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Induction of Haploid Plants from Unpollinated Ovules in *Nicotiana rustica*

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Haploid plants derived from asexual embryos and/or calli were obtained at a high frequency by the culture of unpollinated ovules of *Nicotiana rustica*. The addition of kinetin was found to be a key factor in the induction of gynogenesis. Moreover, histological observations revealed that some of the regenerants were derived from female gametophytes.

Introduction

The culture of unpollinated ovaries or ovules to produce haploid plants met with no success until 1976 when San Noeum reported her first results on the culture of ovaries of *Hordeum vulgare*[1]. As for *Nicotiana*, Zhu and Wu[2] obtained haploid plants from cultured ovaries of *N. tabacum*. Wu and Cheng[3] also obtained haploid plants of *N. tabacum* and *N. rustica* by the culture of unpollinated ovaries. Zhu *et al*[4]. observed the development of asexual embryos within the unpollinated ovaries of *N. tabacum*. These reports are noteworthy but none of the methods described can be considered as a general technique for the induction of haploid *N. rustica* since the frequency of gynogenesis is very low, merely 8% of the ovaries cultured. This paper describes a more efficient method for induction of gynogenesis from the unpollinated ovules of *N. rustica*. Factors affecting the frequency of induction gynogenesis in this system are also discussed.

Materials and Methods

1. *Plant material*

Unpollinated ovules of *Nicotiana rustica* cv. Rustica were used throughout the experiments. To investigate differences in response in various genotypes, the following 8 genotypes of *N. rustica* were also used: cv. No. 1, cv. Soviet No. 4, cv. Qumduz 2, cv. Maruba tabako, cv. K. P. 8, var. *humilis*, var. *brasilia*, var. *pavonii*. All plants were grown in a greenhouse from seeds obtained from the Plant Breeding and Genetics Research Laboratory of Japan Tobacco Inc. (Toyoda, Shizuoka, Japan).

2. *Sterilization*

Flower buds of greenhouse grown plants were sterilized by immersion in 70% ethanol (30 sec.), then in 1% sodium hypochlorite (10 min.), and washed once with sterile water.

3. *Ovule culture*

Ovules with placenta were isolated from the flower buds, which were 5-20 mm in length, and

cultured on a solid medium in a test tube (Diameter: 3 cm; Height: 10 cm). The induction of gynogenetic development was performed mainly on N6 medium[5] supplemented with 5% sucrose and plant growth substances. The pH of the medium was adjusted to 5.8 with 0.1 N KOH before autoclaving. As for the plant growth substances, kinetin and IAA were used either alone or in combination. Cultures were kept at 27°C under a 12 h-light/12 h-dark cycle (white fluorescent lamps, 6 $w \cdot m^{-2}$).

4. *Ploidy level*

The ploidy of the regenerants was checked under a light microscope by squashing root tips stained with acetic orcein. In addition, the number of chloroplasts in the guard cells of the stomata of the lower epidermis was counted using an epifluorescence microscope (Olympus BH-2) with BP 490 excitation filter and a DM 500 dichroic mirror.

5. *Histological observations*

Ovules with placenta were collected at 3, 9, 12 and 21 days after inoculation, and fixed in a solution of formalin/acetic acid/95% ethanol/distilled water (2:1:10:7) for 24 h. Then the samples were dehydrated, embedded in paraffin, sectioned to 12-14 μm, and stained with hematoxylin. The stained materials were then dehydrated with ethanol, dewaxed with xylol, mounted directly on glass slides, and fixed with Canada balsam.

For scanning electron microscopic observations, the cultured ovules were fixed in 0.1 M sodium phosphate buffer (pH7.4) with 1% glutaraldehyde for 24 h at 4°C. This procedure was followed by postfixing in 0.1 M sodium phosphate buffer (pH7.4) with 1% osmic acid for 2 h. The ovules were then dehydrated in an ethanol series, treated with isoamyl acetate and subjected to critical point drying in CO_2. After being coated with gold with an ion sputter (JEOL JFC-1100), they were observed with a scanning electron microscope (JEOL T220A).

Results and Discussion

1. *Ovule culture*

Ovules were cultured on N6 medium supplemented with 8 mg/*l* kinetin, 0.5 mg/*l* IAA and 5% sucrose. During the first 14 days of culture, the excised ovules became markedly large and turned brown (**Fig. 1**). After 3 to 4 weeks of culture, small asexual embryos and/or calli appeared (**Fig. 2**). Most of asexual embryos developed into plantlets. We observed two types of callus. One of them regenerated shoots and another formed asexual embryos which developed into plantlets at a high frequency. When these shoots were transferred to Nitsch and Nitsch (NN) medium[6] containing 2% sucrose, and 0.8% agar, root formation was observed. To investigate the ability of the ovules at various developmental stages to produce asexual embryos and/or calli, ovules excied from flower

