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Chromosome Stability of Cell Suspension Cultures of *Nicotiana* spp.

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ABSTRACT

Cell suspension cultures of Nicotiana were initiated using conditions designed to selectively favor stable chromosome number. These conditions included use of leaf explants to initiate cultures, growth of cells in culture medium containing 2,4-D, and transfer of cells with short subculture intervals. Four cell lines derived from Nicotiana tissue with 2n = 24, 48, or 72 were established and retain stable chromosome number. Each line could be regenerated to recover plants that retained the somatic chromosome number during culture. Establishment of haploid and diploid cell lines with stable chromosome number is important for mutant isolation and protoplast fusion.

INTRODUCTION

As cell suspension cultures have been used frequently for mutant isolation and protoplast fusion, the establishment of cell suspension cultures with stable chromosome number is an essential prerequisite for regeneration of stable haploid and diploid plants. Plant cells grown in culture vary in the ability to attain a constant chromosome number (Bayliss, 1980). Tobacco has been used as a model system for plant tissue culture studies, but no long term cell cultures with stable chromosome number have been reported for tobacco. In some cases, chromosome variation has been attributed to the presence of endoreduplicated and aneuploid cells in the explants used to initiate cell cultures, such as pith tissue (Matthews and Vasil, 1975). However, chromosome instability has also been observed in cultures established from other chromosomally stable tissues in the plant (Bayliss, 1980). In some plant species, evidence has been reported that suggests that only diploid plants are regenerated from mixoploid cultures (D'Amato, 1978). However, in tobacco, and other species of polyploid origin, a wide range of aneuploid plants has been observed among plants regenerated from callus and cell suspension cultures (e.g. Sacristan and Melchers, 1977). As conventional genetic analysis of aneuploid plants is difficult, it is important to establish cultures from which euploid plants can be regenerated if tobacco is to be routinely used for mutant isolation and protoplast fusion.

Three conditions have been identified as being of significance in the establishment of a single dominant karyotype among cultured cells of Nicotiana. (1) Evidence suggests that even though cultures eventually become unstable, the diploid chromosome number predominates shortly after the initiation of cultures derived from diploid tobacco leaves (Marchetti, et al., 1976). As leaf tissue of diploid tobacco has been shown cytophotometrically to have only diploid cells (Dons, et al., 1974), this tissue is preferable to pith tissue for the initiation of cell cultures. (2) Growth regulator concentrations have also been shown to effect chromosome number in cultured cells (D'Amato, 1978). Evidence suggests that use of 2,4-D as opposed to kinetin reduces the frequency of polyploidization (Singh, 1976). (3) Selection pressure in favor of diploid cells has been obtained by establishing cell suspension cultures maintained with short intervals between subculture (Singh and Harvey, 1975; Bayliss, 1977). We have established cell suspension cultures with stable chromosome numbers for 4 genotypes of Nicotiana using leaf or young shoot tissue, cultured on 2,4-D, with short subculture intervals.

MATERIALS AND METHODS

Culture Media and Growth Conditions

Three different media were used for maintenance of all cell suspension cultures (Table 1). The 0.5 MS is composed of the mineral salts of Murashige-Skoog (1962) and vitamins according to Gamborg, et al. (1968) with 2.2 μM 2,4-D. The 1 B5 medium is that of Gamborg, et al. (1968) with 4.5 μM 2,4-D and 1 B5 C contains all components of 1 B5 but includes 200 mg/l N-Z Amine. Shoot cultures of each of these cell lines were maintained on MS medium with 1 or 5 μM 6BA.

