STUDIES ON PLANT CELL AND TISSUE CULTURE

II. Effect of Different Kinds of Media on the Variation of Chromosome Numbers in Tabacco Callus

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Introduction

Two approaches of plant cell and tissue cultures seem to be feasible in their application to crop improvement: (1) callus and cell cultures allow for rapid clonal multiplication and selection of desirable genotypes among the increased genetic variations induced by means of appropriate mutagens, (2) haploid induction from anther culture allows for speeding up the achievement of homozygosity which promises a considerable contribution to the plant breeding program.

It has been reported by a number of investigators using different species of plants that the callus tissues and the cultured cells sometimes may alter their chromosome numbers to produce polyploid cells, or cells with chromosomal aberrations such as pseudochiasmata, chromosome breaks, reunions, anaphase bridges and fragments. Some of the studies in which such considerable chromosomal variations were observed in the cultured calluses and cells are those in *Haplopappus gracilis* (MITRA and STEWARD, 1961), *Nicotiana tabacum* and *Nicotiana alata* (NISHIYAMA and TAIRA, 1966), *Nicotiana tabacum* (SHIMADA and TABATA, 1967), *Triticum aestivum* (SHIMADA *et al.*, 1969), *Nicotiana tabacum*, *Triticum aestivum* and *Triticum dicoccum* (SHIMADA, 1971), *Saccharum* (HEINZ *et al.*, 1969), and *Triticum monococcum*, *Triticum aestivum*, *Glicine max* and *Melilotus alba* (KAO *et al.*, 1970).

Furthermore, some of the regenerated plants from callus tissues and cells possessing such chromosomal variations indicate various chromosome numbers, such as polyploid and aneuploid (NIIZEKI and GRANT, 1971; NISHIYAMA and TAIRA, 1966). In the case of anther culture, from which the production of haploid plants are expected, frequent occurrences of polyploid plants as well as haploid plants were also reported in *Oryza sativa* (NISHI

and MITSUOKA, 1969). In addition, Tuleke (1953, 1957) observed an occurrence of polyploid and aneuploid cells in a culture of haploid calluses obtained from the pollen grain of *Ginkgo biloba*. Thus, chromosomal instability in the callus and cell culture may likely be such a common characteristic that it may be considered as an important problem, when the callus and cell culture technic is applied to crop improvement.

The question arises as to how the polyploidy, aneuploidy and various other chromosomal aberrations originate in the cultured calluses and cells. There is evidence tnat single-celled clones initially diploid, or tetraploid, may increase in ploidy during the course of culture. Cooper *et al.* (1964) examined cytologically the clones of tissues of single-cell origin, isolated from the crown gall callus of *Nicotiana tabacum* which was previously grown on a medium supplemented with α-naphthylacetic acid, coconut milk, 2, 4–D and calcium panthothenate for eight years. Chromosome count revealed numbers of 48, 96, and 192 in the clones. On the other hand, Torrey (1961) observed that when seedling roots of *Pisum sativum* were cultivated on a medium containing yeast extract and auxin (2, 4–D), or kinetin, callus tissues were predominantly composed of the tetraploid chromosome number even though the initial tissues were from roots known to consist of cells with diploid chromosome numbers. On a medium containing a mixture of vitamins, amino acids, amides and urea, the entire population of cells remained diploid. Therefore, he concluded that certain growth regulators used in the culture medium, such as yeast extract, 2, 4–D, or kinetin, induce mitosis in dormant polyploid cells that are present in a tissue when it is introduced into the culture.

These findings suggest that the causativeness in the widespread occurrence of polyploidy in the cultured calluses and cells may be preferential promotion of division in polyploid cells either arising during normal cell expansion or were already present in the original explant and that this promotion may be a function of growth regulators in the culture medium. If this is true, it would be possible that a certain medium containing proper growth regulator(s) would selectively induces the division of cells with particular desired chromosomal constituents, which will be usefull for the purpose of crop improvement.