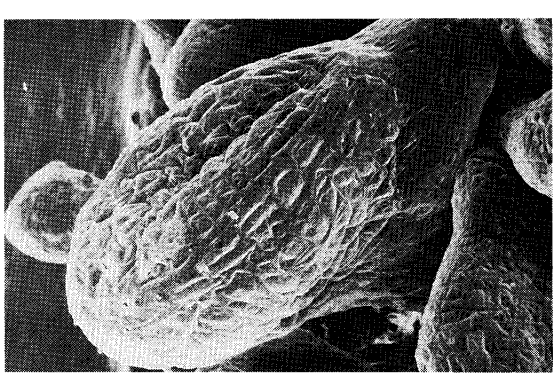

Fig. 1 A scanning electron micrograph of an expanded ovule cultured for 2 weeks.

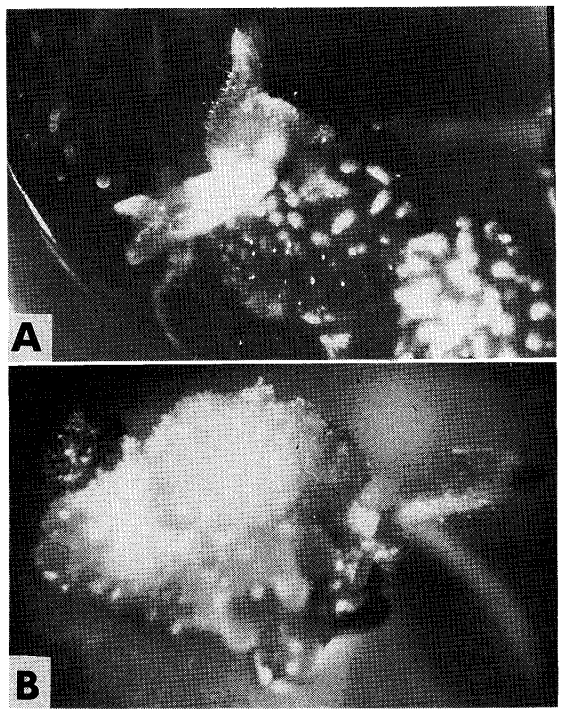

Fig. 2 Ovules cultured for 4 weeks.
A: asexual embryos arising from ovules.
B: callus induced from an ovule, regenerating a plantlet.

Table 1. Effects of length of flower bud on induction of asexual embryos and/or calli from cultured unpollinated ovules.

Flower bud length (mm)	No. of explanted ovaries	Induction frequency (%)	No. of ovaries*1	
			+	++
6-10	10	100	2	8
11-20	10	100	0	10

Ovules with placenta were cultured on N6 medium supplemented with 8 mg/*l* kinetin, 0.5 mg/*l* IAA, 5% sucrose and 0.2% Gelrite (pH 5.8).
Formation of asexual embryos and/or calli was determined after 4 weeks of culture.
*1 Number of ovaries formed 1-3 calli and/or asexual embryos (+) or more than 4 (++).

Table 2. Effects of plant growth substances on induction of asexual embryos and/or calli from cultured unpollinated ovules.

Plant growth substances (mg/*l*)		No. of explanted ovaries	Induction frequency (%)	No. of ovaries*1	
				+	++
KT*2 0	IAA 0	10	0	0	0
4	0.5	10	40	4	0
8	0.5	20	100	2	18
16	0.5	10	0	0	0
8	0	10	90	1	8
8	4	10	50	0	5
8	8	10	60	2	4

Basal medium used was N6 salts supplemented with 5% sucrose and 0.2% Gelrite (pH 5.8).
Formation of asexual embryos and/or calli was determined after 4 weeks of culture.
*1 Number of ovaries fromed 1-3 calli and/or asexual embryos (+) or more than 4 (++).
*2 KT: kinetin

Table 3. Effects of the duration of kinetin treatment on the induction of asexual embryos and/or calli from cultured unpollinated ovules.

Duration of treatment	No. of ovaries forming AE or CA*1	No. of ovaries forming	
		CA	AE
0 (days)	0	0	0
3	7	6	2
12	9	4	6

After kinetin (8 mg/*l*) treatment, ovules with placenta were cultured on N6 medium containing 5% sucrose and 0. 2% Gelrite (pH 5. 8).
Formation of asexual embryos and/or calli was determined after 4 weeks of culture.
Ten ovaries were tested for each treatment.
*1 AE: asexual embryos, CA: calli

Table 4. Induction of asexual embryos and/or calli from cultured unpollinated ovules of 9 genotypes of *Nicotiana rustica*.