Cell Suspension Cultures and Plant Materials

Four cell lines of Nicotiana were initiated in 1978 for this study. In each case, rapidly growing friable callus that was subcultured frequently was used to initiate cell suspension cultures. Nicotiana tabacum homozygous for the sulfur mutation (Su/Su) has been maintained as shoot cultures on MS medium containing 1 or 5 μM 6BA for 3 years. Callus initiated from leaf segments of regenerated plantlets was used to establish the Su-677 suspension culture that has been used for protoplast fusion and plant regeneration (Gamborg, et al., 1979; Evans, et al., 1980). Plants regenerated from the Su-677 suspension culture had 48 chromosomes and regenerated plants were maintained as shoot cultures. Callus was reinitiated on 1 B5 medium from leaf tissue of the albino plants regenerated from the Su-677 culture. The rapidly growing friable callus was subcultured to new 1 B5 medium 2 weeks after the leaf explants

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Table 1. Analysis of Chromosome Number in Four Cultures of Nicotiana

Plant Species	Suspension Culture	Initiated	Explant	Sampling Interval in Months	Suspension Culture 2n=
N. tabacum 2n = 48	Su-378	3/78	leaf	0-2-4-11-22-34	48
N. tabacum (haploid) 2n = 24	HSu	4/78	leaf	0-1-2-4-10	24
N. glauca 2n = 24	Ng	4/78	leaf	0-1-3-10	80% = 24, 20% = 48
N. tabacum + N. glauca 2n = 72	S283	4/78	young shoots	0-1-5	72

were placed on callus medium. This callus was then maintained by subculturing the rapidly growing white callus to new medium once a week. After 6 weeks, callus cells were subcultured to liquid 1 B5 to initiate the Su-378 suspension cultures. The Ng culture was established 2 weeks after placing leaf explants of N. glauca onto 0.5 MS medium and was then subcultured weekly for 6 weeks prior to initiation of cell suspension cultures. The S283 culture was initiated by transferring a young shoot regenerated 4 weeks following protoplast fusion of N. glauca + N. tabacum (Su/Su) (Evans, et al., 1980) from MS medium with 1 μM 6BA onto 1 B5 C culture medium. Callus began to form at the base of the shoot within 2 weeks and the callus was subcultured to fresh 1 B5 C medium 1 week later. After 6 weeks, the rapidly growing callus was used to initiate a cell suspension culture. Anthers of Su/su (light green) N. tabacum were cultured on hormoneless MS medium as described by Burk (1970) and haploid albino plants were selected and maintained as shoot cultures on MS medium with 1 μM 6BA. Leaf sections derived from shoot cultures of the haploid albino tobacco were placed on 0.5 MS medium and callus that had formed after 4 weeks was subcultured weekly to fresh 0.5 MS media. Eight week old callus was used to initiate cell suspension cultures. To initiate each new suspension culture, callus cells (1 week after subculture) were placed in liquid callus medium in a 60 x 15 cm Petri dish and rotated at 40 rpm. Fresh culture medium was added every four days and during the second addition of culture medium, cells were transferred to a 250 ml Erlenmeyer and rotated at 150 rpm. After the cell volume reached 50 ml, cells were subcultured twice weekly as described below.

Culture Conditions

Suspension cultures were maintained on New Brunswick G10 gyratory shakers at 150 rpm. Temperature and light were maintained constant (25°C and 1500 lux, respectively). Suspension cultures were subcultured twice weekly by transferring 10 ml of cells to 50 ml of fresh liquid medium. The growth rate of S283 was greater than either Ng or Su 378 so that a smaller cell volume (ca. 5 ml) was used for subculture of this cell culture.

Cytology

As most cultures appeared to have a single population with one chromosome number, colchicine (1 mg/ml) was routinely added at 24 h post subculture for 4 h to ascertain chromosome number of the culture. The cells were fixed and stored overnight in ethanol: acetic acid (3:1, v/v). Cells were hydrolysed for 4-6 h at room temperature in 1.0 N HCl, then stained in modified carbol fuchsin (Kao, 1975). Between 20 and 100 cells were counted to ascertain chromosome number of each culture. Mitotic index (MI) was ascertained as reported earlier from suspension cultures that were not pretreated with colchicine (Evans and Gamborg, 1979).