Materials and Methods

Aseptic anthers of *Nicotiana tabacum* (cv. Wisconsin 38) containing individualized uninucleate pollen grains were planted on Miller's basic medium (1963) supplemented with three growth regulators, α-naphthylacetic acid

TABLE 1. Shoot and callus formation from anthers containing individual uninucleate pollen grains

Medium type*	Number of anthers used	Number of anthers forming plantlets	Number of anthers forming calluses
I-1	12	2 (16.2)	0 (0)
I-2	12	1 (8.3)	5 (41.7)
I-3	12	0 (0)	9 (75.0)

* I-1, 0.1 mg/l IAA and 0.1 mg/l BAP; I-2, 4.0 mg/l IAA and 2.0 mg/l BAP; I-3, 4.0 mg/l NAA and 2.0 mg/l BAP.
( ) indicates percentage.

(NAA), indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP). The formation of plantlets or calluses are shown in Table 1. On the medium containing a low level of concentration of growth regulators, 0.1 mg/l of IAA and 0.1 mg/l of BAP, only haploid plantlets were induced directly from pollen grains. On the other hand, both haploid plantlets and haploid calluses were induced on a medium containing a high level of concentration of growth regulators, namely 4.0 mg/l of IAA and 2.0 mg/l of BAP and only haploid calluses were induced on the medium containing 4.0 mg/l of NAA and 2.0 mg/l of BAP. One month after the calluses were induced, pieces of the callus were transplanted to a series of MILLER's basic media containing or not containing the growth regulators as follows; 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 kinetin and 2.0 mg/l of IAA (Medium D). One month after the first subculture, the calluses cultured on Medium D were again transplanted to the same series of media as above. All cultures were kept under dark condition at 26 ± 0.5°C.

Chromosome determination of callus tissues was carried out one month after the first and the second transplantation. For the purpose of accumulation of cells at the metaphase stage, pieces of callus tissue were transferred and incubated for 5 hours on media containing 0.1% colchicine. They were then 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% pectinase for 6 to 12 hours at room temperature, and then stained with alcoholic hydrochloric acid-carmine (Snow, 1963) for about 12 hours. Slides were prepared by the routine squashing method.

Results

Primary calluses derived from the individualized uninucleate pollen grains

by anther culture consisted of only haploid cells of 24 chromosomes. Even at the first transplantation at three weeks after the induction of the calluses, they did not show any variation in chromosome numbers in all cells examined.

The callus growth after the first transplantation was mainly affected by the addition or lack of growth regulators. The greatest growth was observed on the calluses cultured on Medium D containing both IAA and kinetin. Medium B and C containing IAA and kinetin, respectively, promote the callus growth to a lesser extent than Medium D. The calluses on Medium A containing no growth regulator grew very slowly and appeared to become gradually quiescent.

One month after the first transplantation, the callus tissues cultured on Medium A containing no growth regulator did not show any variation in chromosome numbers and consisted of cells with only 24 somatic chromosomes. On the other hand, a considerable range of variation in chromosome numbers was observed in the calluses cultured on Medium B, C and D (Fig. 1, 3). The widest variation in chromosome numbers occurred in the callus tissues on Medium D. There were two peaks in the distribution of the

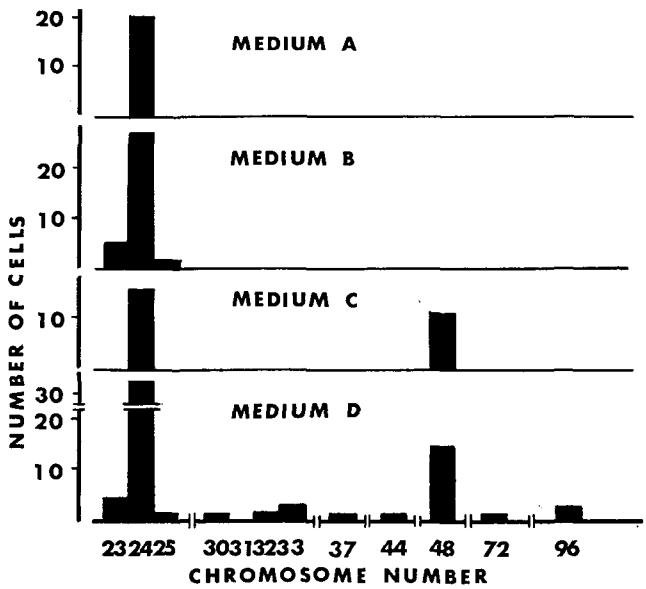


Fig. 1. Somatic chromosome numbers in the first subculture of callus tissues induced by anther culture of *N. tabacum* (cv. Wisconsin 38). Medium A, no growth regulator; Medium B, 4 mg/l kinetin; Medium C, 4 mg/l IAA; Medium D, 2 mg/l kinetin and 2 mg/l IAA.