Genotype	No. of explanted ovaries	Induction frequency (%)	No. of ovaries*1	
			+	++
cv. Rustica	20	100	2	18
cv. No. 1	10	0	0	0
cv. Soviet No. 4	10	40	4	0
cv. Qumduz 2	10	0	0	0
cv. Maruba tabako	10	50	4	1
cv. K. P. 8	10	10	1	0
var. *humilis*	10	20	2	0
var. *brasilia*	10	0	0	0
var. *pavonii*	10	0	0	0

Ovules with placenta were cultured on N6 medium supplemented with 8 mg/*l* kinetin, 0. 5 mg/*l* IAA, 5% sucrose and 0. 2% Gelrite (pH 5. 8).
Formation of asexual embryos and/or calli was determined after 4 weeks of culture.
*1 Number of ovaries formed 1-3 calli and/or asexual embryos (+) or more than 4 (++).

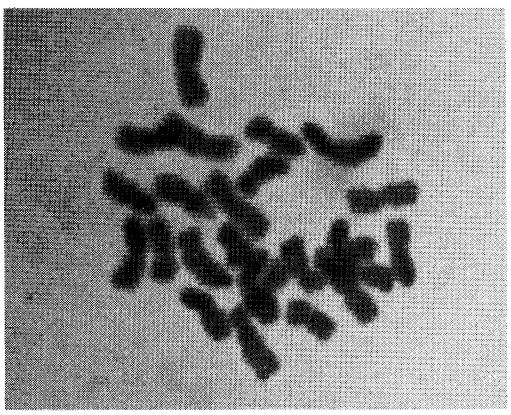

Fig. 3 Root-tip squash of a plantlet obtained from unpollinated ovules showing haploid number of chromosomes ($2n=24$).

buds with various lengths were cultured. Observation of sectioned ovules excised from the flower buds revealed a positive correlation between the length of flower bud of *N. rustica* and the developmental stage of the ovule in the flower bud (data not shown). However, the stages of the ovules from megaspore mother cell to 8-celled embryo sac were found to be unrelated to frequency

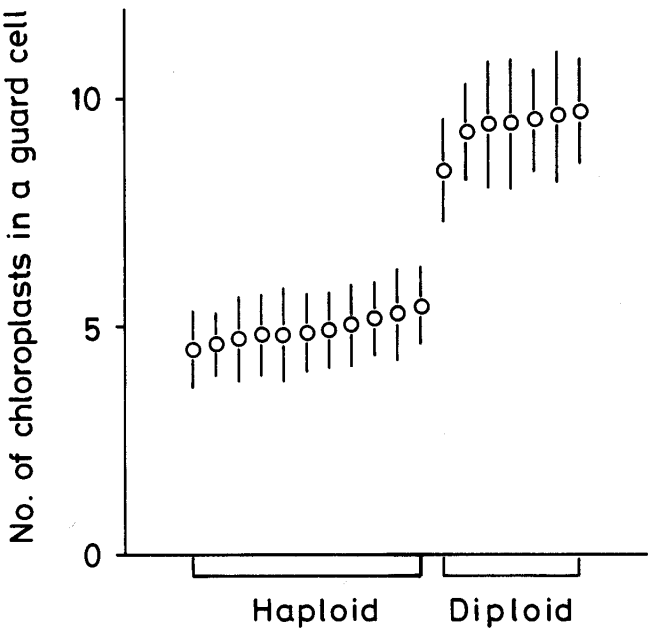

Fig. 4 Average number of chloroplasts per guard cell in leaf epidermis of the plantlets produced via unpollinated ovule culture.
Vertical line represents standard deviations.

of induction (**Table 1**). It seems that ovules in a wide range of stages are able to produce asexual embryos and/or calli, unlike anther cultures of *N. rustica*, in which mature pollen usually did not show androgenesis[7].

2. *Basic media and plant growth substances*

To study the effects of different basic media, ovules were cultured on Murashige and Skoog (MS)[8], NN and White[9] media. Asexual embryos and/or calli did appear in MS and NN media, but not in White medium (data not shown). The induction frequency of asexual embryos and/or calli per ovary was 80% in MS and NN media. However, none of these media was more effective than N6 medium.

To investigate the effect of plant growth substances on the induction of asexual embryos and/or calli, unpollinated ovules were cultured on a medium containing various concentrations of kinetin and IAA (**Table 2**). The best results were obtained on a medium containing 8 mg/*l* kinetin and 0.5 mg/*l* IAA. In this medium, the frequency of induction of asexual embryos and/or calli was 100%. This value is remarkably higher than the result of Wu & Chen[3] (8% of asexual embryo induction). When 16 mg/*l* kinetin was added, no asexual embryos nor calli were observed. The addition of IAA seems unnecessary for the induction of asexual embryos and/or calli from ovules (**Table 2**). These results show that the presence of kinetin is thought to be an important factor in inducing asexual embryos and/or calli from unpollinated *N. rustica* ovules. Wu & Chen[3] reported that treatment with 2 mg/*l* 6-benzylaminopurine (BA) and 0.5 mg/*l* IAA induced asexual embryos via callus from the unpollinated ovules of *N. tabacum*. Hisajima *et al.*[10] also reported the formation of plantlets from maize ovaries of unfertilized ears on MS medium containing 0.5 μM BA and 0.25 μM indolebutyric acid. In *N. rustica*, asexual embryos and/or calli were also obtained on the medium without auxin.

