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STUDIES ON PLANT CELL AND TISSUE CULTURE

VI. Karyotypic changes in calluses of haploid and diploid plants of *Nicotiana* species

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Introduction

The occurrence of polyploidy, aneuploidy and chromosome aberrations in plant and animal cells cultured *in vitro* are widespread phenomena although the causative mechanism still remains obscure.

The majority of mammalian cell cultures can be grouped into two classes, depending upon the variation in chromosome complements. First homonuclear lines are characterized by the fact that there is very little variation among cells in chromosome complement; next heteronuclear lines, on the other hand, show marked variation. Homonuclear lines can be started from benign (i. e., noncancerous) solid tissue of any individual living body, while heteronuclear lines are initiated quite frequently from malignant tissues. The growth of homonuclear cell lines have in general a finite life time of about 50 generations after the separation of the cells from the explant. Thereafter the generation time gradually becomes prolonged, and the morphology of the cells changes somewhat. Cell lines have been maintained for several years under this condition, but growth did not resume. On the other hand, the growth of heteronuclear cell lines are capable of indefinite life time *in vitro*, mostly with malignant properties, exhibiting chromosome variation even between cells of a single clone (see rev.: KROOTH, *et al.*, 1968).

In contrast to mammalian culture, plant cell cultures are capable of indefinite growth *in vitro* even without tumorous transformation such as crown-gall tumor or the habituation. Furthermore, these normal cultures of plant cells frequently exhibit an extensive chromosome variation. There are now a considerable amount of data indicating that the chromosome stability of plant cultures is profoundly affected by cultural conditions and particularly by the composition of the culture media (TORREY, 1958; DE TOROK and WHITE, 1960; MITRA, *et al.*, 1960; COOPER, *et al.*, 1962;

BLAKELY and STEWARD, 1964; DEMOISE and PARTANEN, 1969; VAN'T HOF and McMILLAN, 1969; SINGH, *et al.*, 1972). Especially, the presence of constituents of growth regulators in the medium plays an important role in the chromosome state in the cultured callus tissues (NIIZEKI, 1973, 1974b).

This chromosome instability generally found in cultured plant tissues may be considered sometimes as a serious barrier, when the cultured cells are applied to several approaches such as somatic cell genetics and mutation research which require the preservation of a stable chromosome state. The difficulty to obtain a callus culture which is cytologically stable may partly depend upon the plant species. In other words, the chromosome stability or instability of plant callus cultures is subject in part to plant species from which these cultures originated.

The present study reports an investigation of the comparison in nuclear behaviour and karyotypic changes in the haploid and diploid plants in three *Nicotiana* species, *N. tabacum*, *N. sylvestris* and *N. glutinosa*.

Materials and Methods

Haploid plants of three species, *N. tabacum* ($2n=48$), *N. sylvestris* ($2n=24$) and *N. glutinosa* ($2n=24$) were induced from anther culture on the medium of NITSCH and NITSCH (1969) supplemented with 0.1~2 mg/l indole-3-acetic acid (IAA) and 0.1~2 mg/l kinetin singly or in combination (Table 1). Calluses of these three species of haploid plants and the parental diploid plants were induced from young stem cultures on the basic medium of MILLER (1963) which was supplemented with 2mg/l of IAA and kinetin. These obtained calluses were thereafter subjected to subcultures at intervals of about one month on the same medium which was used for the induction

TABLE 1. Anther culture of three *Nicotiana* species

Species	Medium type	No. of anther used for culture	No. of anther forming plantlets	% of anther forming plantlets	Size of flower bud used for anther culture (mm)	Size of flower bud forming plantlets (mm)
<i>N. tabacum</i>	A	120	69	57.50	5-25	9-23
<i>N. sylvestris</i>	A	100	22	22.00	8-85	15-30
	C	100	18	18.00	6-85	18-35
<i>N. glutinosa</i>	A	275	2	0.73	4-26	16-22
	B	250	0	0	4-27	
	C	250	1	0.40	4-28	12
	D	65	1	1.54	5-17	17

A, 0.1 mg/l IAA and 0.1 mg/l kinetin; B, 1 mg/l IAA and 1 mg/l kinetin; C, 2 mg/l IAA; D, 2 mg/l kinetin.

of these calluses. All cultures were kept under dark condition at $26 \pm 0.5^\circ\text{C}$.

