Induction of Haploid Plant From Anther Culture

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Haploid plants are of special interest to the geneticist and plant breeder because by chromosome doubling completely homozygous diploid plants can be obtained from haploid plants. Owing to the great theoretical and applied value of haploids, many methods have been tried to induce their occurrence. Various techniques of artificial haploid production, such as treatment with irradiated pollen, delayed pollination, utilization of alien cytoplasm or pollinator, distant hybridization, polyembryony, twin seedlings and physical and chemical treatments, have been employed. As Katayama and Nei (1964) described, however, most brilliant result of haploid formation will be brought from the success of pollen culture.

In India, Guha and Maheshwari (1964, 1966) succeeded in the production of haploid seedlings of *Datura innoxia* Mill. by the culture of mature anthers. This is the first report of the production of embryoids and seedlings from the pollen grains of plant. In the case of *Datura*, in Nitsch's medium with 1,000 ppm yeast extract or casein hydrolysate the anthers produce callus, while on the same medium with 5, 15 or 30 per cent coconut milk or 10^{-6} M kinetin the callus masses differentiate to produce embryoids and plantlets. The fact that these are haploids and easily differentiate *in vitro* giving rise eventually to plantlets, further enhances its value for use in problems of genetics, cell physiology, morphogenesis and plant breeding.

Recently, the production of haploid plants from pollen culture was reported in tobacco and rice plant both in Japan. This report describes the methods for obtaining haploid plant from anther culture in these two species.

Tobacco (*Nicotiana tabaccum* L.)

At Hatano Tobacco Experiment Station, Nakata and Tanaka (1968) succeeded in the production of haploid tobacco plants from anther culture. Tobacco plant belongs to the same Solanaceae as *Datura*.

Tobacco varieties tested were Bright yellow, Hicks 103 and Shirodaruma. Flower buds varying in stages of development were harvested from tobacco plants. One of five anthers from each flower bud was used to determine the stage of pollen development. According to this procedure all flower buds were classified into nine groups ranging from the stage of archesporial cells to that of mature pollen grains. The remaining anthers from each flower bud of every groups were sterilized in 95 per cent ethanol for 2-3 seconds, in 10 per cent (v/v) sodium hypochlorite solution for 10-15 minutes and then rinsed in sterile distilled water. The anthers were then placed aseptically on the surface of agar media in a 150 ml of Erlenmyer flask. Cultures were maintained in a room at 25°C with continuous illumination with fluorescent lamp ranging from 3,500 to 4,000 lux.

The medium used was revised tobacco C medium, revised RM-1964 medium and modified White medium (Table 1.). On the revised RM-1964 medium, no roots differentiate and so seedlings on this medium must be transplanted on the modified White medium for root initiation. On the revised tobacco C medium, thirty days after the inoculation of anthers, roots appeared from the inside of dehisced anther which had been cultured from pollen grains of tetrad or uninuclear stage and then 10-20 days embryoids

appeared. These embryoids developed later into visible buds having dicotyledons. On this medium, 20-30 seedlings developed from an anther and showed no callus formation. However, around the basal part of the buds differentiated on the revised RM-1964 medium, callus tissues were formed.

Microscopic observation of cultured germ cells revealed their morphological changes into the following types : 1) Ellipsoidal, shrivelled pollen grains, 3) Hypertrophied pollen grains with transparent cell walls and abundant starch grains, 4) Pollen grains developing into yellowish, multicellular bodies which later give rise to embryoids.

Chromosome counts conducted on a squashed of the leaf primordia or root-tips revealed that a young plant had 24 somatic chromosomes of haploid number. This fact and that the embryoids appeared from the inside of the dehisced anther and were easily removed from the wall suggest that they might be originated from developing germ cells.

Haploid seedlings were transplanted to the pots filled with the sterilized soil and farmyard manure and brought into a greenhouse. Haploid plants had selender pale green leaves and were completely sterile. In their meiosis, it was found that in almost all cells univalents were observed. Twenty-four diploid tobacco plants were obtained from 34 haploid plants after continuous colchicine treatments.

Rice (*Oryza sativa* L.)

The first successful induction of haploid plant from anther culture in monocotyledons was reported in rice plant by Niizeki and Oono (1968) of The National Institute of Agricultural Sciences. In this case, haploid plants were produced from haploid callus tissues proliferated from pollen grains.

