STUDIES ON PLANT CELL AND TISSUE CULTURE

X. Diploid plant production by callus culture of haploid *Nicotiana* species

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Introduction

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 and aneuploid cells[10,11,12,14,20,21]. MURASHIGE and NAKANO[9] obtained tetraploid plants from the callus with polyploidized cells which were originally raised from single cells isolated from the pith of diploid tobacco plants. This observation leads us to believe that the technique of tissue culture may be useful in the production of homozygous diploid plants from the callus tissues derived from haploid plants. Indeed, KOCHHAR *et al.*[6] succeeded on induction of diploid plants from the cultured haploid callus which originated from the stem of haploid plantlets produced by anther culture of the tobacco plant. Thus, it was postulated that these diploid plants were differentiated from cells that have undergone autopolyploidy during the growth of the calluses. This rapid method of producing homozygotes by callus culture may be potentially of importance in plant breeding and in programmes of crop improvement.

Materials and Methods

Haploid plants of two species, *Nicotiana tabacum*, var. Wisconsin 38 ($2n=48$) and *N. sylvestris* ($2n=24$) were induced from anther culture on the medium described by NITSCH and NITSCH[16]. About 2-4 mm of young stems disected from the 3-4 cm of plantlets derived by anther culture were cultured on the basic medium of MILLER[8] supplemented with 2.0 mg/l of IAA (indole-3-acetic acid) and 2.0 mg/l of kinetin. About 3 to 4 weeks after culture, whitish calluses proliferated from the stems of both species. These obtained calluses were thereafter subjected to subcultures at intervals of about

one month on the same medium which was used for the induction of these calluses. All cultures were kept under dark condition at 26±0.5°C. After several subcultures, these calluses were transplanted to a series of Miller's basic media containing or not containing the growth regulators as follows; no growth regulator (medium M-1), 4.0 mg/l of kinetin (medium M-2), 4.0 mg/l of IAA (medium M-3) and 2.0 mg/l of kinetin and 2.0 mg/l of IAA (medium M-4). All callus cultures were then transferred into light condition at 26± 0.5°C. After 10 to 20 days of sustained culture, shoots or shoot primordia developed from the calluses. The regenerated shoots 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 generally proliferated a number of roots, and thereafter the obtained plantlets were transplanted into pot. The methods of determination of chromosome numbers of the callus cells and the regenerated plants were carried out by the same way as described in a previous paper[13].

Results

Chromosome numbers of callus tissues

N. tabacum

Chromosome numbers of the calluses are shown in Table 1. The callus tissues cultured on medium M-1 without growth regulators showed the most stable chromosomal state maintaining the constituent of cells with only 24 (n) and 48 ($2n$) chromosomes. No aneuploid cell was found in this callus tissues. On the other hand, considerable variations including polyploid and

TABLE 1. Frequencies (%) of different chromosome numbers in callus tissues of *Nicotiana tabacum* cultured on four types of media

Medium type	Number of cells observed	% of cells			
		chromosome number			
		24 (n)	48 ($2n$)	96 ($4n$)	others*
M-1	25	64.0	36.0	—	—
M-2	31	48.4	29.0	12.9	9.7
M-3	28	21.4	25.0	17.9	35.7
M-4	28	10.7	39.3	21.4	28.6

M-1, no growth regulator; M-2, 4 mg/l kinetin; M-3, 4 mg/l IAA; M-4, 2 mg/l kinetin and 2 mg/l IAA.

*, including higher polyploids than tetraploid and various aneuploids.

aneuploid chromosome numbers were observed in the callus tissues cultured on medium M-2, M-3, or M-4, which contained IAA and kinetin singly or in combination. The most frequent polyploidization was found in the callus tissues cultured on medium M-4 containing both IAA and kinetin and chromosome numbers exceeding 300 were sometimes counted in several of the cells. The callus tissues cultured on medium M-3 containing IAA alone also showed a considerable polyploidization in the chromosome numbers. The calluses on medium M-2 containing kinetin alone showed a less polyploidization than those cultured on medium M-3 and M-4.

N. sylvestris

Chromosome numbers of the calluses of this species are shown in Table 2. The callus tissues cultured on medium M-1 and medium M-2 showed the constituent of cells with 12 (n), 24 ($2n$) and 48 ($4n$) chromosomes and no aneuploid cell was observed in this callus tissues. The widest variation in chromosome numbers and the most frequent aneuploidization were found in the callus tissues cnltured on medium M-4 supplemented with both IAA and kinetin. The calluses on medium M-3 containing IAA alone indicated a somewhat smaller variation than the calluses on medium M-4. However, the cells of the most frequent appearance were diploid ($2n=24$) in any calluses cultured on all four types of media. Therefore, a high frequency of the multiplication of chromosome number from haploid to diploid on any different media was considered as a conspicuous characteristic in the callus tissues of this species.

