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STUDIES ON PLANT CELL AND TISSUE CULTURE

V. Effect of Different Kinds of Media on the Variation of Chromosome Numbers in Tobacco Callus and Regenerated Plant

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Introduction

Haploids from higher eukaryotes are of great importance for studies on genetics, cytogenetics, and plant breeding. The isolation of haploids from higher plants is generally very difficult. The technique of anther culture, however, has been used to overcome this difficulty, but it has been observed that anther culture leads to calluses consisting of cells of different levels of ploidy (NIIZEKI, 1973). Also, from the calluses induced by anther culture, in which the production of haploid plants is expected, frequent occurrences of polyploids as well as haploids were reported in *Oryza sativa* (NISHI and MITSUOKA, 1969; NIIZEKI and OONO, 1971), *Setaria italica* (BAN, KOKUBU and MIYAJI, 1971), *Solanum nigrum* (HARN, 1972) and *Hordeum vulgare* (CLAPHAM, 1973). Thus, the chromosomal instability in the anther culture has been recognized as a relatively common characteristic that may be considered as an important problem in the maintenance of haploid cell lines. The chromosomal instability is not restricted to only the calluses derived by anther culture but also to calluses originating from somatic tissue of plants, which display more than the usual degree of chromosomal instability when they are removed from the stabilizing environment of the intact organism and transplanted into a culture medium environment.

There is now a considerable amount of data indicating that the chromosomal 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, MAPES and STEWARD, 1960; COOPER, HILDEBRANDT and RIKER, 1962; BLAKELY and STEWARD, 1964; Demoise and Partanen, 1969; VAN'T HOF and McMILLAN, 1969; SINGH, HARVEY, KAO and MILLER, 1972). A particular instance of this is the

preservation of the haploid cells and the diminishing of the diploid cells in tobacco callus cultures by incorporation of para-fluorophenylalanine into the culture medium (GUPTA and CARLSON, 1972; NIIZEKI, 1974). Further critical work is now required to determine to what extent different culture media enhance or diminish the incidence of chromosomal irregularities and how far the apparent stabilization or breakdown of nucleus in different media is due to such media which may be selective or non-selective for particular genotypes.

In a previous study (NIIZEKI, 1973), polyploidization and aneuploidization in the haploid calluses of *Nicotiana tabacum* in early passages of subculture were found to be accelerated by media containing IAA alone or IAA and kinetin in combination. On the other hand, all cells in division were mainly haploid and diploid in the callus tissues in media containing no growth regulator or kinetin singly. The intent of this paper is to investigate the transition of chromosomal stability in different medium conditions in advanced subculturing passages in further detail and to study the relationship of the chromosomal variations between regenerated plants and callus tissues.

Materials and Methods

Haploid callus tissues of *Nicotiana tabacum* (var. Wisconsin 38) were used in this study. The initial callus was established by anther culture on MILLER'S basic medium (1963) supplemented with 4.0 mg/l of indole-3-acetic acid (IAA) and 2.0 mg/l of 6-benzylaminopurine (BAP). Fragments of the callus were transplanted to a series of Miller's basic media containing and/or not containing growth regulators in the following manner; no growth regulator (Medium A), 4.0 mg/l of kinetin (Medium B), 4.0 mg/l of IAA (Medium C), and 2.0 mg/l of IAA and 2.0 mg/l of kinetin (Medium D). The calluses cultured on Medium D were repeatedly subjected to transplantations to fresh media in the same manner as the above-described series of media. The transplantations were carried out at intervals of 30 days throughout the experiment. All cultures were kept in the dark at $26 \pm 0.5^\circ\text{C}$.

Cytological observations of the callus tissues were performed on the 1st, 2nd and 8th subculturing passage. The results on the 1st and 2nd subculture have already been reported in a previous paper (NIIZEKI, 1973). The chromosome numbers were determined after 10, 20, 30 days of the 8th passage of subcultures. 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 room temperature, and then stained with alcoholic hydrochloric acid-carmin (SNOW, 1963) for about 12 hours. Slides were prepared by the routine squashing method.