chromosome numbers, at 24 and 48, with a frequency of 53.2% and 24.2%, respectively. Hexaploid and octoploid cells were also rarely observed. All others were cells of aneuploid chromosome number. The calluses cultured on Medium B mainly consisted of cells of haploid chromosome number of 24, but several cells were aneuploids with numbers such as 23 and 25. In the calluses cultured on Medium C, 40.7% of cells had a diploid chromosome number of 48, while the remainder had a haploid chromosome number of 24. In these calluses, there no aneuploid cells were seen.

Thus, the variation in chromosome numbers was found in the callus lines cultured on media containing IAA alone or both IAA and kinetin, even though they were derived from the same line which originally had uniform cells of 24 chromosomes. Therefore, the polyploidy and aneuploidy

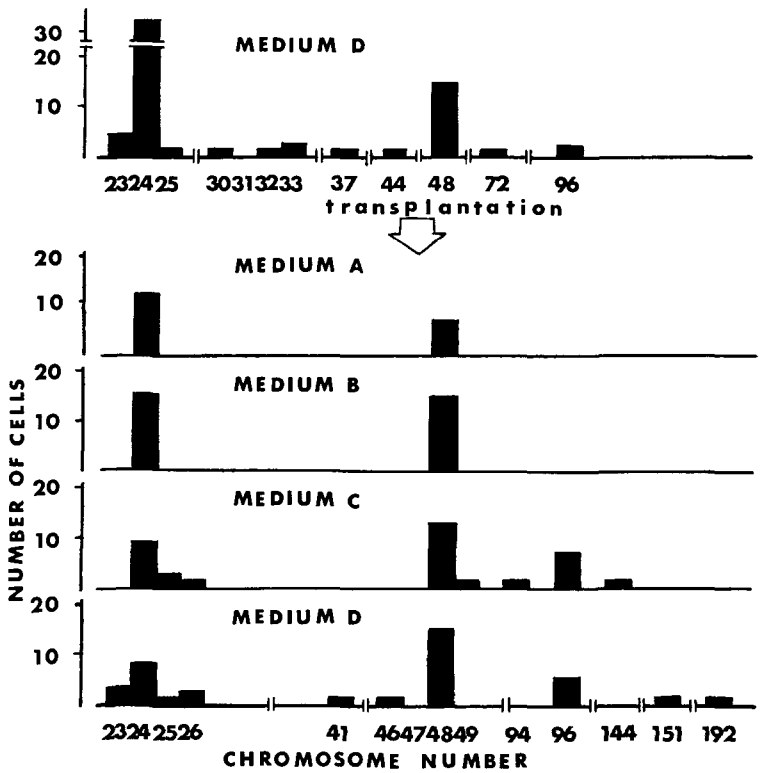


Fig. 2. Somatic chromosome numbers in the second subculture of callus tissues of *N. tabacum* (cv. Wisconsin 38) derived from the first subculture on Medium D. Medium A, no growth regulator; Medium B, 4 mg/l kinetin; Medium C, 4 mg/l IAA; Medium D, 2 mg/l kinetin and 2 mg/l IAA.

