniques involving chromosome counts. Feulgen cytophotometry also permits measurement of ploidy levels in nondividing interphase cells, whereas conventional chromosome counting requires mitotically active cells.

Since the Feulgen technique measures nuclear DNA content and not chromosome numbers, 3C and 4C levels do not directly indicate triploid and tetraploid cells, respectively. Root meristem cells of Pinus banksiana seedlings spend 63% of each interphase cycle in G1, 31% in S, and 6% in G2 (calculated from Miksche 1967), with corresponding 2C, intermediate, and 4C DNA levels, respectively. Assuming similar characteristics for mitotically active loblolly pine tissues, we interpret the distribution of 2C, 3C, and 4C levels which are observed in stem sections (Fig. 2A) and in long-term callus cultures (e.g. Fig. 2E) as representing a replicating diploid cell population, since the average durations of G1, S and G2 would fall in the same relative order. On the other hand, cells with DNA levels greater than 4C are unlikely to be dividing diploid cells.

Stem explants are free from polyploid cells, as discussed above (Fig. 2A). By three weeks in culture, a considerable reduction in the 2C population and an increase in 3C and 4C levels has occurred, along with new 6C and 8C populations (Fig.

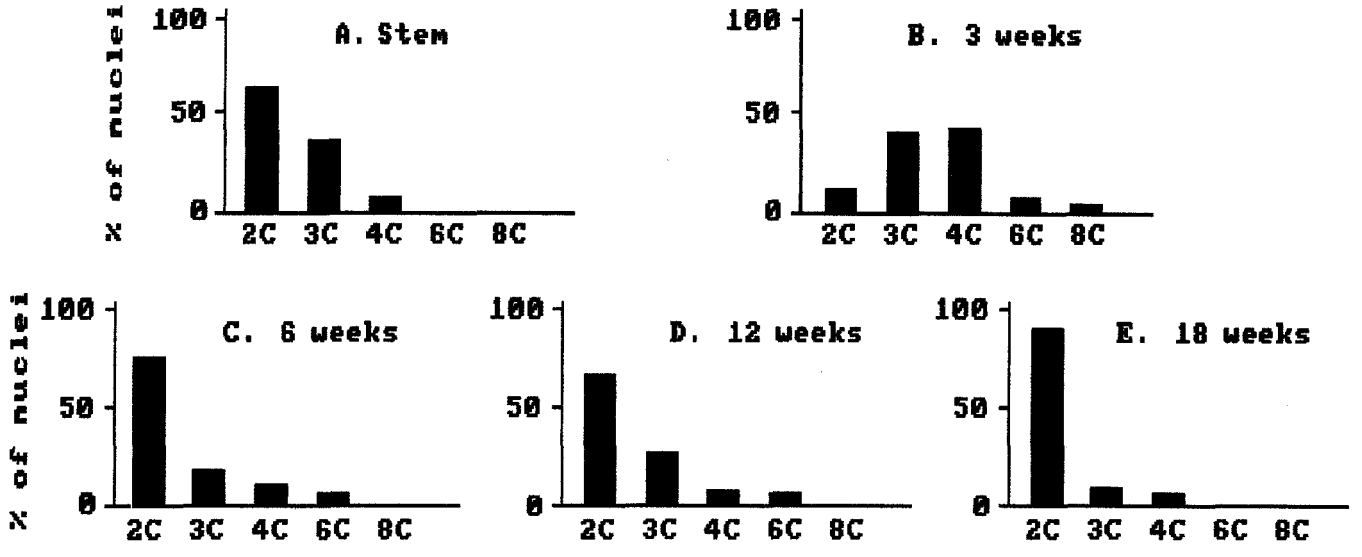

Figure 2. Distribution of nuclear DNA content in loblolly pine stem-derived cultures at different ages from culture initiation on BLG medium. A: Stem discs at the time of explantation (day 0 of culture initiation), B: callus cells 3 weeks after initiation, C: 6 weeks after initiation, D: 12 weeks and E: 18 weeks after initiation when subcultured to fresh medium at 3 week intervals beginning at week 6.

2B). The relative ratios suggest that a tetraploid population of cells was created which produced the 6C and 8C measurements by DNA replication in their cell cycle. Upon successive subculturing, a return to the diploid nuclear DNA distribution occurred, as indicated by a gradual reduction in the higher ploidy levels (Figs 2C, 2D). By 18 weeks, the diploid nuclear DNA distribution was again achieved with a complete elimination of DNA content higher than 4C, indicating that the higher ploidy levels observed during and shortly after callus initiation were totally eliminated (Fig. 2E).

No naturally occurring polyploid Pinus species have been documented, and only three polyploid species are known to occur among the gymnosperms as a whole (Wright 1976). Under experimental conditions colchicine-induced polyploid meristematic cells of slash pine (Pinus elliottii) radicles either die or revert back to diploid condition (Mergen 1959), suggesting a mechanism of selection against polyploid cells. We observe a similar reduction in polyploid cell populations in vitro with loblolly pine callus as it is initiated from stem tissues. The process by which higher ploidy levels originate and then are lost in the cultures is worthy of study, and may be similar to the naturally occurring mechanism in whole plants which selects against polyploidy in the genus. Reversible DNA amplification can account for some changes in DNA content of plant cells, as suggested for fast dividing root apical cells in Azolla filiculoides (Kurth and Gifford 1985) and for germinating soybean seeds (Dhillon and Miksche 1983). However, since these pine cultures can double the nuclear DNA content in some cells at the time of callus initiation, amplification of specific portions of the genome is unlikely.

The diploid nuclear DNA distribution achieved by 18 weeks in culture is maintained even in the oldest callus cultures measured (1.3 years), regardless of media composition or explant source. Gymnosperm cell cultures as a whole seem to have stable ploidy levels, e.g. Picea abies (Hakman et al. 1984), P. glauca (de Torok and White 1960), Pinus gerardiana (Konar and Nagmani 1972), P. lambertiana (Partanan 1963), P. cembra (Salmia 1975), P. roxburghii (Mehra and Anand 1983), and Cryptomeria japonica (Mehra and Anand 1979)], though some exceptions have been reported. One exception is a study of loblolly pine callus, where increased ploidy levels seen at callus initiation continued for at least six months in culture (Renfroe and Berlyn 1985). These workers used growth regulator levels ca. 5 times higher than we used in this study, suggesting an important role for growth regulators in controlling genetic stability in these cultures.

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