After one month of sustained culture, the regenerated shoots from callus tissues cultured on each medium were severed from the calluses and then transplanted onto MILLER's basic medium without any growth regulators. After 10 to 20 days of culturing, the shoots had generally proliferated a number of roots, and thereafter the obtained plantlets were transplanted to pots. The determination of chromosome numbers of these regenerated plants was carried out by the squashing method of root tips which were first fixed in alcohol-acetic acid solution (3:1) for about 12 hours and which were then stained with alcoholic hydrochloric acid-carmin.

Results

Frequencies of cell division in callus tissues

About 1 to 2×10^3 cells of meristematic parts of callus tissues were examined for mitotic frequencies at the time just before transplantation and at two periods after 10 and 20 days of the 8th subculture. The number of cells at the metaphase and anaphase stage in the cell cycle was shown as the relative activity of the mitotic cell division in each callus tissue cultured on four different types of media. The results are shown in Table 1. The frequencies of metaphase and anaphase cells at both periods after 10 and 20 days of incubation were higher in the order of calluses cultured on the Medium D, C, B, and A. In other words, the calluses cultured on the Medium D containing both IAA and kinetin showed the highest frequency of cell division at both periods of culture. The cell division on

TABLE 1. Frequencies of mitotic cells at meristematic parts of callus tissues cultured on four types of media

Medium type	Days in culture		
	0*	10	20
Medium A	0.235%	0.539%	0.315%
Medium B	0.235	1.201	0.587
Medium C	0.235	1.284	0.874
Medium D	0.235	1.390	0.890

Medium A, no growth regulator; Medium B, 4 mg/l kinetin; Medium C, 4 mg/l IAA; Medium D, 2 mg/l kinetin and 2 mg/l IAA.

* at the time just before transplantation to subculture.

Medium B or C containing IAA or kinetin alone was somewhat lower as compared to those on the Medium D. The lowest cell division was observed on the callus tissues cultured on the medium to which on growth regulator was added. As a common characteristic of the calluses cultured on four different types of media, the frequencies of mitotic cell division showed a tendency to decrease with the advanced periods in all cultures.

Chromosome numbers of callus tissues

Determination of chromosome numbers of the calluses was performed after 10, 20, and 30 days of the 8th passage of subcultures. The results are shown in Fig. 1. The callus tissue cultured on Medium A without growth regulators showed the most stable chromosomal state maintaining the constituent of cells with only 24 (n) and 48 ($2n$) chromosomes throughout the entire period of the culture. Only changes such as a slight increase of the cells with 48 chromosomes and decrease of the cells with