Cytological observations of the callus tissues were performed after 10 days of the first or second subculturing passages. The samples of callus tissues were pretreated in 0.002 M 8-hydroxyquinoline for 2 hours and then fixed in alcohol-acetic acid solution (3:1) for about 12 hours. The fixed callus tissues were macerated in 4% of pectinase for 6 to 12 hours at 20°C , and then stained with alcoholic hydrochloric acid-carmin (SNOW, 1963) for about 12 hours. Slides were prepared by routine squashing method.

Results

The following changes in chromosome number and structure in the cultured callus tissues of three *Nicotiana* species were observed:

N. tabacum ($2n=48$)

Haploid callus tissues maintained the original haploid cell ($n=24$) line for more than 70%, 10 days after transplantation onto the subculture (Fig. 1). About 6% of the cells in the callus tissues showed diploid chromosome

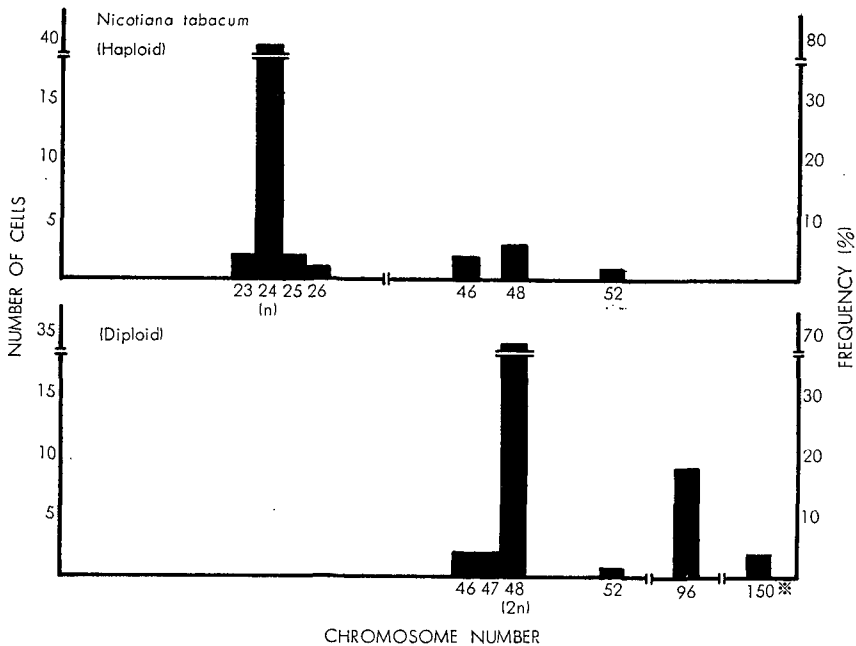


Fig. 1. Frequencies of the cells with various chromosome numbers in haploid and diploid calluses of *Nicotiana tabacum* ($2n=48$).

* More than 150 chromosomes.

numbers ($2n=48$) which might have arisen from the multiplication of the haploid chromosomes by the process of endomitosis. The diploid callus line also maintained the original diploid cells at a high frequency of about 70%, but a considerable number of cells (18%) showed tetraploid chromosome numbers ($4n=96$). In both haploid and diploid callus tissue lines a few aneuploid cells were observed and sometimes cells with more than 150 chromosomes were found in the diploid callus line.

N. sylvestris ($2n=24$)

In the line of haploid callus tissues 50% and 18% of the cells examined for their chromosome number showed diploid ($2n=24$) and tetraploid ($4n=48$), respectively. The cells with the original haploid chromosome number ($n=12$) were, on the other hand, remained at only less than 30% (Fig. 2). In the line of diploid callus tissues 48% and 26% of the cells exhibited tetraploid ($4n=48$) and octoploid ($8n=96$) chromosome numbers, respectively, and about 10% of the cells showed more than 150 chromosomes, while only 4% of the cells preserved the original chromosome numbers of diploid state ($2n=24$). Therefore, a high frequency of the multiplication of chro-