RESULTS AND DISCUSSION

The Su-378 cell culture, which is routinely subcultured every 4 days, was examined in detail. The MI of Su-378 was recorded in cells collected at 4 h intervals between 4 and 96 h following subculture, and data was collected during four subculture intervals. At 0 h, the MI was 3% and the MI continually increased to a maximum at 24 h (Fig. 1). The MI remained between 7-10% until the last day before subculture when the MI decreased. While the MI of Su-378 varied from a low of 3% at 96 h to a maximum of 9.5% at 24 h after subculture, the chromosome number remained constant, 2n = 48, in each sampling. If cells were not subcultured at 96 h, the MI of the culture decreased to less than 0.5% within 32 additional h (Fig. 1, at 128 h). In cell lines of Su-378 that were continually subcultured at 7 day intervals, an extensive lag period of 16-20 h was observed upon subculture to new culture medium before the MI increased. A high frequency of polyploid cells was observed shortly after Su-378 cultures were changed to a 7 day subculture regime. In addition to preliminary evidence suggesting greater chromosome stability, Su-378 cell suspension cultures subcultured every 96 h were ideal for reliable, high frequency release of protoplasts with subsequent recovery of rapidly dividing cell colonies. Consequently, a subculture procedure was established for the HSu, Ng, and S283 suspension cultures that resulted in MI between 2-15% at any time during the subculture cycle. The HSu and Ng growth rates were similar to Su-378, so that for each of these cultures, 10 ml of cells were transferred to 50 ml of culture medium twice a week. The S283 suspension culture grew more rapidly than the other cultures so that 5 ml of cells were transferred to a 50 ml of culture medium twice a week to establish a similar growth cycle.

Chromosome number was ascertained for each culture following the first subculture of each cell suspension. This chromosome count, though the cells had been in callus culture for 8-10 weeks, was designated as time 0 for the cell suspension cultures. The somatic chromosome number was preserved for each of the four Nicotiana cell suspension cul-

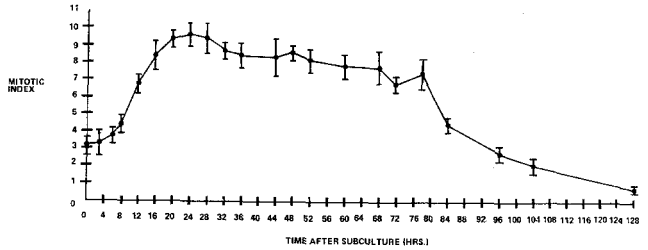

Fig. 1. Mitotic index of N. tabacum (Su/Su) suspension culture sampled following subculture. Values represent the mean of three samples of 200 cells with standard error.