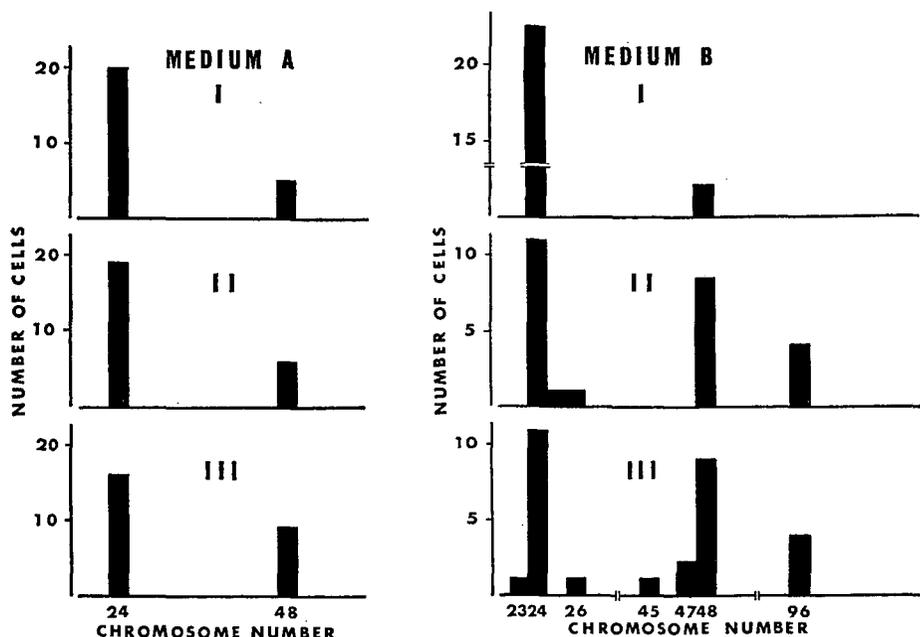


Fig. 1. Somatic chromosome numbers in the 8th subculture of haploid callus tissues of *N. tabacum* (cv. Wisconsin 38). The callus tissues cultured on the Medium D successively transplanted into four types of the next subculturing media at interval of 30 days. Medium A, no growth regulator; Medium B, 4 mg/l kinetin; Medium C, 4 mg/l IAA; Medium D, 2 mg/l kinetin and 2 mg/l IAA. I, 10 days after transplantation; II, 20 days after transplantation; III, 30 days after transplantation.

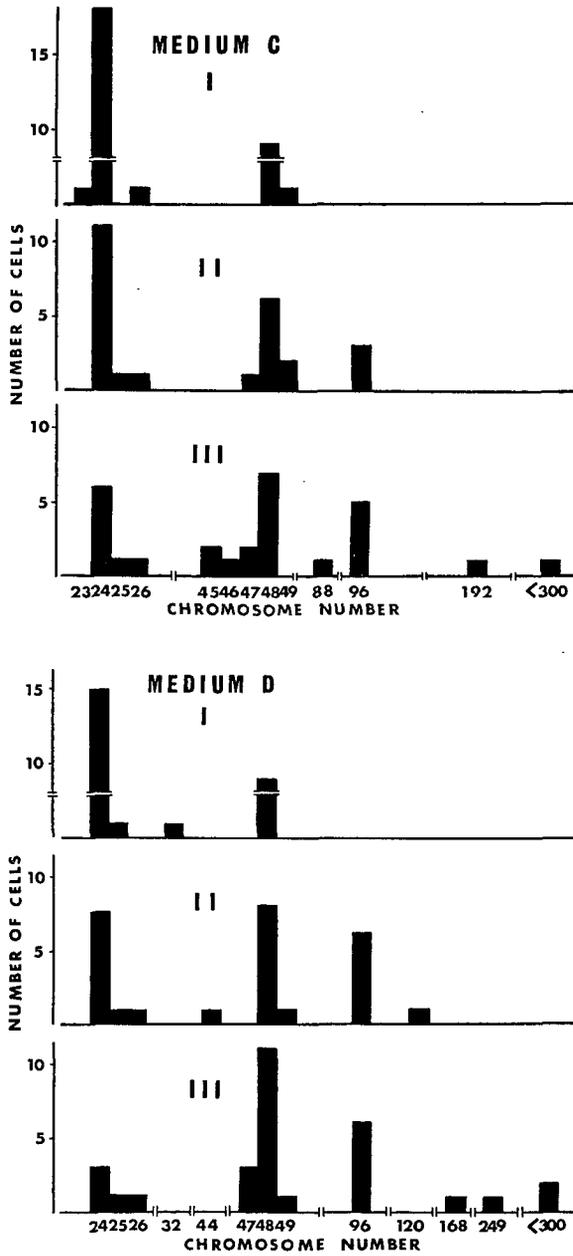


Fig. 1. (Continued)

24 chromosomes were noted. No aneuploid cell was found in this callus tissue. On the other hand, considerable variations in the chromosome numbers were observed in the callus tissues cultured on the Medium B, C, or D, which contained IAA and kinetin singly or in combination. As a common feature of the calluses on these three types of media, the variations of chromosome numbers were generally small in the early stages after transplantation and became wider in advanced stages of the subcultures. Most of the cells of the callus tissues on these media showed 24 and 48 chromosomes and a few cells were aneuploids after 10 days of incubation. However, in late periods of the cultures, namely after 20 to 30 days of incubation, the variations of chromosome numbers drastically increased, including 96 ($4n$), 192 ($8n$) chromosomes, and various aneuploids. The widest variation was observed in the callus tissues cultured on Medium D containing both of IAA and kinetin and chromosome numbers exceeding 300 were sometimes counted in several of the cells. The callus tissues cultured on Medium C containing IAA alone also showed a considerable variation in the chromosome numbers. The calluses on Medium B containing kinetin alone showed a somewhat smaller variation than those cultured on Medium C and D.

