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Title	Studies on Plant Cell and Tissue Culture : IV. Effect of para-fluorophenylalanine on haploid and diploid cells of tobacco plant in vitro
Author(s)	NIIZEKI, Minoru
Citation	Journal of the Faculty of Agriculture, Hokkaido University, 57(4), 349-356
Issue Date	1974-09
Doc URL	https://hdl.handle.net/2115/12885
Type	departmental bulletin paper
File Information	57(4)_p349-356.pdf

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STUDIES ON PLANT CELL AND TISSUE CULTURE

IV. Effect of Para-fluorophenylalanine on Haploid and Diploid Cells of Tobacco Plant *in vitro*

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Received Oct. 24, 1973

Introduction

Haploid plants are generally recognized because of their usefulness in various approaches to genetics, cytogenetics, and plant breeding research. Haploid cells *in vitro* are also considered to play an important role in somatic cell genetics and mutation research.

Numerous investigators using various artificial methods have attempted to induce haploids from higher plants, but no satisfactory means have been found to circumvent the difficulties encountered in order to obtain haploids at a high frequency and freely at will (KIMBER and RILEY, 1963). Recently, however, the induction of haploid plants and callus tissues by means of anther cultures seems to show some promise for removing some of these difficulties in haploid production (GUHA and MAHESHWARI, 1964, 1966; BOURGIN and NITSCH, 1967; NAKATA and TANAKA, 1968; NIIZEKI and OONO, 1968; KAMEYA and HINATA, 1970; NITZSCHE, 1970; CLAPHAM, 1971; NARAYANASWAMY and CHANDY, 1971; ZENKTELER, 1971; BAN, KOKUBU and MIYAJI, 1971; KIMATA and SAKAMOTO, 1971, 1972 and IRIKURA and SAKAGUCHI, 1972).

It has been observed that the induced callus tissues by the anther cultures are not only haploidy but also have different levels of polyploidy and aneuploidy (NARAYANASWAMY and CHANDY, 1971 and NIIZEKI, 1973). This chromosomal instability in the callus tissues may be considered as a serious barrier, when the callus cultures are applied to several approaches such as somatic cell genetics and mutation research which require the maintenance of pure haploid cell lines.

An amino acid analogue, para-fluorophenylalanine (PFP), has been reported as an inducer of mitotic haploidization in diploid lines of some genera of fungi (LHOAS, 1961). GUPTA and CARLSON (1972) found that this PFP specifically inhibits the growth of the diploid callus tissues and has

no effect on the haploid callus tissues of higher plants, *Nicotiana tabacum, in vitro.*

This paper reports the results of an experiment attempted to define the response of PFP to the haploid and diploid calluses and to exploit the possibility of selection of the haploid cell lines from calluses containing various polyploids in the tobacco plant.

Materials and Methods

Aseptic immature anthers of *Nicotiana tabacum* (cv. Wisconsin 38) were planted on the medium described by NITSCH and NITSCH (1969). The medium contains 0.1 mg/l of kinetin and 0.1 mg/l of indole-3-acetic acid (IAA). Numerous haploid plantlets were produced from these anthers after approximately one month of incubation. The haploid plantlets derived from the anthers and the diploid plantlets grown from seeds were planted on MILLER's basic medium (1963) supplemented with 2 mg/l of kinetin and 2 mg/l of IAA. Callus tissues vigorously proliferated from both of the stems of haploid and diploid plantlets during two to four weeks. Pieces of 400 mg of both of the haploid and diploid calluses were transplanted on the MILLER's basic medium (1963) supplemented with 2 mg/l of kinetin, 2 mg/l of IAA and 0–200 mg/l of PFP. The haploid calluses were retransplanted after one month of subculturing for five passages and all cultures were kept under dark conditions at 26 ± 0.5°C. For the purpose of determining the chromosome numbers the callus tissues were pretreated in 0.002*M* of 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 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

Growth of haploid and diploid calluses on media containing PFP.

400 mg of the haploid and diploid calluses were inoculated in the media supplement with 4 concentration levels of PFP, namely 0, 50, 100 and 200 mg/l. Callus weight on each medium was examined after one month of incubation. The results are indicated in Table 1. On the medium containing no PFP, as the control, the growth of diploid calluses were very rapid and resulted in a 18.250 fold increase of the initial callus weight while the haploid calluses showed a 13.708 fold increase. The growth of

TABLE 1. Growth of haploid and diploid calluses of *Nicotiana tabacum* (cv. Wisconsin 38) on media containing various concentrations of PFP

	Concentrations of PFP (mg/l)	Weight of initial callus (mg)	Weight of callus after one month (mg)	Fold increase	Rate of growth inhibition (%)
Haploid	0	400	5,483	13.708	0
	50	400	4,800	12.000	12.5
	100	400	3,417	8.543	37.7
	200	400	1,033	2.583	81.2
Diploid	0	400	7,300	18.250	0
	50	400	3,733	9.332	48.9
	100	400	2,900	7.250	60.3
	200	400	633	1.583	91.3

diploid calluses, however, was progressively inhibited at increasing levels of the PFP concentrations. On the other hand, the haploid calluses was less inhibited in growth than the diploid calluses by the presence of PFP. For example, the diploid calluses were suppressed by 48.9 and 60.3% of their growth by the presence of 50 and 100 mg/l of PFP, while the haploid calluses were suppressed only by 12.5 and 37.7%, respectively. High concentrations of the PFP such as 200 mg/l, however, remarkably inhibited the growth of both haploid and diploid calluses which eventually led to death after one month of incubation.

