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Effects of Various Culture Conditions on Developmental Patterns of Tobacco Ovules

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We have found three simple ways for changing the developmental patterns of mature ovules of *Nicotiana tabacum* L. using various types of stimuli. Cytokinin treatment induced the integument cells to differentiate into style-like and stigma-like structures, and the embryo sac to collapse. Sucrose starvation treatment induced callus formation from ovules while it inhibited the differentiation of style-like and stigma-like structures from integuments. Plants were regenerated from the callus. Pollination with X-ray-irradiated pollen grains resulted in asexual embryogenesis from the ovules when they were cultured 3-5 days after the pollination. This pseudofertilization did not cause the integuments to develop into style-like and stigma-like structures but rather into a seed coat.

Introduction

In angiosperms, the ovule is composed of an embryo sac, nucellus and one or two integuments which envelop these cells and tissues. After fertilization, the embryo develops in the embryo sac and the integuments develop into seed coat of mature seed. Generally, once an ovule reaches maturity, its development ceases unless it is fertilized.

In tobacco plants, there are a few reports on the control of the developmental pathway of unpollinated immature ovules[1-3]. However, there is no report on that of mature ovules. In the course of our work to establish an effective method to obtain haploid plants from ovules, we have succeeded in inducing formation of style-like and stigma-like structures, callus, and haploid embryos from mature ovules of tobacco. In this paper, we described the abnormal development of mature tobacco ovules. This is the first report on the induction of haploid plants from mature ovules of tobacco by starvation treatment.

Materials and Methods

1. *Plant material*

Tobacco plants (*Nicotiana tabacum* L. cv. BY-4) were grown in a greenhouse. To investigate the origin of diploid plants obtained from ovules, a heterozygous kanamycin-resistant line of BY-4 was used. This line is a hybrid between normal BY-4 and a homozygous kanamycin-resistant line of BY-4. The latter was obtained by self-pollination of a transgenic BY-4 into which was introduced a kanamycin-resistant gene (neomycin phosphotransferase II gene) and by testing the Kanamycin resistance of their self-pollinated progeny.

2. *Ovule culture*

The flower buds were surface-sterilized with 70% ethanol for 30 sec., 1% sodium hypochlorite for 10 min. and rinsed with sterilized water. Ovaries were excised from the flower buds under aseptic conditions. The ovary wall was removed to expose the ovules, and ovules with placenta were cultured on various media. The pH was adjusted to 5.8 with 0.1 N KOH before autoclaving. Cultures were kept at 27°C under a 12 h-light/12 h-dark cycle (white fluorescent lamps, 6 w • m^{-2}) for 6 weeks.

3. *Cytokinin treatment*

Ovules with placenta were cultured on NN medium[4] supplemented with 2 mg/*l* kinetin or 6-benzylaminopurine (BA), 2% sucrose and 0.8% agar without pretreatment.

4. *Starvation treatment*

For the nutrient starvation treatment, ovules were culturd in a medium without any nutrient for 2-4 days. These ovules were then transferred to a second culture medium composed of major and minor salts of N6[5] or SK[6] supplemented with 2 mg/*l* kinetin, 0.5 mg/*l* 2-methyl-4-chlorophenoxy-acetic acid (MCPA), 5% sucrose and 0.2% Gerlite.

5. *Pseudofertilization*

Flowers were emasculated one day before anthesis, and then pollinated with pollen grains which were irradiated with 100 kR of soft X-rays (OHMIC OM-100 R). Ovaries were collected 3-5 days after the pollination. Ovules with placenta were cultured on N6 medium containing 5% sucrose and 0.2% Gerlite.

For checking the ability of irradiated pollen grains to germinate on stigmas, to elongate into styles and to penetrate into ovules, the irradiated pollen grains were applied to the stigmas. After 24 h, these stigmas with styles were stained with aniline blue reagent[7] and squashed. On the other hand, 4 days after pollination with irradiated pollen grains, ovaries were collected, sectioned by a cryotome (Yamato CR 502) and stained with 0.01 mg/*l* propidium iodide in 0.1 M sodium phosphate buffer (pH 7.4). These were observed under an epi-fluorescence microscope (Olympus BH-2) with a BP 490 excitation filter and a DM 500 dichroic mirror.