Chromosome numbers of regenerated plants

30 plants regenerated from each callus tissue cultured on four types of media were determined for their chromosome numbers by using root tips (Table 2). Totipotency of the callus tissues seems to be affected by different medium types. A high potentiality of regeneration of plants from calluses appeared to occur on Medium A and B, while Medium C and D containing

TABLE 2. Number of regenerated plants with different chromosome numbers from callus tissues cultured on four different types of media

Medium type	Number of regenerated plants			Total
	24 (n)	Chromosome number		
		48 ($2n$)	96 ($4n$)	
Medium A	28	2	0	30
Medium B	18	9	3	30
Medium C	27	3	0	30
Medium D	23	7	0	30

Medium A, no growth regulator; Medium B, 4 mg/l kinetin; Medium C, 4 mg/l IAA; Medium D, 2 mg/l kinetin and 2 mg/l IAA.

IAA led to a considerable decrease in the ability to produce shoot initiation of the calluses. On Medium A containing no growth regulator, 28 individuals of regenerated plants had 24 (n) chromosomes and the remaining 2 had 48 ($2n$) chromosomes. On Medium B containing kinetin alone, 18, 9, and 3 of the regenerated plants had 24, 48 and 96 ($4n$) chromosomes, respectively. These various ploidy levels of regenerated plants derived from the callus on Medium B could be naturally expected from callus tissues containing various polyploid cells. On the other hand, 27 and 3 of the regenerated plants had 24 and 48 chromosomes, respectively, on Medium C containing IAA, 23 and 7 of the regenerated plants had 24 and 48 chromosomes, respectively, on Medium D containing IAA and kinetin. Tetraploid plants and much higher levels of polyploidy did not regenerate from the callus tissues on Medium C and D even though these calluses had more frequent of the higher levels of polyploid cells than those on Medium B. No single aneuploid plant was derived from all calluses cultured on the above described four types of media.

Discussion

It is clear now that the growth regulators added to the culture medium have a determinative role of both polyploidization and aneuploidization regarding the nuclear behaviors in callus tissues, although the governing mechanisms contribute to the above phenomena are not known at present. SUNDERLAND (1973) reported that a suspension culture of *Haplopappus* cotyledon cells grown in a medium containing 2, 4-D changed entirely from diploid to entirely tetraploid over a period of less than 6 months of subculture. Therefore, it may be concluded that 2, 4-D as an auxin component contributes by accelerating the rate of polyploidization. In this study the occurrence of chromosomal changes, such as diploid, tetraploid, octoploid and various aneuploids diverted from a haploid state, was also frequent in the calluses cultured on media containing an auxin of IAA while the callus cultured on medium without growth regulators maintained only haploid and diploid cells. The highest occurrence of polyploid and aneuploid cells was observed in the calluses cultured on medium supplemented with IAA in combination with kinetin. The frequencies of the metaphase and anaphase cells which may indicate the relative activity of the mitosis and may be closely related to the rate of callus growth were significantly high on media containing IAA as compared to those on medium containing no growth regulator or kinetin alone. The highest cell division was observed in the callus tissues on medium containing IAA and kinetin in combination.

Therefore, it seems to be a incontrovertible fact that a condition such as the medium containing IAA as an auxin enhances the growth of calluses and simultaneously affects the variations of chromosome numbers. However, it still remains unclarified as to whether the association of the rapid callus growth and the high chromosomal instability depends upon a mere coincidence of two independent phenomena in a common environment or has a certain cause and effect relationship in such a way that the higher callus growth strikingly gives rise to wider variations in the chromosome numbers.