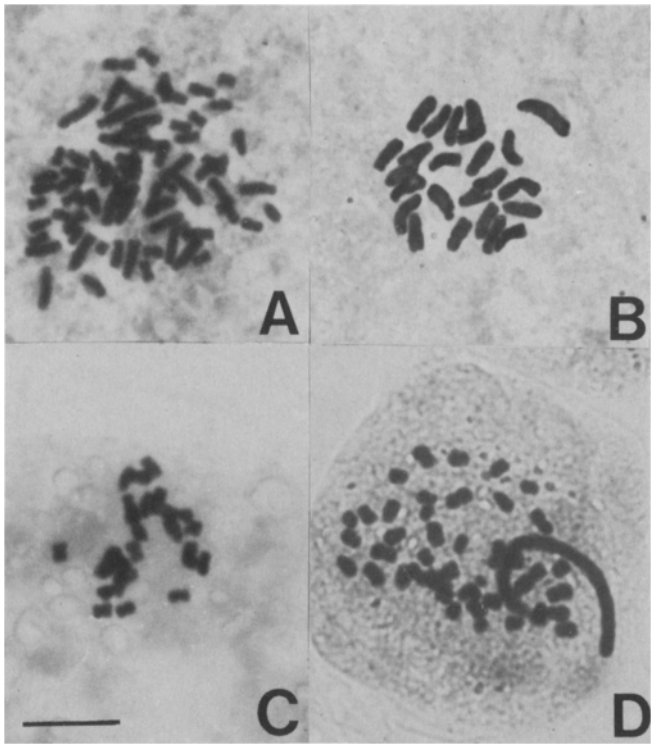

Fig. 2. Representative metaphase cells from plant suspension cultures of Nicotiana: (A) Somatic hybrid of N. tabacum + N. glauca, 2n = 72; (B) N. glauca, 2n = 24; (C) Haploid N. tabacum, 2n = 24; and (D) N. tabacum, 2n = 48, including one megachromosome. Bar = 10 μM.

tures despite the range of ploidies and species that was examined (Table 1). The Su-378 cell suspension culture has retained a chromosome number of 48 when 89 cells were counted in cultures that were examined 34 months after initiation of the suspension culture. All ten plants that were regenerated from this culture, 4 months after the culture was established, had 48 chromosomes. Protoplasts that were isolated from this suspension culture have been fused with leaf mesophyll protoplasts (Evans, et al., 1981). All interspecific hybrid plants that were recovered using the Su-378 suspension culture had only the amphiploid summation chromosome number (Evans, et al., 1981). This Su-378 line has also been used to isolate mutant cell lines that retain 48 chromosomes (Flick, et al., 1981). The availability of chromosome stability in mutants and somatic hybrids isolated using this culture will greatly facilitate the genetic analysis of the regenerated plants. The S283 suspension culture derived from the somatic hybrid retained stable chromosome number during the 5 months that it was monitored (Fig. 2A). Ten plants were regenerated from this cell culture after 5 months and each plant was stable with 72 chromosomes. The Ng suspension culture contained the only variation in chromosome number. When examined after the first subculture, no polyploid cells were observed. One month later, 19% of the 75 cells counted were tetraploid, while 49 cells (81%) were diploid, and at 10 months when cells were transferred to regeneration medium, 20% of the cells that were counted were tetraploid. This behavior is consistent with induction of tetraploidy by a component of the culture medium as no tetraploids were visible during initial examination. All 9 regenerated plants were diploid (Fig. 2B), suggesting diploid cells have selective advantage for regeneration. The RSu culture had stable chromosome number during the 10 months in which it was monitored. At 10 months after initiation cells were transferred to regeneration media. All 10 shoots regenerated had the haploid chromosome number (Fig. 2C).

While chromosome structure was not monitored in detail, the data collected on these suspension cultures suggest that structural rearrangements were minimal. When MI was monitored in the cell suspension cultures, anaphase cells were examined for bridges and fragments that have frequently been reported in aneuploid tobacco cultures (e.g. Dimitrov and Zagorska, 1976). However, anaphase aberrations were not detected in any of these cultures. Only one abnormal metaphase containing a megachromosome (Fig. 2D) was observed among the 89 cells counted in the 34 month old Su-378 culture. Megachromosomes have been reported in root tips of certain Nicotiana hybrids and may reflect differential replication of heterochromatin rather than chromosome structural rearrangements (Burns and Gerstel, 1973). Finally, all plants regenerated from these suspension cultures had the expected phenotype, while structural rearrangements would probably alter plant phenotype. While this evidence is inconclusive, it suggests that abnormal structural rearrangements were minimal in these cell cultures.

Cell cultures of Nicotiana tabacum frequently contain aneuploid cells and prior to this report, no haploid tobacco cell lines had been isolated with stable chromosome number. This method, for initiation of cell cultures of Nicotiana with stable chromosome number, relies on initiation of cultures from leaf or young shoot explants that contain homogeneous chromosome number and applies the selection methods proposed for Triticum and Haplopappus (Singh, et al., 1975) that favor establishment of a predominant cell population with uniform chromosome number.

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