6. *Ploidy level*

The number of chromosomes of the regenerants was counted using root tip cells according to the standard method of aceto-orcein staining. Ploidy level was also estimated by counting the number of chloroplasts in stomatal guard cells of young leaves of the regenerated plants[8,9].

7. *Kanamycin resistance test*

Resistance to kanamycin of S_1 (first generation of self-pollinated offspring) plants of diploid regenerants obtained from the ovules of heterozygous kanamycin-resistant plants were checked by culturing them on MS medium[10] supplemented with 200 μg/*l* kanamycin and without plant growth substances.

Results

1. *Development of style-like and stigma-like structures from integuments*

When mature ovules were cultured on a medium containing 2 mg/*l* kinetin or BA for 2 to 3 weeks, style-like and stigma-like structures were formed from the integument tissue, and the collapse of the embryo sac was observed (**Fig. 1-a, b, c**). In the absence of cytokinin, ovules died after slight expansion. The morphological characteristics of the style-like and stigma-like structures were similar to those of normal stigmas and styles, and their functions were also the same, that is, pollen grains germinated and pollen tubes elongated into style-like structures after applying them on the

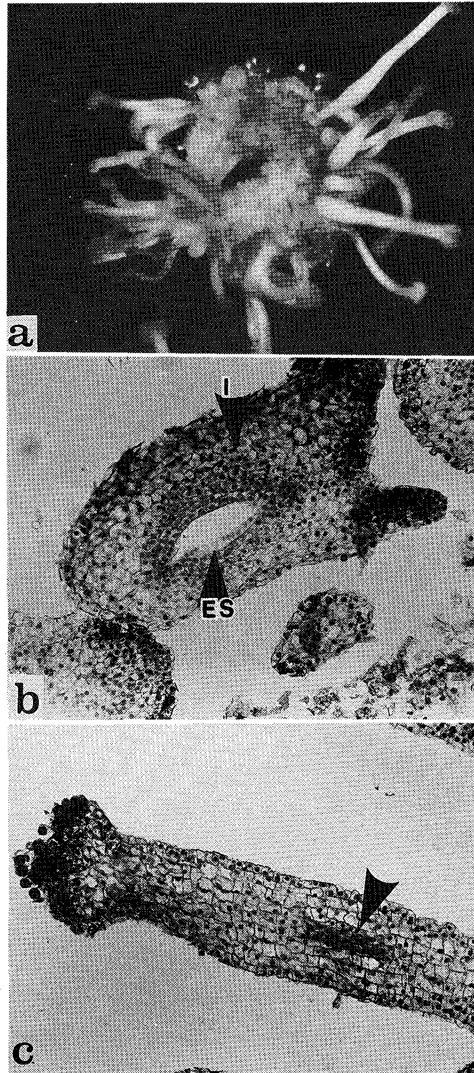

Fig. 1 a : Style-like and stigma-like structures differentiated from cultured ovules.
b : Longitudinal section of cultured ovule. Arrow heads show embryo sac (ES) and integument (I).
c : Longitudinal section of style-like and stigma-like structure. Arrow head shows collapsed embryo sac.
Ovules were cultured on NN medium supplemented with 2 mg/*l* kinetin.

stigma-like structures.

2. *Callus induction and plant regeneration by starvation treatment*

When ovules were cultured in distilled water for several days (starvation treatment) and then transferred to a second culture medium containing 2 mg/*l* kinetin, 0. 5 mg/*l* MCPA and 5% sucrose, five to ten calli per one ovary were formed from ovules after about 6 weeks (**Fig. 2-a**). When the calli were transferred and cultured on NN medium supplemented with 2 mg/*l* kinetin, 0. 5 mg/*l* IAA, 2% sucrose and 0. 8% agar, shoots were regenerated from the calli (**Fig. 2-b**). The maximum frequency of callus formation was achieved when the starvation treatment in distilled water continued for 3 days at 35°C (**Table 1**). When ovules were subjected to starvation treatment at a lower temperature (25°C), a longer treatment was required to obtain a high frequency of callus formation. Strong treatment (such as long duration *i. e.* at 25-35°C for 5 days) led to the death of