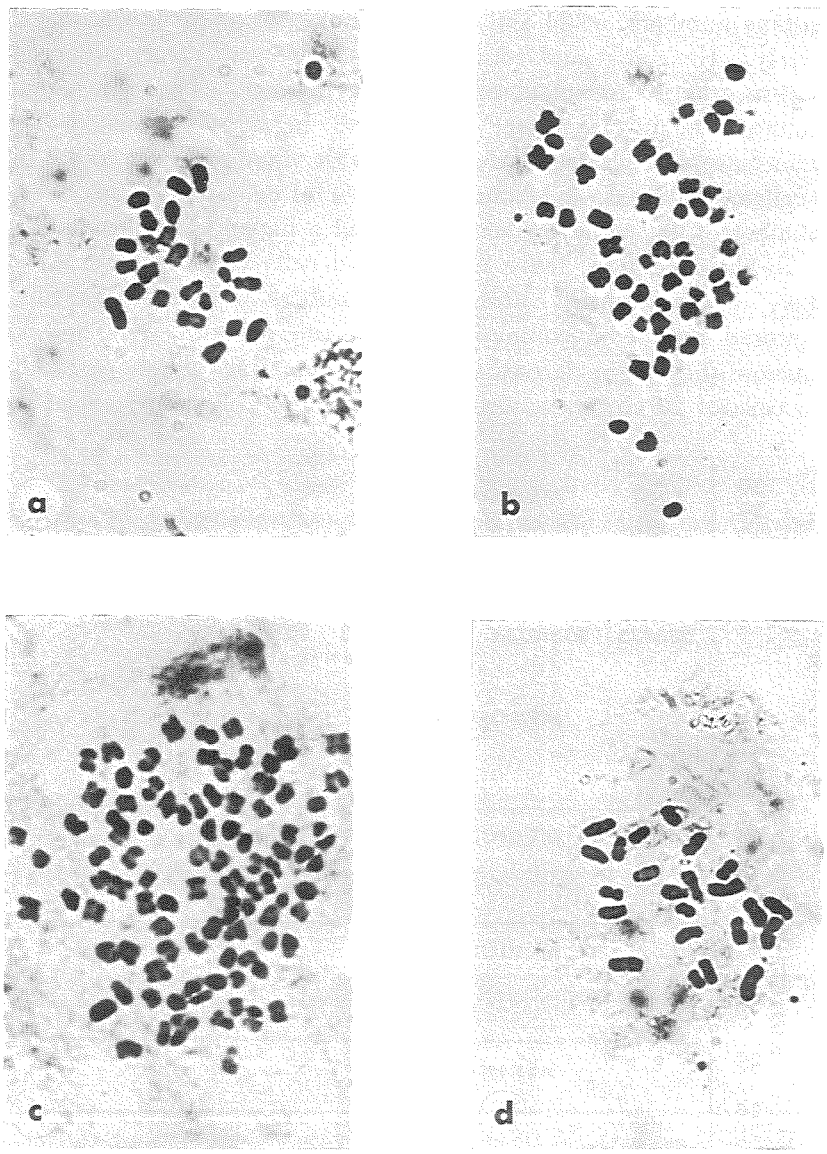
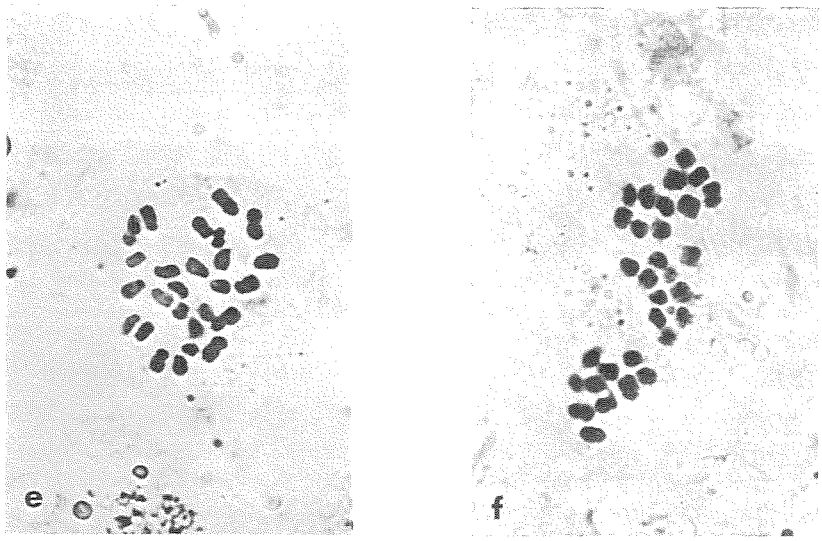


Fig. 3. Different chromosome numbers in the first and the second subculture of callus tissues induced by anther culture of *N. tabacum* (cv. Wisconsin 38). × ca. 1300.

a. 24 chromosomes.
b. 48 chromosomes.
c. 96 chromosomes.
d. 23 chromosomes.



e. 26 chromosomes.
f. 32 chromosomes.