TABLE 2. Frequencies (%) of different chromosome numbers in callus tissues of *N. sylvestris* cultured on four types of media

Medium type	Number of cells observed	% of cells			
		chromosome number			
		12 (n)	24 ($2n$)	48 ($4n$)	others*
M-1	50	16.0	80.0	4.0	—
M-2	50	22.0	58.0	20.0	—
M-3	50	22.0	56.0	14.0	8.0
M-4	50	20.0	56.0	8.0	16.0

M-1, no growth regulator; M-2, 4 mg/l kinetin; M-3, 4 mg/l IAA; M-4, 2 mg/l kinetin and 2 mg/l IAA.

*, including higher polyploids than tetraploid and various aneuploids.

Chromosome numbers of regenerated plants

N. tabacum

A high potentiality of regeneration of plants from calluses occurred on medium M-1 and M-2, while medium M-3 and M-4 containing IAA led to a considerable decrease in the ability to produce shoot initiation of the calluses (Table 3).

On medium M-1 containing no growth regulator, most of regenerated plants had 24 (n) chromosomes and a few plants had 48 ($2n$) chromosomes (Table 4). On medium M-2 containing kinetin alone, the regenerated plants showed 24, 48 and 96 ($4n$) chromosomes. These various ploidy levels of

TABLE 3. Frequencies of regenerated plants from callus tissues cultured on four types of media

Medium type	Frequencies of regenerated plants	
	N. tabacum	*N. sylvestris*
M-1	++	+++
M-2	+++	++
M-3	+	++
M-4	+	+

M-1, no growth regulator; M-2, 4 mg/l kinetin; M-3, 4 mg/l IAA; M-4, 2 mg/l kinetin and 2 mg/l IAA.
+++, high frequency; ++, medium frequency; +, low frequency.

TABLE 4. Frequencies (%) of regenerated plants with different chromosome numbers from callus tissues of *N. tabacum* cultured on four types of media

Medium type	Number of regenerated plants observed	% of regenerated plants			
		chromosome number			
		24 (n)	48 ($2n$)	96 ($4n$)	others*
M-1	30	93.3	6.7	—	—
M-2	30	60.0	30.0	10.0	—
M-3	30	90.0	10.0	—	—
M-4	30	76.7	23.3	—	—

M-1, no growth regulator; M-2, 4 mg/l kinetin; M-3, 4 mg/l IAA; M-4, 2 mg/l kinetin and 2 mg/l IAA.
*, including higher polyploids than tetraploid and various aneuploids.

regenerated plants derived from the callus on medium M-2 could be naturally assumed from the callus tissues containing various polyploid cells. On the other hand, the plants with haploid chromosome number ($n=24$) were predominantly regenerated from the calluses cultured on medium M-3 and M-4 containing IAA, even though the callus tissues had a high frequency of diploid and tetraploid cells. No single aneuploid plant was derived from all calluses cultured on the above described four types of media.

N. sylvestris

A high ability of plant regeneration from callus tissues occurred on medium M-1, M-2 and M-3 (Table 3). Medium M-4 containing both IAA and kinetin appeared to decrease the production of shoot from the calluses.

On the contrary to *N. tabacum* which mainly produced the haploid plants, about 70 to 90% of the regenerated plants from calluses cultured on all media indicated diploid ($2n=24$) chromosome number in *N. sylvestris* (Table 5). The regenerated haploid plants were less than 30% on any media. This result could be expected from the callus tissues containing a high frequency of diploid cells. No single tetraploid and aneuploid plant was regenerated, even though the callus tissues cultured on medium M-3 and M-4 had a considerable number of high levels of polyploid and aneuploid cells.

TABLE 5. Frequencies (%) of regenerated plants with different chromosome numbers from callus tissues of *N. sylvestris* cultured on four types of media

Medium type	Number of regenerated plants observed	% of regenerated plants			
		chromosome number			
		12 (n)	24 ($2n$)	48 ($4n$)	others*
M-1	18	22.2	77.8	—	—
M-2	22	18.2	81.8	—	—
M-3	17	29.4	70.6	—	—
M-4	19	5.3	94.7	—	—

M-1, no growth regulator; M-2, 4 mg/l kinetin; M-3, 4 mg/l IAA; M-4, 2 mg/l kinetin and 2 mg/l IAA.