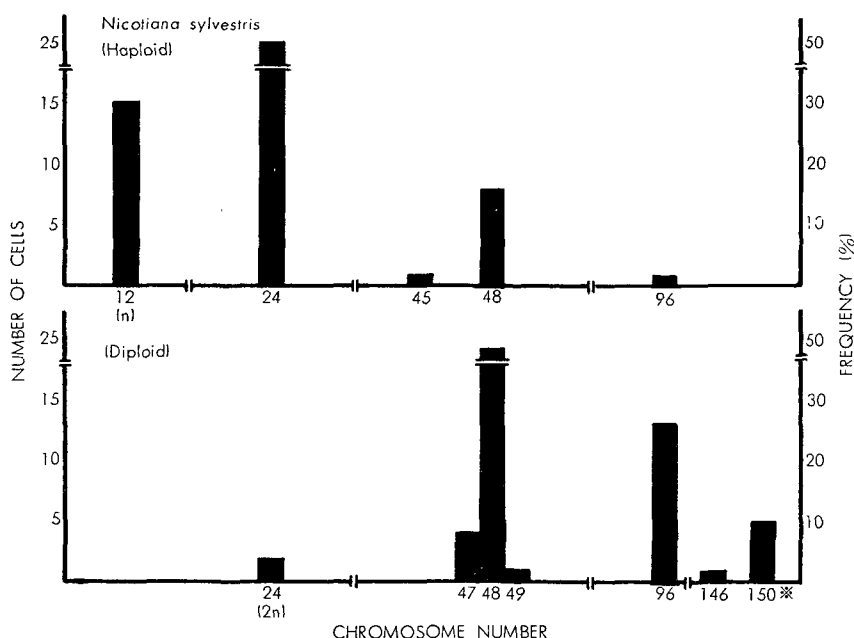


Fig. 2. Frequencies of the cells with various chromosome numbers in haploid and diploid calluses of *Nicotiana sylvestris* ($2n=24$).

* More than 150 chromosomes.

mosome numbers was considered as a conspicuous characteristic in both ploidy levels of the callus cultures of this species.

N. glutinosa ($2n=24$)

The line of haploid calluses contained 22%, 22% and 6% of the haploid ($n=12$), diploid ($2n=24$) and tetraploid ($4n=48$) cells, respectively (Fig. 3). The most remarkable characteristic of the calluses of this species was the highly frequent appearance of aneuploid cells such as 13 ($n+1$), 25 ($2n+1$) and 26 ($2n+2$) chromosomes. Especially, cells with 13 chromosomes showed 22% of the same frequency as the original euhaploid cell with 12 (n) chromosomes (Fig. 4). In the diploid callus line 38%, 12% and 4% were diploid ($2n=24$), tetraploid ($4n=48$) and octoploid ($8n=96$) cells, respectively. The occurrences of aneuploid cells were also significantly high in this diploid callus line, especially 23 ($2n-1$) and 47 ($4n-1$) which were 30% and 8%, respectively. Chromosome structural changes which showed dicentric chromosomes were observed as a special feature in this diploid callus line (Table 2 and Fig. 4). Cells with one dicentric chromosome were most frequently observed, but sometimes a few of the cells showed two

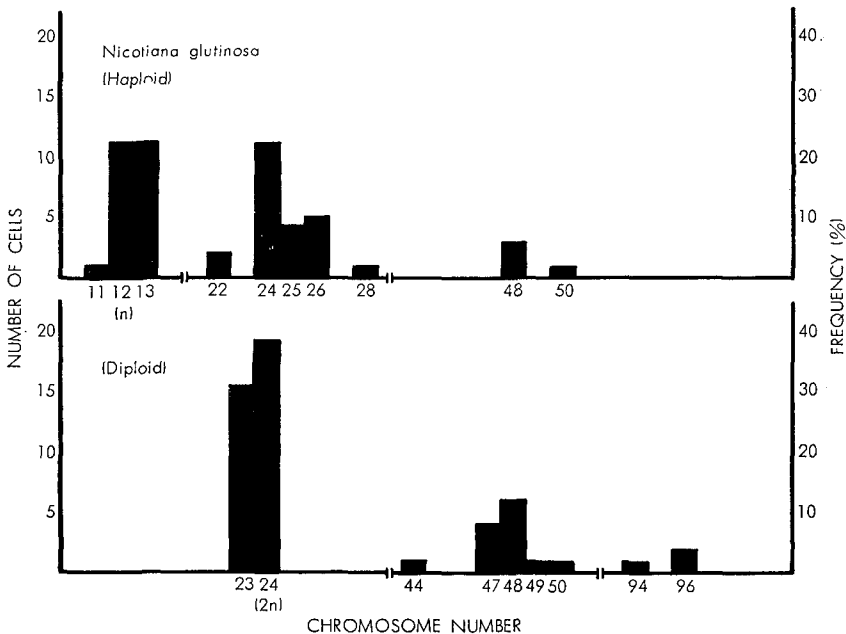


Fig. 3. Frequencies of the cells with various chromosome numbers in haploid and diploid calluses of *Nicotiana glutinosa* ($2n=24$).

