GENETIC SCREENING OF TISSUE CULTURE FOR USE IN TREE IMPROVEMENT PROGRAMS 1/

Michael H. Renfroe and Graeme P. Berlyn

Abstract. --Plant tissue culture has many applications for forest tree improvement programs., However, genetic variation arising during the tissue culture phase of plant regeneration programs may limit the usefulness of in vitro propagation for certain forestry practices. Tissue cultures and regenerated plants should be routinely screened for abnormal nuclear conditions. Using two different tissue culture systems we regenerated buds from loblolly pine embryos. Microspectrophotometric analysis revealed that one system produced buds with a diploid nuclear DNA content but that the other system produced buds with nuclei containing abnormal amounts of DNA. Methods of genetic screening are compared.

Additional keywords: Pinus taeda, microspectrophotometry, nuclear DNA.

INTRODUCTION

Routine genetic screening of tissue cultures is essential to maximizing benefits of tissue culture techniques for forest tree improvement. As in agronomy and horticulture, the many benefits of tissue cultures for forest tree improvement programs will be increasingly realized and exploited. Although variants produced in tissue cultures can be useful to propagators, most benefits are largely contingent upon genetic stability in the cultured tissues.

Rapid multiplication of trees of superior genotype could provide clones for inclusion in seed orchards without the necessity of grafting, for the study of genotype and environment interaction, and for inclusion in short-rotation coppice plantations for production of fiber, fuel and chemical feed-stocks. Through parasexual hybridization, hybrids could be made of species with marginal or no sexual compatibility and triploids could be produced directly without production of tetraploids and backcrossing with diploids. In vitro techniques can be used for germplasm preservation and for screening plants for tolerance and resistance with subsequent regeneration of plants of desirable qualities. However, should genetic instability arise during the tissue culture phase, the benefits of these techniques would be reduced or eliminated.

Among the more commonly reported genetic aberrations occurring in plant tissue cultures are aneuploidy and polyploidy (Bayliss 1980; Partanen 1963; Sheridan 1975; Skirvin 1978). We have developed methods for organ

1 This research was partially supported by grants from the Weyerhaeuser Company, Tacoma, WA, and The Great Mountain Forest Corporation, Litchfield, CT.

regeneration for several members of the Pinaceae. In order to investigate the genetic stability of pine tissues grown in our systems, we have used visible light microspectrophotometry of Feulgen-stained nuclei (Berlyn and Cecich 1976).

MATERIALS AND METHODS

Loblolly pine (Pious taeda L.) seeds were surface sterilized by immersion in 50% Clorox for 10 min. After seeds had been rinsed in sterile, distilled water and allowed to imbibe overnight, seed coats were removed and megagametophytes were excised under aseptic conditions. Embryos were cultured on basal medium or basal medium supplemented with plant growth regulators. The basal medium consisted of mineral salts as specified by Campbell and Durzan (1975) with the micronutrients diluted 10-fold (Berlyn and Beck 1980), and 10 mg thiamine-HC1, 5 mg nicotinic acid, 1 mg pyridoxine-HC1, 100 mg myo-inositol, 30 g sucrose and 8.0 g Bacto agar per 1 of medium. The medium was adjusted to pH 5.6-5.7 before addition of agar. Medium was autoclaved at 121 C for 15 min. For bud induction medium, basal medium was supplemented with 1.0, 2.0, 3.0, 4.0 or 5.0 mg l^{-1} benzylaminopurine (BAT). For callus induction medium, basal medium was supplemented with 2.0 mg l^{-1} BAP and 2.0 mg l^{-1} naphthaleneacetic acid (NAA). Callus was maintained by subculturing on fresh medium at monthly intervals. Cultures were grown with constant light (ca. 75 $\mu E\ m^{-2}\ s^{-1}$) and temperature (25+2 C).

Tissue samples were removed periodically from cultured embryos, regenerated buds and callus. Samples were fixed for 1 hr in Carnoy's fluid No. 2 (Berlyn and Miksche 1976), hydrated, and macerated in 2% pectinase in 0.01 M sodium citrate (45+2 C, pH 5.0) (Berlyn et al. 1979). Macerated cells were adhered to microscope slides along with chicken erythrocytes which served as an internal standard (Dhillon et al. 1977) so that absolute amounts of DNA per nucleus or mitotic figure could be calculated. Slides were stained by the Feulgen method (Berlyn and Miksche 1976) using an hydrolysis time of 30 min in 5N HC1 at 25 C. DNA content was measured with a modified Leitz MPV1 microspectrophotometer (Patel and Berlyn 1982) using the two-wavelength method (Berlyn and Cecich 1976) or with a Vickers M85 scanning microdensitometer (Miksche et al.1979). For the two-wavelength method, wavelenths of maximum and half-maximum optical density were determined to be 560 and 490 nm, respectively, for pine cells and 560 and 503 nm, repectively, for erythrocytes. The 2C and 4C values of loblolly pine was determined from measurements on a total of 49 metaphase and telophase figures, with other C values based on these standards. For all other samples, at least 50 pine nuclei and at least 20 erythrocytes were measured per slide.