*, including higher polyploids than tetraploid and various aneuploids.

Discussion

The growth regulators such as auxin and cytokinin play an important role of chromosomal behaviour in callus tissues or cultured cells[1,2,3,4,5,7,22]. SUNDERLAND[21] reported that a suspension culture of *Haplopappus* cotyledon

cells grown in a medium containing 2, 4-D (2, 4-dichlorophenoxyacetic acid) changed entirely from diploid to tetraploid over a period of less than 6 months of subculture. Therefore, it may be stated that 2, 4-D as an auxin component contributes to accelerating the rate of polyploidization. In this study on haploid calluses of *N. tabacum*, the occurrence of polyploid cells such as diploid and tetraploid was frequent in the calluses cultured on the media containing IAA alone or IAA and kinetin in combination. Also, the appearance of aneuploid cells on the media containing IAA or both IAA and kinetin was more frequent than any other media. In contrast, almost all cells cultured on the medium containing no growth regulator resulted in haploid and diploid cells. It is, therefore, concluded that IAA, a kind of auxin, has a function related to the formation of polyploid and aneuploid cells and that kinetin, a kind of cytokinin, intensifies the function of auxin. On the other hand, the chromosomal stability and instability of callus cultures may at time depend upon plant species[15]. Indeed, chromosomal behaviour of *N. sylvestris* in this study did not show so much drastic response to the differences of medium types as found in *N. tabacum*, while a high frequency of the diploidization of haploid cells occurred on any media used for the calluse cultures. Therefore, a high degree of multiplication in chromosome numbers appears to be one of the attribute of *N. sylvestris*.

In the study of *Lotus corniculatus*, 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[14]. In the case of *N.* tabacum, the regenerated plants from the calluses cultured were mainly haploid, even though the callus tissues showed a high degree of polyploidization of the chromosome numbers. Only the medium containing kinetin alone appeared to be favourable in the production of diploid plants and even tetraploid plants, but the haploid plants were still predominantly regenerated on this medium. On the other hand, in *N. sylvestris*, the regenerated plants from the calluses cultured on any media were mainly diploid plants and a few of them were haploid plants. Therefore, it is possibly assumed which plants of haploid and diploid preferentially produced from the callus tissues mainly depend upon the differences of plant species, but not upon the medium types.

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 a high aneuploidy in both species. Cells such as aneuploids in the calluses might be naturally screened for survival under *in vitro* cultures, and their genetic systems required for totipotency had no selective

advantage under these conditions and could not be regenerated from the cultured cells. Furtheremore, it can be considered that the loss of chromosomes or the loss of a part of chromosomes might be a partial, but not total explanation for the loss of totipotency. On the other hand, the regeneration of plants with grossly aneuploid chromosome complements was succeeded from a number of long established aneuploid tobacco calluses[19]. In addition, chromosomal chimeric tobacco plants with various aneuploid cells were obtained from a mutant callus tissue[17,18]. These provide 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 occurrence of polyploid cells on haploid calluses of *N. tabacum* was frequent on the media containing IAA. Also, aneuploid cells on these media were more frequent than any other media. In contrast, almost all cells of callus tissues cultured on the medium containing no growth regulator resulted in only haploid and diploid. Thus, it was concluded that the chromosomal behaviour of callus tissues in *N. tabacum* may be largely affected by the growth regulators in medium. On the other hand, chromosomal behaviour of callus tissues in *N. sylvestris* did not strongly response to the growth regulators, but a high frequency of diploidization of chromosome numbers occurred on any medium conditions. Therefore, the multiplication of chromosome numbers from haploid to diploid was considered as a conspicuous characteristic in this species.

The regenerated plants from the callus tissues of *N. tabacum* were mainly haploid, even though the callus tissues showed a high degree of polyploidization of the chromosome numbers. Although the medium containing kinetin alone appeared to be somewhat favourable in the production of diploid plants, the frequency of regenerated haploid plants was still clearly higher than any other polyploid plants. On the other hand, the regenerated plants from the callus tissues of *N. sylvestris* were mainly diploid and a few of them were haploid. Therefore, it was concluded what kinds of ploidy level of plants produced predominantly from callus tissues are largely due to the plant species, but not to the medium types.

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