Anthers of 10 rice varieties and 9 F_1 hybrids were placed aseptically on the surface of agar medium in the test tubes and incubated in the dark at 28°C. Twenty-thirty anthers with pollen grains of uninuclear stage were put in a culture. A piece of pale yellow callus proliferated from the inside of the dehisced anther after about 4 weeks of planting (Fig. 1). The formation of callus tissues was most abundant in

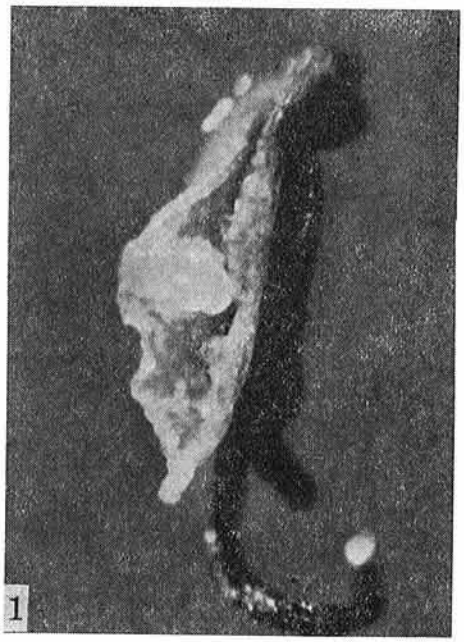

Fig. 1. A callus tissue proliferated from the inside of the dehisced anther of rice plant

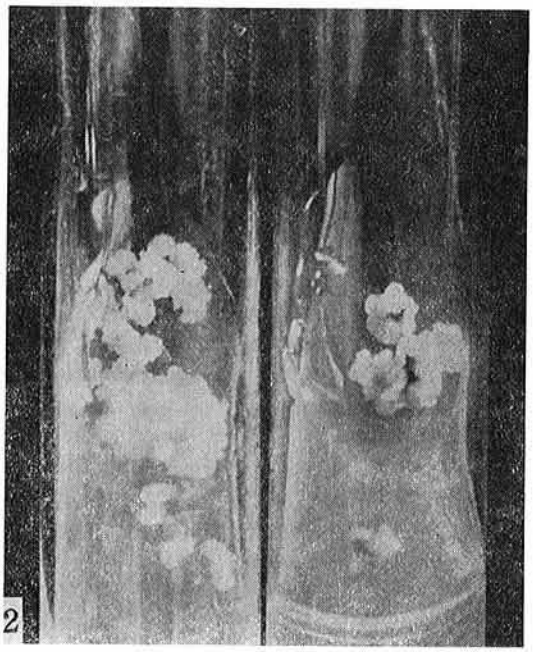

Fig. 2. Subculture of callus tissues of rice plant Right : Haploid callus culture, Left : Diploid callus culture obtained from the seed of the same variety.

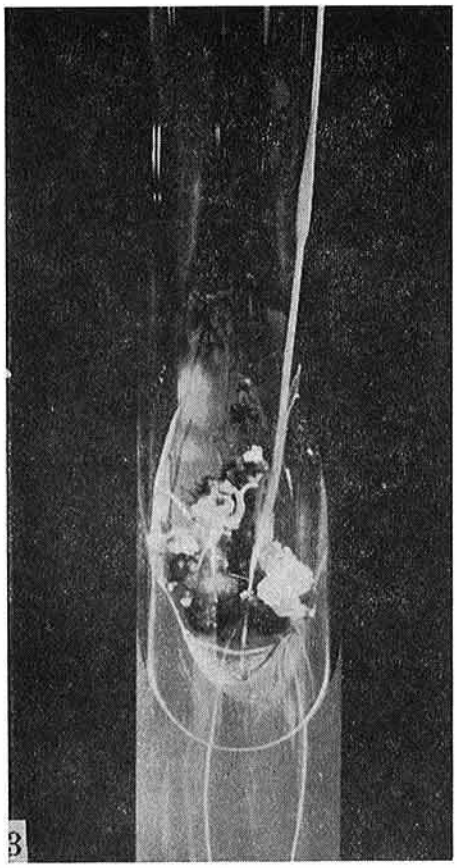

Fig. 3. A rice seedling differentiated from haploid callus culture.

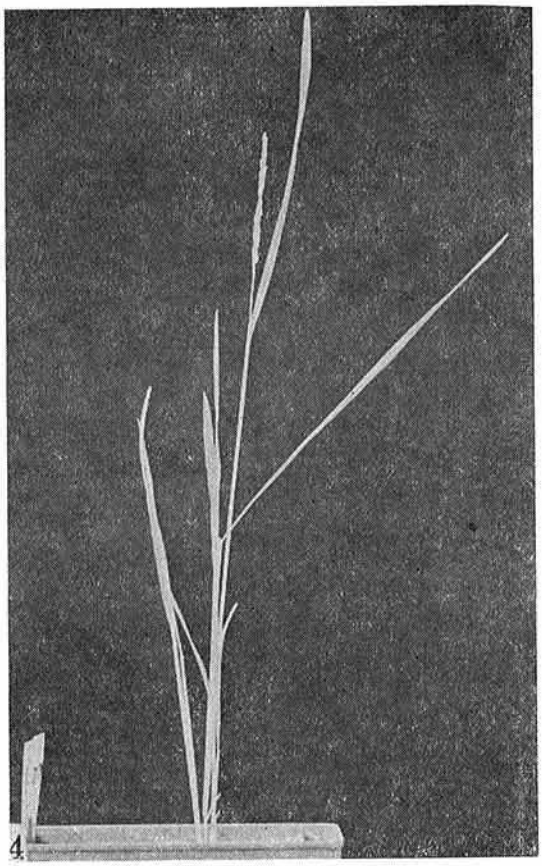

Fig. 4. A haploid rice plant with completely sterile spikelets (The variety, Norin 20).