RESULTS

The 2C amount of DNA, which corresponds to the amount of DNA in a diploid nucleus in the G_1 stage of the cell cycle, was determined to be 26 pg. Shoot apices from embryos germinated and grown on basal medium for 5 weeks contained only cells at or between a 2C and 4C DNA content (fig. 1, a), representing the normal diploid cell cycle. These results were comparable with those obtained from analysis of shoot apices from 5-week-old and 10-week-old pot-grown seedlings.

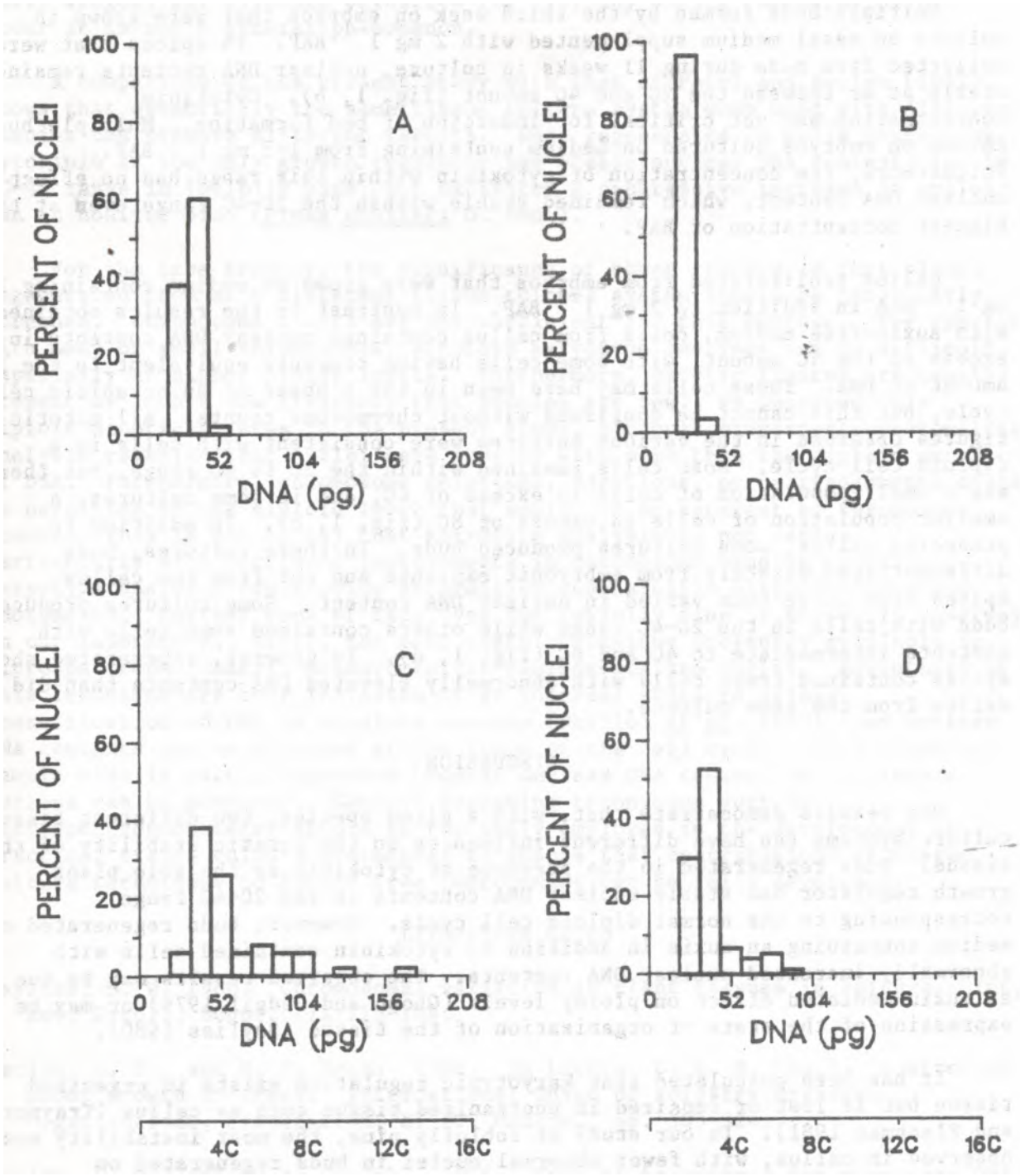

Figure 1. Nuclear DNA content of loblolly pine cells. (A) Embryonic shoot apices grown on basal medium for 5 weeks. (B) Shoot apices from buds regenerated on medium with cytokinin and no auxin. (C) Callus tissue grown on medium with cytokinin and auxin. (D) Shoot apices from buds regenerated on medium with cytokinin and auxin.