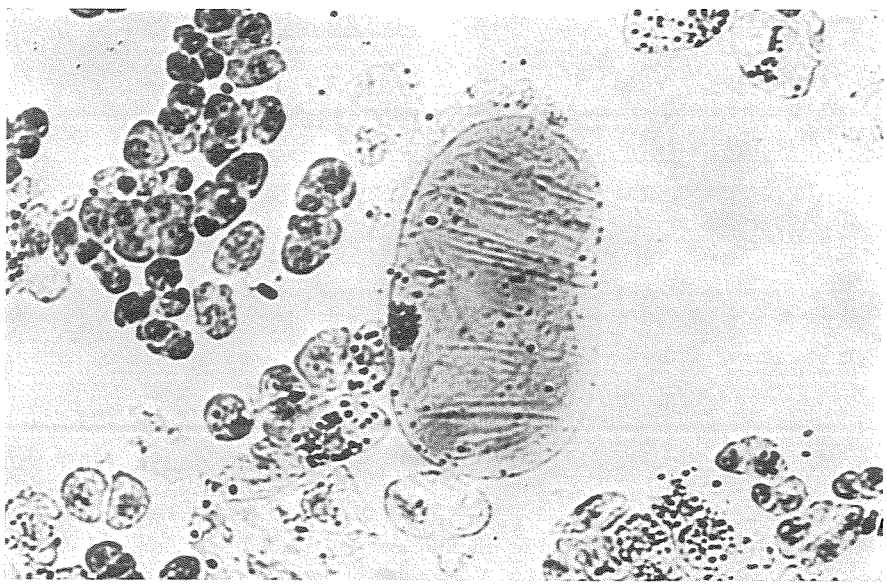


Fig. 4. Giant cell found in the first subculture of callus tissues induced by anther culture of *N. tabacum* (cv. Wisconsin 38). × ca. 200.