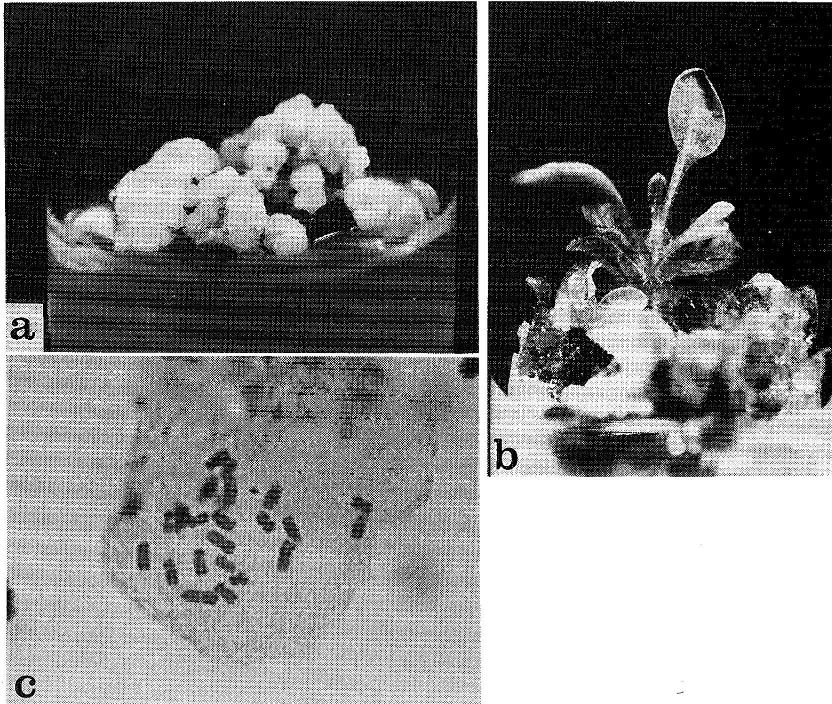

Fig. 2 a : Calli arising from unpollinated ovules.
b : Plantlets regenerated from callus.
c : Chromosomes of a plantlet in b.
Ovules were cultured on N6 medium supplemented with 2 mg/*l* kinetin and 0. 5 mg/*l* MCPA after starvation treatment. Calli obtained in ovule cultures were transferred to NN medium supplemented with 2 mg/*l* kinetin and 0. 5 mg/*l* IAA.

Table 1. Effects of temperature and duration of starvation treatment on callus formation from cultured ovules.

Duration of starvation	Temperature (°C)	Callus formation*1 −	+
2 days	25	10	0
	30	10	0
	35	10	0
3 days	25	10	0
	30	6	4
	35	0	10
4 days	25	6	4
	30	4	6
	35	7	3

Ovules were cultured on N6 medium supplemented with 2 mg/*l* kinetin and 0. 5 mg/*l* MCPA after starvation treatment.
Haploid plants were regenerated from a piece of these calli.
Ten ovaries were tested for each treatment.
*1 − sign represents ovaries without callus formation.
+ sign represents ovaries formed callus.

Table 2. Effects of various starvation treatments on callus formation from cultured ovules.

1 st culture medium	Callus formation*3 −	+
D. W.*1	0	10
D. W.+mineral salts*2	2	8
D. W.+2. 7% mannitol	4	6
D. W.+5% sucrose	10	0

Ovules were cultured for 3 days in each 1st culture medium mentioned at 35°C and then transferred to N6 medium supplemented with 2 mg/*l* kinetin and 0. 5 mg/*l* MCPA. Ten ovaries were tested for each treatment.

*1 D. W.=distilled water.

*2 Mineral salts of N6 medium.