Multiple buds formed by the third week on embryos that were grown in culture on basal medium supplemented with 2 mg l^{-1} BAP. In apices that were collected from buds during 11 weeks in culture, nuclear DNA contents remained stable at or between the 2C and 4C amount (fig. 1, b). Cytokinin concentration was not critical for induction of bud formation. Multiple buds formed on embryos cultured on medium containing from 1-5 mg l^{-1} BAP. Furthermore, the concentration of cytokinin within this range had no effect on nuclear DNA content, which remained stable within the 2C-4C range even at the highest concentration of BAP.

Callus proliferated from embryos that were grown on medium containing 2 mg l^{-1} NAA in addition to 2 mg l^{-1} BAP. In contrast to the results obtained with auxin-free medium, cells from callus contained nuclear DNA contents in excess of the 4C amount, with some cells having contents equivalent to the 14C amount of DNA. These cells may have been in the S phase of an octaploid cell cycle, but this cannot be confirmed without chromosome counts. All mitotic figures observed in the various cultures were consistent with cells in a diploid cell cycle. Most cells remained within the 2C to 4C range, but there was a small population of cells in excess of 4C, and in some cultures, a smaller population of cells in excess of 8C (fig. 1, c). In addition to producing callus, some cultures produced buds. In these cultures, buds differentiated directly from embryonic explants and not from the callus. Apices from these buds varied in nuclear DNA content. Some cultures produced buds with cells in the 2C-4C range while others contained some cells with contents intermediate to 4C and 8C (fig. 1, d). In general, regenerated shoot apices contained fewer cells with abnormally elevated DNA contents than did callus from the same culture.

DISCUSSION

Our results demonstrate that, with a given species, two different tissue culture systems can have different influences on the genetic stability of the tissue. Buds regenerated in the presence of cytokinin as the sole plant growth regulator had stable nuclear DNA contents in the 2C-4C range, corresponding to the normal diploid cell cycle. However, buds regenerated on medium containing an auxin in addition to cytokinin contained cells with abnormally increased nuclear DNA contents. The observed results may be due to an auxin-mediated effect on ploidy levels (Ghosh and Gadgil 1979) or may be an expression of the state of organization of the tissue (Bayliss 1980).

It has been postulated that karyotypic regulation exists in organized tissue but is lost or impaired in unorganized tissue such as callus (Traynor and Flashman 1981). In our study of loblolly pine, the most instability was observed in callus, with fewer abnormal nuclei in buds regenerated on callus-induction medium, and no instability observed in buds regenerated on shoot-induction medium. Restoration of diploidy during organogenesis and plant regeneration has been observed in some studies (Mitra et al. 1960; Mehra and Mehra 1974). However, tetraploid and mixoploid organs and plants (Torrey 1965, 1967; Cionini et al. 1978; Furner et al. 1978) and aneuploid organs and plants (Furner et al. 1978; Wenzel et al. 1979; Sacristan and Melchers 1969) have also been regenerated from tissue cultures. This demonstrated the necessity of screening later developmental stages of plant growth as well as

early stages, for although selection for diploidy during regeneration can occur it is not a general phenomenon.

A comparison of the present study with that of Patel and Berlyn (1982) shows that essentially the same tissue culture system when used with different species can produce different results. Buds regenerated on media containing cytokinin as the only growth regulator had stable nuclear DNA contents in the 2C-4C range in loblolly pine, but exhibited a progressive increase in nuclear DNA in coulter pine (Pinus coulteri D. Don.).

For the tree breeder, the significance of these results is that plants regenerated from each different tissue culture system should be genetically screened. Chromosome counts are not sufficient for this purpose. For species with many or small chromosomes, accurate karyotype analyses can be difficult, especially at higher ploidy levels. Furthermore, mitotic figures are usually present at such a low frequency that samples are few. We observed only diploid mitotic figures in the present study, although microspectrophotometric analysis revealed that nuclei were present with more than the diploid amount of DNA. Furthermore, chromosome deletions, additions, or rearrangements could be occurring at the diploid level that would not be apparent by chromosome counts. This is not to say that karyotype analysis is not useful. Particularly with continued improvements in chromosome banding techniques, karyotype analyses can provide information unobtainable with microspectrophotometry. Further work of this type for loblolly pine is presently underway in our laboratory. Still, the most useful technique presently available is microspectrophotometric measurement of Feulgen-stained nuclei. Advantages of this technique are that inclusion of an internal standard allows quantification of DNA in absolute amounts (Dhillon et al. 1979), and nuclear DNA contents can be measured at any stage of the cell cycle. Thus potential sample size is vastly increased because nuclear DNA content of any intact nucleus can be measured. Genetic screening techniques such as microspectrophotometry should be routinely employed in the development of practical tissue culture procedures to ensure that contributions of tissue culture techniques to forest tree improvement can be fully developed.