1-2 month.

The basic medium used was the same as that described by Blaydes except growth regulators (Table 1). The appropriate media for callus formation from the anther were found to be the basic medium supplemented with 10^{-7}-5×10^{-4}M 2, 4-dichlorophenoxyacetic acid (2, 4-D) or 10^{-5}-5×10^{-5}M α-naphthalene acetic acid (NAA). The effect of kinetin on callus formation was not observed. In experiments carried out so far, the ratio of successive callus formation was ranging from 8. 1 to 0. 5 per cent (the average was 2. 9 per cent).

The callus tissue pieces were fixed in acetic alcohol (1 : 3) containing 0. 5 per cent ferric chloride for more than 3 hours, then stained with aceto-carmine for 12-24 hours. Chromosome counts on a squashed preparation of the callus tissue proved that all the cells were haploid (2n =12).

Callus tissues could be successfully sub-cultured on the medium of similar composition to that which gave callus formation. But these haploid calluses grew more slowly than the diploid calluses obtained from the seed of the same variety (Fig. 2).

Attempts to induce shoots and roots from the haploid callus on the medium with various combination of indole-3-acetic acid (IAA) and kinetin were made. A piece of callus was transferred to the above medium containing no 2, 4-D and was incubated in the light at 28°C for 1-2 months. Shoots and roots formation was not induced by IAA and kinetin alone, but certain combination of IAA and kinetin (10^{-5}M IAA and -2×10^{-5}M kinetin) was found to be optimal for organ formation.

About 4 weeks after the transplanting to this medium, small plants with a coleoptile occurred. Then they developed leaves normally (Fig. 3).

Table 1. Media used for the anther culture

Salts mg/l	Tobacco			Rice
	revised tobacco C medium	revised RM-1964 medium	modified White medium	Blaydes medium
KCl	65		65	65
$CaCl_2 \cdot 2H_2O$		440		
KNO_3	80	1,900	80	1,000
$Ca(No_3)_2 \cdot 4H_2O$	400		300	347
$MgSO_4 \cdot 7H_2O$	180	370	720	35
Na_2SO_4	800		200	
NH_4NO_3		4,950		1,000
KH_2PO_4		510		300
$NaH_2PO_4 \cdot H_2O$	33		16.5	
$Fe_2(SO_4)_3$			2.5	
$FeSO_4 \cdot 7H_2O$	27.8	27.8		
Na-Fe-EDTA				32
Na_2-EDTA	37.3	37.3		
$MnSO_4 \cdot 4H_2O$	0.45	22.3	7	4.4
$ZnSO_4 \cdot 7H_2O$	0.6	8.6	3	1.5
H_3BO_3	0.00375	6.2	1.5	1.6
KI	0.03	0.83	0.75	0.8
$CuSO_4 \cdot 5H_2O$		0.025	0.001	
MoO_3			0.0001	
$Na_2MoO_4 \cdot 2H_2O$		0.25		
$CoCl_2 \cdot 6H_2O$		0.025		
Glycine			300	2
Thiamine·HCl	0.4	0.4	10	0.1
Pyridoxine·HCl			10	0.1
Myo-inositol		100	100	
Nicotinic acid			50	0.5
Sucrose	20,000	30,000	20,000	30,000
IAA		2	2	1.75
NAA	0.1			
Kinetin		2-4	0.05	2.2-4.4
Coconut milk	150ml/l			
Agar	6,000	10,000	10,000	10,000
pH	5.8	5.6	5.06	6.0

Their root-tip cells had a haploid number of chromosomes. So far, 70 haploid seedlings have been grown from calluses of rice varieties, Fujisaka 5, Minehikari, Norin 20, Norin 32 and Toride 2. Usually these plants were normal ones with green leaves, but albino plants were rarely found.

These haploid seedlings were transferred to pots containing sterilized soil and brought in a greenhouse. All the haploid plants were markedly dwarf and produced short ears with small spikelets (Fig. 4). The leaves were also smaller as compared with the leaves of the diploid relatives. Haploid rice plants could be remarkably distinguished from the diploid relatives in lacking of ligules. Pollen grains of these plants were completely sterile and produced no seeds. Rarely diploid plants occurred spontaneously from haploid seedlings of the variety Toride 2.

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