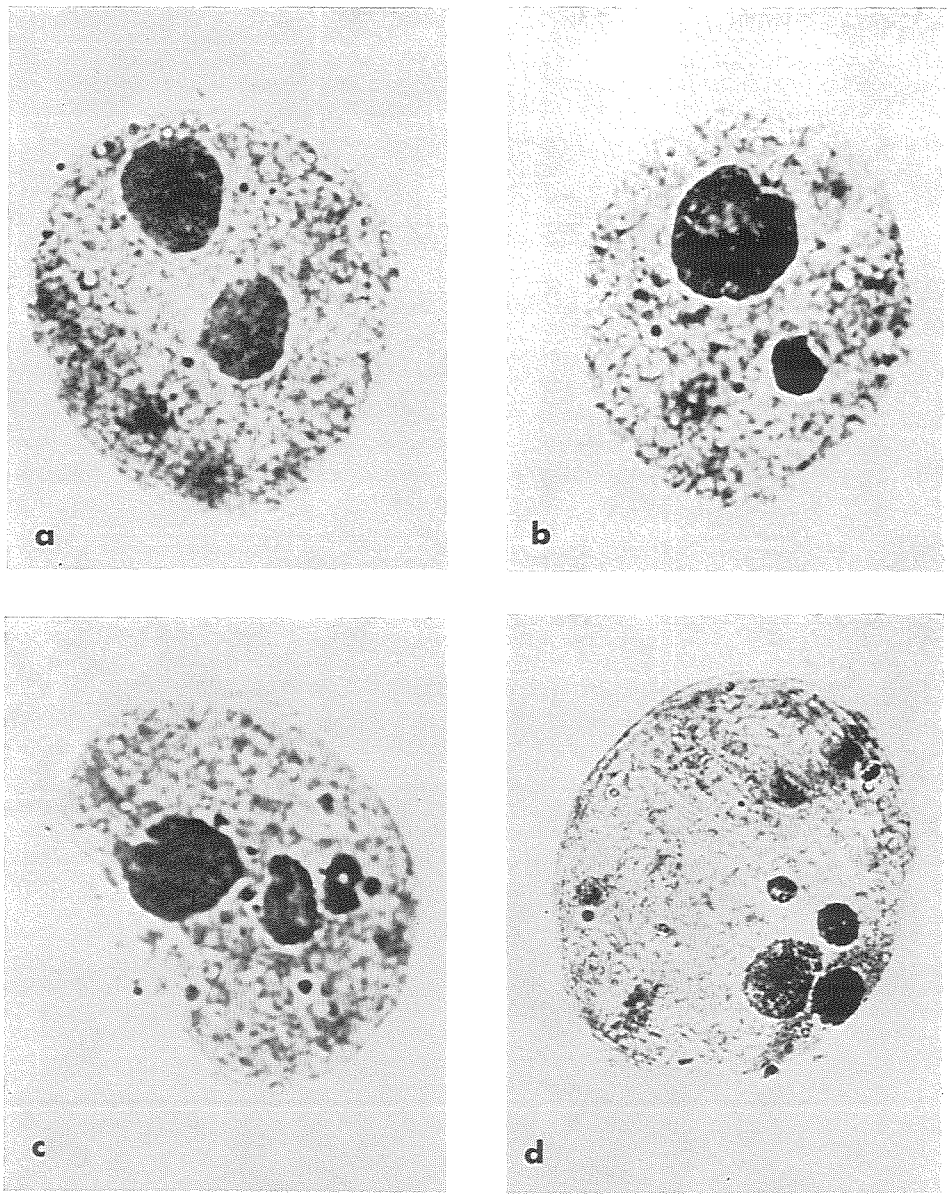


Fig. 5. Cells with multinuclei in the first and the second subculture of callus tissues of *N. tabacum* (cv. Wisconsin 38) derived from cultured anthers.

a. two nuclei in equal size. × ca. 1600.
b. two nuclei in different size. × ca. 1600.
c. three nuclei in different size. × ca. 1600.
d. four nuclei in different size. × ca. 800.

are possibly interpreted as a result of response to the growth regulator of IAA. The supplement of kinetin to IAA promotes further increase of the chromosome numbers than the addition of IAA alone to a medium.

The calluses, which were cultured for one month on Medium D and which had the widest variation of chromosome numbers, were again transferred to the same series of media as those of the first subculture. One month after the second transplantation, the determination of chromosome numbers of callus tissues on each medium revealed the same tendency of chromosome variation as those of the first subculture (Fig. 2, 3). In the calluses cultured on Medium A and B, only haploid and diploid chromosome numbers of 24 and 48, were observed, even though the calluses on the first subculture contained cells of a higher level of polyploids and various aneuploids. This means that only the cells of 24 and 48 chromosomes are active in cell division on Medium A and B. On the other hand, Medium C and D promote the division of the cells of various chromosome numbers including polyploids and aneuploids. Furthermore, on these media, the variation of chromosome numbers increased more than those of the first subculture, and the highest number of 192 ($8n$) was counted on Medium D.

Many types of alterations of cell morphology in the cultured calluses were observed. The prominent one was the so-called giant cell which sometimes increased from 5 to 20 times of cell size of ordinary cells (Fig. 4). The other prominent cell morphology was the change of nuclear behaviors. Multinuclei were frequently observed in either the giant cells or the ordinary cells (Fig. 5). They might have originated from the failure of cytokinesis. However, it was not ascertained as to how they were precisely originated. Some cells consist of up to four nuclei and in some cases they were not equal in size and some of them had an appearance of micronuclei. Frequently, nuclear fusion was observed in the multinucleate cells.

Discussion

It has been well established that the growth regulators such as auxin and cytokinin play a role in the polyploidization of cultured tissues or cells (BLAKELY and STEWARD, 1964; COOPER, HILDEBRANDT and RIKER, 1962; De TOROK and RODERICK, 1961, 1962; De TOROK and WHITE, 1960; MITRA, MAPES and STEWARD, 1960; TORREY, 1958). One explanation of the occurrence of polyploidy in the cultured tissues or cells is the failure of cytokinesis in mitosis, which leads to cells of multinuclei which sometimes fuses to uninucleus in a later stage (MITRA and STEWARD, 1961). Another possible explanation is endoreduplication, which involves chromosomal repro-

duction during the interphase and is made manifest by the presence of diplochromosomes (4-chromatids), quadruplochromosomes (8-chromatids), or polychromosomes (polyteny) (D'AMATO, 1952). This endoreduplication is observed commonly in differentiated tissues of higher plants and is regarded as the most widespread mechanism of somatic polyploidization. The observation of the multinuclei in the present study on the haploid callus tissues of tobacco, however, agrees with the former explanation. The precise mechanism by which growth regulators induce such a change, however, are not well understood. STERN (1960) gave an interpretation of the physiological events which surround chromosomal behavior in the cultured tissues or cells. Factors which determine respiration, oxygen and energy utilization and which have been invoked to explain different phases of mitotic and meiotic activity, may lead to abnormal chromosomal behaviors. However, the extent to which such ideas may be applicable to the observation made on the cultured haploid callus tissues of tobacco remains to be seen.