*3 −sign represents ovaries without callus formation. +sign represents ovaries formed callus.

ovules, whereas weak treatment (at 25-35°C for 2 days or at 25°C for 3 days) led to the expansion and browning of ovules on the second medium (Data not shown here). The development into style -like and stigma-like structures from the ovules also occurred at low frequency after weak treatment.

To clarify what type of nutrient starvation was necessary for the induction of callus from ovules, ovules were cultured on the second culture medium described above after culturing them in either of the followings: distilled water, inorganic salts of N6 medium, 2. 7% mannitol solution or 5% sucrose solution (**Table 2**). Calli were formed when ovules were cultured in distilled water, inorganic salts of N6 medium or 2. 7% mannitol solution. In contrast, calli did not appear when ovules were cultured in 5% sucrose solution.

Thirty four regenerants were obtained from calli which were induced from ovules cultured on SK medium supplemented with 2 mg/*l* kinetin and 0. 5 mg/*l* MCPA and 5% or 11% sucrose. The ploidy level of the regenerants was checked by counting the number of chromosomes in the root tip cells and the number of chloroplasts in stomatal guard cells. Out of 34 plants, 4 plants were haploid ($2n=24$, **Fig. 2-c**), 28 plants were diploid and 2 plants tetraploid or aneuploid. Haploid plants were regenerated from the calli induced on the medium containing 11% sucrose (**Table 3**). To clarify the origin of diploid regenerants obtained from ovules, regenerants induced from ovules of plants of heterozygous kanamycin-resistant line of BY-4 were examined for their resistance to kanamycin. All of these regenerants were resistant to kanamycin. S_1 plants germinated from seeds which were obtained from regenerants by self-crossing were cultured on a medium containing 200 μg/m*l* kanamycin. The phenotype of kanamycin resistance (KR) of these S_1 plants segregated. The average segregation ratio was 2. 4 (KR) : 1 (normal). These results indicate that diploid regenerants from ovules originated from somatic cells.

3. *Pseudofertilization*

When ovules pollinated with X-ray-irradiated pollen grains were cultured, about 30% of the cultured ovules expanded (**Fig. 3-a**). In some of these, asexual embryos appeared after 3 or 4 weeks of culture (**Fig. 3-b**). This embryogenesis induced by pseudofertilization did not require any treatment with plant growth substances. The average number of plantlets per ovary was 1. 14, and most of them were haploid. The integuments of pseudofertilized ovules did not develop into style -like and stigma-like structures on a medium containing 2 mg/*l* kinetin, as was the case for

Table 3. Ploidy levels of plantlets obtained from cultured ovules.

Sucrose (%)	No. of haploid	No. of diploid	No. of others*1
5	0	15	2
11	4	13	0

Plantlets were regenerated from calli obtained in ovule cultures on SK medium supplemented with 2 mg/l kinetin and 0.5 mg/l MCPA after starvation treatment. Ploidy levels of regenerants were checked by counting the number of chromosomes in the root tip cells and the number of chloroplasts in stomatal guard cells.

*1 These plantlets were estimated as aneuploid or tetraploid.

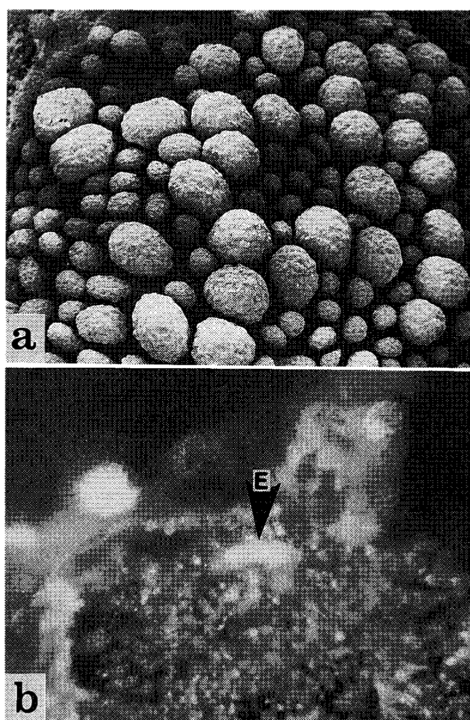

Fig. 3 a : Pseudofertilized ovules on placenta 6 days after pollination. The photograph was taken before *in vitro* culture.
b : An asexual embryo arising from pseudofertilized ovule after *in vitro* culture. Arrow head shows the embryo (E).

unfertilized ovules. By microscopic observations, it was confirmed that tubes of irradiated pollen grains elongated into styles and penetrated into ovules.