Studies by TORREY (1959), and MITRA, MAPES and STEWARD (1960) have shown that root meristems induced from highly polyploid cultured tissues are normally diploid. In our previous study of *Lotus corniculatus* (NIIZEKI and GRANT 1971), most of the regenerated plants from the calluses containing a number of the various polyploid and aneuploid cells were diploid and only a few of them were tetraploid. In this study, media containing IAA, which was employed for accelerating the polyploidization of chromosome numbers, appeared to have a considerably high inhibiting effect on the regeneration of plants from the calluses as compared to the medium containing kinetin alone or no growth regulator. Furthermore, the regenerated plants from the calluses cultured on media containing IAA were mainly haploids and a few of them were diploids, even though the callus tissues showed a high degree of polyploidization of the chromosome numbers. On the other hand, the callus cultured on medium containing kinetin alone produced a considerable number of diploids and even tetraploids, although the calluses did not indicate so high polyploidization as those on media containing IAA. These facts suggest that the conditions which are favorable for polyploidization of the calluses probably are unfavorable on the morphogenesis of higher levels of polyploids. In contrast, it seems that suppressible conditions of polyploidization of the callus tissues are rather preferential to morphogenesis of polyploid levels of plants.

Another feature of the morphogenesis of the calluses found in this study is that no single plant showing aneuploidy was obtained even from the calluses showing high aneuploidy. MURASHIGE and NAKANO (1965) reported that in tobacco pith cultures advanced age was associated with a decline in differentiation of the roots and shoots. In addition, they noted that the callus lines in the morphogenetically reduced state were associated with considerable variations in chromosome numbers, especially, aneuploidy, while initial lines were either diploid or tetraploid. From these observations it may be concluded that in cultured tissues there is a progressive accumulation of irregularities of chromosomes which increases with the

aging process and that these tissues have a reduced potentiality for morphogenesis. Cell populations such as aneuploids might be naturally screened for survivals under *in vitro* culture, while genetic systems necessary for totipotency had no selective advantage under these conditions and could be lost from the population. Furthermore, it can be possibly assumed that the loss of chromosomes or the loss of a part of chromosomes might be a partial, and not total explanation for the loss of totipotency. On the other hand, SACRISTAN and MELCHERS (1969) succeeded in the regeneration of plants with grossly aneuploid chromosome complements from a number of long established aneuploid tobacco calluses. This provides striking evidence that even though cell populations may reach a highly aneuploid state, they are still able to retain their morphogenetic potentiality. In this case, it could be concluded that they still have a whole set of genes required for the totipotency in spite of high chromosomal irregularities of the callus tissues.

Summary

The effect of four types of media containing or not containing growth regulators of the callus growth and the chromosome numbers of callus tissues or of regenerated plants were studied by using the subculturing haploid calluses of *Nicotiana tabacum* (cv. Wisconsin 38). The relative callus growth measured by the frequencies of the metaphase and anaphase cells was significantly high on media containing an auxin of IAA as compared to those on media containing no growth regulator or kinetin alone. Furthermore, the growth regulator, IAA added to the culture media, showed its effect by accelerating the rate of polyploidization and aneuploidization while the medium without any growth regulators maintained a considerable stability of original nuclear state. Thus, it may be concluded that a condition such as the medium containing IAA as an auxin promotes the callus growth and simultaneously causes variations of the chromosome numbers.

Most of the regenerated plants from the calluses cultured on the media to which IAA was added were haploids and the remaining were diploids, even though the callus tissues showed high polyploidization. On the other hand, the callus cultured on medium containing kinetin alone produced higher numbers of diploids and even tetraploids, although the calluses did not show such high polyploidization as those on media containing IAA. These facts suggest that the conditions which are favorable for high polyploidization are presumably different from the conditions affecting the morphogenesis of higher levels of polyploid plants.

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