The cultured tissues or cells were found not only to contain polyploid cells, but also to show, more or less infrequently, various chromosomal aberrations and aneuploids. MITRA, MAPES, and STEWARD (1960) observed di- and tricentric bridges in mitosis of cultured carrot cells. Similarly, nuclei showing pseudochiasmata, chromosome breaks, reunions, and bridges were observed in suspension cultures of *Haplopappus gracilis* by MITRA and STEWARD (1961). TORREY (1958, 1959, 1961, 1965) in his studies on polyploidy in pea root callus found that, in older cultures maintained on media containing yeast extract or kinetin, there could be detected not only various degrees of polyploidy up to $12n$ but also aneuploids (especially around $4n+1$) and cells showing anaphase bridges, chromosome loops, and rings. Whether the mechanism for the occurrence of such chromosomal aberrations and aneuploids are the same as those which have undergone structural alterations as a result of treatment with irradiations and/or other agents which promote chromosomal breakage is uncertain. It does seem, however, probable that division, and subsequent multiplication of such aberrant nuclei, is promoted in culture medium containing growth regulators such as auxin and cytokinin.

It is clear now that the growth regulators in the culture medium have a determinative role of chromosomal behavior in callus tissues, although the precise mechanism (s) are uncertain at present. In this study, the occurrence of polyploid cells such as diploid, tetraploid and octoploid were frequent in the calluses cultured on the media containing IAA alone or IAA and kinetin in combination. Also, aneuploid cells on the medium containing both IAA

and kinetin were more frequent than any other media. On the contrary, almost all cells cultured on the media containing no growth regulator or only kinetin resulted in haploidy. Furthermore, when the callus tissues containing many polyploid and aneuploid cells transferred onto the media containing no growth regulator or kinetin alone, only haploid and diploid cells were active in mitosis. It is, therefore, concluded that IAA, a kind of auxin, has a function of the formation of polyploid and aneuploid cells and that kinetin, a kind of cytokinin, intensifies the function of auxin. Therefore, it may be stated that chromosomal constituents of callus tissues and cultured cells are possibly controlled by such media containing appropriate growth regulators and particular cells of desired chromosomal constituents can be selected in the cultured calluses and cells. It is, however, a problem in future study to maintain or select only haploid cells from any other cells of chromosomal constituent.

Summary

Effect of four types of media containing or not containing growth regulators on the chromosome numbers was studied by using haploid calluses induced by means of anther culture of *Nicotiana tabacum* (cv. Wisconsin 38). The occurrence of polyploid cells in the callus tissues was promoted by the media containing IAA alone or IAA and kinetin in combination. In addition, various kinds of aneuploid cells were observed, especially in the callus tissues cultured on the medium containing both IAA and kinetin. Several types of aberrations of nucleus such as multinuclei (multikaryon), micronucleus and nuclear fusion were accompanied by the occurrence of the polyploid and aneuploid cells. On the contrary, on the media containing no growth regulators or kinetin alone, the haploidy of the callus tissues were maintained with the exception of a few aneuploid cells.

The callus tissues having cells of wide variation in chromosome numbers, which were promoted on medium containing both IAA and kinetin, were again transplanted to the four types of media. The degree of polyploidization was further progressed on the media containing IAA alone or IAA and kinetin in combination and some of them showed a count of as much as 192 chromosomes ($8n$). The frequency of the occurrence of aneuploid cells was also as high as those of the first subculture. On the other hand, all cells in division were haploid and diploid in the callus tissues on the media containing no growth regulator or kinetin alone. No other cells of a higher level of polyploid and aneuploid were observed. This result indicates that only haploid and diploid cells among various polyploid and

aneuploid cells undergo selectively to division under these medium conditions. Thus, by using these appropriate media, it is possible to maintain or promote the cell division of desirable chromosome number which may contribute to breeding programs.

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