TABLE 2. Frequencies (%) of different chromosome numbers in calluses cultured on media containing various concentrations of PFP

		Concentrations of PFP (mg/l)						
		0	5	10	20	40	80	200
10 days in culture	Haploid (24)	50	62	62	50	60	60	96
	Diploid (48)	38	32	26	39	34	34	4
	Others*	12	6	12	11	6	6	0
20 days in culture	Haploid (24)	42	50	58	55	82	74	—
	Diploid (48)	44	44	36	45	16	24	—
	Others*	14	6	6	0	2	2	—

* including various polyploids and aneuploids.

— no more callus growth occurred.

Chromosome numbers of callus cells on media containing PFP.

Determination of chromosomal variations in cells of the haploid calluses was performed on media supplemented with 7 levels of PFP, 0, 5, 10, 20,

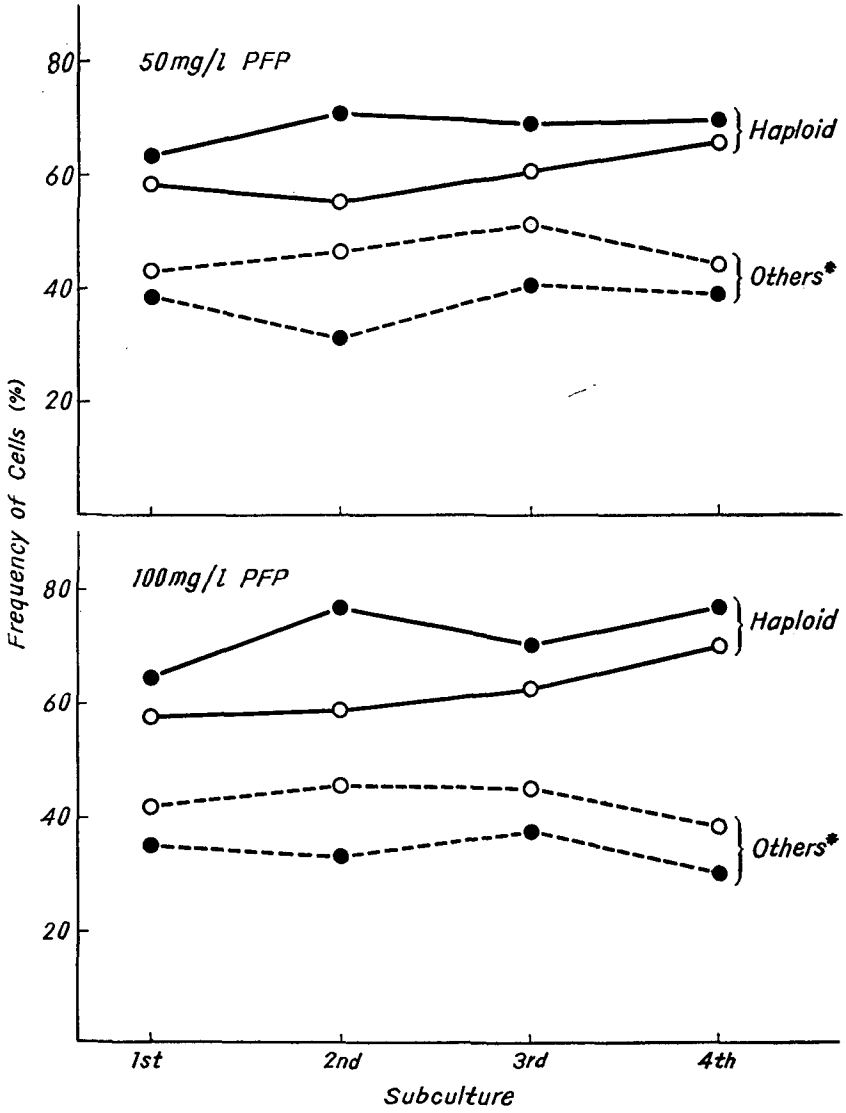

Fig. 1. Changes of variation in chromosome numbers after 10 days of subculture.
● containing PFP, ○ containing no PFP.
* including various polyploids and aneuploids.

40, 80, and 200 mg/l. 50 metaphase cells of the callus tissues on each medium were subjected to examinations for their chromosome numbers after 10 and 20 days of incubation. The results are indicated in Table 2. The cells of the haploid chromosome number in the callus tissues cultured on the control medium containing no PFP amounted to 50% and 42% at 10 days and 20 days after respective incubation. The others were mostly diploid, polyploid, and a few aneuploid cells. On the other hand, the haploid cells on the media containing PFP were raised in their frequencies at increasing levels of PFP concentrations. Especially, the haploids after 20 days in the cultures occupied over 70% of the callus cells on the media containing more than 40 mg/l of PFP. The haploid cells on the medium containing 200 mg/l of PFP showed an extremely high frequency at 10 days in the early stage of the culture, but the callus tissues eventually turned black and appeared to die.