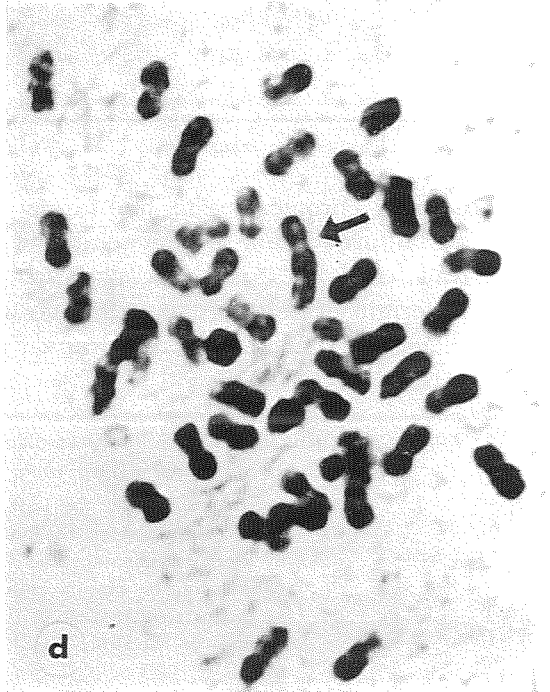
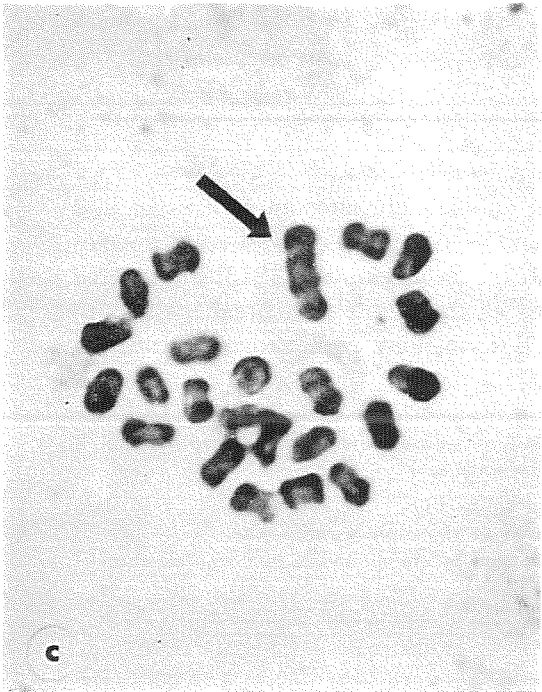


TABLE 2. Changes in chromosome number and structures in the cells of *Nicotiana glutinosa* ($2n$) in callus culture

Chromosome no. of cell	No. of cells	Remarks
21	1	All monocentric
22	3	All monocentric
22	1	Two dicentric
23	19	All monocentric
23	14	One dicentric
24	55	All monocentric
44	1	One dicentric
46	1	One dicentric
46	1	Two dicentric
47	4	All monocentric
47	9	One dicentric
48	24	All monocentric
48	1	Two dicentric
48+F*	1	
49	1	All monocentric
50	1	Two dicentric
54	1	All monocentric
94	1	All monocentric
96	2	All monocentric

* chromosome fragment.

dicentric chromosomes. The formation of such chromosomes in tissue and cell cultures is not a rare event and has been reported in several species (KAO, *et al.*, 1970 and SUNDERLAND, 1973). SUNDERLAND (1973) interpreted the origin of this dicentric chromosome as the result of a breakage-fusion-bridge cycle similar to that originally described by McCLINTOCK (1951) in maize.

Fig. 4. Changes in chromosome number and structure were found in haploid and diploid callus lines of *Nicotiana glutinosa*.

- 13 chromosomes in the haploid callus line.
- Normal chromosome complement in the diploid callus line.
- One dicentric chromosome (indicated by arrow) and 22 monocentric chromosomes in the diploid callus line.
- One dicentric chromosome (indicated by arrow) and 46 monocentric chromosomes in the diploid callus line.