Discussion

Style-like and stigma-like structures were formed by culturing immature ovules of *N. tabacum* in a medium without plant growth substance[1]. In *N. tabacum*, gynogenesis was also induced from immature ovules by the addition of 0.5 mg/l IAA with 2 mg/l kinetin or BA[2,3]. However, these combinations of plant growth substances did not reveal such an effect in mature ovules. In cultured

mature ovules of *N. tabacum*, the integuments differentiated into only the style-like and stigma-like structures (**Fig. 1**). Obtained style-like and stigma-like structures have similar forms and functions to those of normal stigmas and styles. These characteristics were same as those of style-like and stigma-like structures differentiated from receptacles of tobacco[11].

As shown in **Table 2**, the addition of mannitol to starvation medium did not inhibit callus formation whereas the addition of sucrose inhibited it. It is well known that mannitol is not utilized as a carbon source. These facts suggested that the effect of starvation was not caused by the low osmotic potential due to sucrose starvation. It seems that the level of carbohydrate utilized as a carbon source in ovule tissues is one of critical factors to induce callus formation.

Starvation treatment before culturing ovules in the second medium was an effective way of inducing haploid plants. In immature tobacco pollen grains, starvation treatment was required for the induction of pollen embryogenesis[12]. In our experiment, asexual embryogenesis was not observed in cultured ovules. On the other hand, in our experiment, some calli were obtained from haploid cells in the embryo sac and haploid plants were regenerated from the calli obtained by starvation treatment. Thus, it can be concluded that the developmental pathway of both male and female gametes in tobacco is affected by starvation treatment.

As shown in **Table 3**, haploid plants were obtained from callus which were induced on the second medium supplemented with 11% sucrose. Yang *et al.*[13] reported that in *Helianthus*, the optimum sucrose concentrations were 1%, 3-9% and about 12%, for induction of callus formation from integument, asexual embryogenesis from endothecium, and gynogenetic embryogenesis, respectively. These results show that sucrose concentration is an important factor for the induction of gynogenetic competence.

Pseudofertilization is defined as the fertilization between an egg cell in an ovule and a sperm cell from a pollen grain irradiated with X-rays. In this case, zygotic embryogenesis does not occur because the nucleus of the sperm cell is inactivated. In the report of Pandey[14], pollination of *N. tabacum* with X-ray-irradiated pollen grains of *N. glutinosa* also produced predominantly haploid plants in about 50% of pollinated ovaries. In the experiment, fruit and seed development after pollination were achieved in a greenhouse not using *in vitro* technique. The result indicates that in tobacco, pseudofertilization has the potential to induce haploid embryogenesis and to make haploid plants without *in vitro* culture. In our experiment, using *in vitro* culture of ovules after pollination with X-ray-irradiated pollen grains, we obtained 1.14 haploid plants per ovary. This value was higher than the result of Pandey[14] who obtained 0.5 plants per ovary. Probably, some asexual embryos were rescued by ovule culture.

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《和文要約》

タバコ (*Nicotiana tabacum*) 胚珠の発生パターンに及ぼす各種培養条件の影響

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タバコの胚珠に異なった刺激を与えて培養することにより，胚珠がさまざまな形態へと分化方向を変えうることを見出した．培地にサイトカイニンを添加して培養した場合，胚珠の珠皮組織が柱頭様，花柱様組織へ分化した．飢餓処理後，サイトカイニン及びオーキシンを含む培地で培養した場合，胚珠からカルスが出現した．得られたカルスからは半数体及び二倍体の植物が再分化した．偽受精後，胚珠を培養した場合は，不定胚形成を経て半数体植物が出現した．