Chromosomal variations in subcultures on media containing PFP.

The callus tissues cultured on the media containing 0, 50, and 100 mg/l of PFP were subjected to serial transplantation until 4th subculture. The chromosome determination of callus cells was carried out after 10 days in each subculture. The results are shown in Figure 1. The frequencies of haploid cells of the callus tissues on the media containing 2 levels of the PFP were significantly higher than those on the medium containing no PFP throughout 4 subcultures and the frequencies of the other cells including the diploids, polyploids, and aneuploids were the opposite. The haploid cells of the callus tissues, however, did not appear to increase at the advanced passages of the subculture and never attained more than 80% in frequency.

These results clearly indicate the effectiveness of the PFP on the preferential selection of haploids from a mixed population of varying ploidy cells, although it seems to be incapable of establishing a completely pure population consisting of only haploid cells.

Discussion

Bacteria have contributed for long time to sophisticated genetic analysis for several reasons, namely because they generally have a quite short life cycle and are haploid. Genetic analysis of eucaryotes, by contrast, has been hindered by a much longer life cycle and by the diploid condition of the somatic cells. If haploid cell lines could be produced and maintained, half of the hindrance would be removed.

A previous report (NIIZEKI, 1973) indicated that some culture media supplemented with the defined growth regulators render the callus tissues to reduce the increased variations of chromosome numbers. Indeed, only haploids and diploids among various polyploid and aneuploid cells undergo selectively to divide under such medium conditions as the supplementation of none or only kinetin. It is, however, still unknown as to how to select and maintain only haploid cells from a mixed population of various chromosomal constituents.

Para-fluorophenylalanine has been used occasionally to induce the haploidization of some species of fungi (LHOAS, 1961). GUPTA and CARLSON (1972) reported that this amino acid analogue specifically kills the callus of diploid cells of tobacco plants and does not exert any damaging effect on the haploid calluses aud aids in maintaining a vigorous growth. The present study substantially supports their results as to the function of PFP. However, the present results are somewhat in controversary with them, namely the PFP not only hinders the growth of diploid but also haploid callus tissues. In other words, even haploid calluses are not freed from the considerable suppression of their growth at increasing concentrations of PFP, although the haploid calluses are less damaged than the diploid calluses. The cause of this difference is somewhat obscure, but this may be at least in part due to the impurity of the initial implanted callus tissues used in the present work. The present starting material probably contained a considerable number of polyploids and aneuploids besides the haploid cells.

Cytological observations of the callus tissues revealed that the haploid cells effectively increase in frequencies at some range of concentration of the PFP such as 40 to 100 mg/l and the haploid cells attain more than 70%, while the haploid cells were less than 50% of the control medium containing on PFP. This may be caused by the prevention of polyploidization of the haploids and simultaneously the preferential growth of haploid cells. Therefore, it may be suggested that the possibility of using this amino acid analogue may result not only in the promotion of preferential growth of the haploid calluses but also to select and maintain the haploid cells from the mixed populations of varying polyploids.

To date, the present study and also the study of Gupta and CARLSON (1972) have used only tobacco plant cells, but if the effect of PFP is not specific to this species, they may well be a generally useful method of maintenance of haploid cells of higher plants in culture passages. Further work should be performed to determine the extent of applicability of PFP

for the preferential selection of haploid cells of other species *in vitro*.

Summary

Haploids of higher plants are generally recognized for their usefulness in genetic studies, especially somatic cell genetics.

Haploid callus tissues from plantlets derived by anther cultures of tobacco and diploid callus tissues from tobacco seedlings were compared for their responses to the presence of different concentration levels of PFP. The diploid callus tissue growth was proportionally inhibited by increasing levels of PFP, while the haploid callus tissues were less inhibited by the presence of PFP. Cytological observations indicated that the haploid callus tissues cultured on the media containing PFP showed increased frequencies of the cells of haploid chromosome number, especially the calluses on the media containing over 40 mg/l of PFP attained more than 70% of the haploid cells. In contrast, the haploid cells of the callus tissues cultured on the control medium without PFP addition were less than 50% and the other cells were polyploids and aneuploids. High concentrations of PFP such as 200 mg/l showed an extremely selective effect on the haploid cells, but simultaneously caused serious damage to the growth of calluses. Continuous subculture passages were made in an attempt to obtain pure cell lines of the haploidy did not show any accumulative effects from passage to passage and the haploid cells did not exceed 80%.

In conclusion, certain defined concentrations of PFP may suppress the growth of diploidy and may promote preferential selection of haploid cells from mixed populations of various chromosomal constituents, although it seems to be difficult to establish a complete pure cell line of haploidy.

Acknowledgments

The auther wishes to extend his sincere appreciation to Dr. M. TAKAHASHI and Dr. F. KITA, Proffessors of Faculty of Agriculture, Hokkaido University, for the guidance and encouragement for carring out this study.

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