Discussion

Although the results presented here did not show a sufficiently stable culture line with regard to the nuclear state in the three *Nicotiana* species, it may still be considered important to pay attention to the fact that some cultures in certain species actually remain stable without any visible chromosomal changes. For instance, REINERT and KÜSTER (1966) observed no variation in chromosome number after one year in cells derived from *Crepis capillaris* L. SACRISTÁN (1971) also reported that in the same species 34.1% of the subcultures derived from the haploid plants and 78.5% from the diploid plants preserved the original ploidy levels for more than a year. Therefore, there is still hope of achieving chromosome stability in certain species for considerable long periods of subculturing.

There have been reported, on the other hand, many instances of unescapable chromosome variation in cell and tissue cultures of plants in many species. There is no fixed pattern of the chromosome variation in those cultured populations of plant cells and tissues. The variation pattern changes not only from one species to another species, but also the pattern differs among the same species in the different culture conditions. This inconstancy may be partly due to different nutrient media, especially varied growth regulators such as auxin and cytokinin and different periods of subculture (NIIZEKI, 1973, 1974 b). The fundamental causes of chromosomal changes in the course of the culture, however, largely remain unknown. The question inevitably arises as to whether or not perpetual maintenance of the stable nuclear state can be achieved by the culture techniques alone at present time. Insofar as the past experiments by many investigators are concerned the answer appears to be very pessimistic. Therefore, we must seek alternative methods, for instance, preserving clones in non-growing condition or the use of some specific chemicals which have a selective effect on a particular nuclear state. CAPLIN (1959) succeeded in maintaining cultures for long periods without loss of viability by covering them with a layer of mineral oil. More recently some successes have been reported in freezing of cultures in the presence of protecting agents such as glycerol or liquid nitrogen (LATTA, 1971, and NAG and STREET, 1973). Such a controlled freezing and low temperature storage of the cultured plant cells might enable the initial characters of newly established cultured to be preserved. An amino acid analogue, para-fluorophenylalanine (PFP), has been reported as a specific inhibitor for the growth of the diploid callus tissues in *Nicotiana tabacum* (GUPTA and CARLSON, 1972). Furthermore, NIIZEKI (1974 a) ob-

served that certain defined concentrations of PFP may suppress the growth of diploid cells and may promote preferential selection of haploid cells from mixed population of various chromosome constituents, although it seems to be difficult to establish a complete pure cell lines of haploidy.

Because tissue cultures are useful in various types of study on genetics and plant breeding, further research for conditions by which original genomes can be maintained and chromosome instability minimized seems to be required. Especially, control is essential in the prevention of polyploidization of haploid cell and tissue cultures because it plays an important role on somatic cell genetics and mutation research.

Summary

Recent advanced tissue culture techniques in eukaryotes are very useful, because the cultured tissues or cells are highly advantageous for studies in many fields of genetics which have been mainly carried out by the use of prokaryotes. However, the occurrence of chromosome instability such as polyploidy, aneuploidy and chromosome aberrations in the cultured plant tissues is a widespread phenomenon and may be considered sometimes as a serious barrier for several approaches such as somatic cell genetics and mutation research.

For the purpose of seeking for a stable cultured line of nuclear state, chromosome behaviours and karyotypic changes were investigated in the calluses of haploid and diploid plants in three *Nicotiana* species, *N. tabacum* ($2n=48$), *N. sylvestris* ($2n=24$) and *N. glutinosa* ($2n=24$). Both haploid and diploid cultured calluses in *N. tabacum* showed a considerably high stability in the nuclear state as compared with the other two species, although a considerable amount of the cells with polyploid and aneuploid chromosomes were also observed in both ploidy levels of this species. On the other hand, in *N. sylvestris* the degree of polyploidization of the haploid or diploid cultures was significantly higher than those of the other species. The original haploid and diploid cells in each line were maintained at rate of only 30% and 4%, respectively. In *N. glutinosa*, the appearance of aneuploid cells was very remarkable, especially the cells with $n+1$ or $2n+2$ chromosomes in haploid and $2n-1$ or $4n-1$ chromosomes in diploid culture line observed at high frequencies. Furthermore, dicentric chromosomes which might have originated from chromosome rearrangement were frequently observed in the diploid cultured callus of this species. As far as in this experiment is concerned, in conclusion, no single line of cultured calluses having a sufficient stability in the nuclear state was found in these

cultured calluses in the three species, although the degree and pattern of chromosomal variation was quite different among them.

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