LITERATURE CITED

Bayliss, M. W. 1980. Chromosomal variation in plant tissues in culture. Int. Rev. Cytol., Suppl. 11A:113-144.

Berlyn, G. P., and R. C. Beck. 1980. In Little, C. H. A. (ed.). Control of shoot growth in trees. International Union of Forestry Research Organizations, Fredericton, N. B., Canada. pp. 305-324.

Berlyn, G. P., and R. A. Cecich. 1976. Optical techniques for measuring DNA quantity. In Miksche, J. P. (ed.). Modern methods in forest genetics. Springer-Verlag, N.Y. pp. 1-18.

Berlyn, G. P., S. S. Dhillon, and J. P. Miksche. 1979. Feulgen cytophotometry of pine nuclei. II. Effect of pectinase used in cell separation. Stain Technol. 54:201-204.

Berlyn, G. P., and J. P. Miksche. 1976. Botanical microtechnique and cytochemistry. Iowa State Univ. Press, Ames, IA. pp. 32, 246-248.

Campbell, R. A., and D. J. Durzan. 1975. Induction of multiple buds and needles in tissue cultures of Picea glauca. Can J. Bot. 53:1652-1657.

Cionini, P. G., A. Bennici, and F. D'Amato. 1978. Nuclear cytology of callus induction and development in vitro. I. Callus from Vicia faba cotyledons. Protoplasma 96:101-112.

Dhillon, S. S., G. P. Berlyn, and J. P. Miksche. 1977. Requirement of an internal standard for microspectrophotometric measurements of DNA. Amer. J. Bot. 64:117-121.

Furner, I. J., J. Kinf, and 0. L. Gamborg. 1978. Plant degeneration from protoplasts isolated from a predominantly haploid suspension culture of Datura innoxia (Mill.). Plant Sci. Letters 11:169-176.

Ghosh, A., and V. N. Gadgil. 1979. Shift in ploidy level of callus tissue: a function of growth substances. Ind. J. Exp. Biol. 17:562-564.

Mehra, A., and P. N. Mehra. 1974. Organogenesis and plantlet formation in vitro in almond. Bot. Gaz. 135:61-73.

Miksche, J. P., S. S. Dhillon, G. P. Berlyn, and K. J. Landauer. 1979. Nonspecific light loss and intrinsic DNA variation problems associated with Feulgen DNA cytophotometry. J. Histochem. Cytochem. 27:1377-1379.

Mitra, J., M. O. Mapes, and F. C. Steward. 1960. Growth and organized development of cultured cells. IV. The behavior of the nucleus. Amer. J. Bot. 47:357-368.

Partanen, C. R. 1963. Plant tissue culture in relation to developmental cytology. Int. Rev. Cytol. 15:215-243.

Patel, K. R., and J. P. Berlyn. 1982. Genetic instability of multiple buds of Pinus coulteri regenerated from tissue culture. Can. J. For. Res. 12:93-101.

Sacristan, M. D., and G. Melchers. 1969. The caryological analysis of plants regenerated from tumorous and other callus cultures of tobacco. Molec. Gen. Genet. 105:317-333.

Sheridan, W. F. 1975. Plant regeneration and chromosome stability in tissue cultures. In Ledoux, L. (ed.). Genetic manipulations with plant materials. Plenum, N.Y. pp. 263-295.

Skirvin, R. M. 1978. Natural and induced variation in tissue culture. Euphytica 27:241-266.

Torrey, J. G. 1965. Cytological evidence of cell selection by plant tissue culture media. In White, P. R., and A. R. Grove (eds.). Proc. Int. Conf. Plant Tissue Culture. McCutchan Pub. Corp. Berkeley. pp. 473-484.

Torrey, J. G. 1967. Morphogenesis in relation to chromosomal constitution in long-term plant tissue cultures. Physiol. Plant. 20:265-275.

Traynor, P. L., and S. M. Flashman. 1981. Hormone-induced caulogenesis in long-term tobacco cell lines and its effect on nuclear DNA content. Theor. Appl. Genet. 60:31-36.

Wenzel, G., 0. Schnieder, T. Przewozny, S. K. Sopory, and G. Melchers. 1979. Comparison of single cell culture derived Solanum tuberosum L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:419-55.