To investigate the effect of the duration of the kinetin treatment, ovules were cultured on a medium with 8 mg/*l* kinetin for 3 or 12 days, then transferred to a kinetin-free medium after

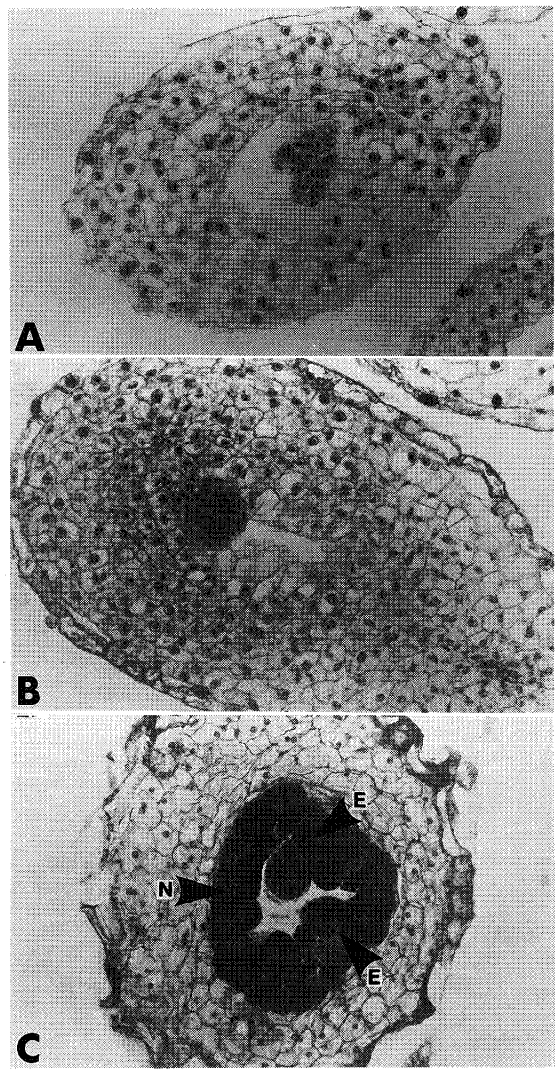

Fig. 5 Asexual embryogenesis in the embryo sac of cultured unpollinated ovules of *N. rustica*.
A: multicellular proembryos on 9th bay.
B: a globular embryo on 9th day.
C: somatic embryos (E) arising from swollen nucellus (N).

washing with sterile water, and cultured for 6 weeks. Formation of asexual embryos was observed after a 3-day treatment (**Table 3**). Although it is not clear whether kinetin remained in the ovules, this result suggests that kinetin was necessary only in the initial period of ovule culture. It seems that the role of kinetin is mainly for the induction of gynogenetic competence.

3. *Importance of genotype*

To investigate differences in response in various genotypes, 9 genotypes of *N. rustica* were examined, and the frequency of the induction of asexual embryos and/or calli was observed to cover a wide range from 0% to 100% (**Table 4**). The frequency of induction of asexual embryos and/or calli varied depending on the genotypes as seen in the unpollinated ovule culture of rice[11] and sugarbeet[12].

4. *Ploidy level*

The ploidy level of various regenerants was checked by counting the number of chromosomes in

the root tips. It was confirmed that haploid plants were obtained from asexual embryos and calli ($2n=24$, **Fig. 3**). Out of 18 *N. rustica* cv. Rustica plants, 11 were haploid, and the rest were diploid. The number of chloroplasts in the guard cells of the stomata of the lower epidermis of young leaves in the regenerants was also checked (**Fig. 4**), and showed a positive correlation to the ploidy level. The number of chloroplasts in haploid guard cells was 4-5, while for diploid it was 8-10.

5. *Histological observation*

Ovules at various growth stages from megaspore mother cells to 8-celled embryo sacs were cultured and observed under a microscope on the 3rd, 9th, 12th, 21st days of culture. On the 9th day, asexual embryos and/or calli with various sizes and shapes were observed at the micropylar regions of some embryo sacs (**Fig. 5**). It is uncertain whether they originated from an egg cell or a synergid. The formation of endosperm was not observed. This means that, in the *in vitro* development of asexual embryos, endosperm formation is inevitable. In some cases, nucellus cells were induced to mitotic division, and formed a structure similar to an embryo (**Fig. 5-C**). It seems that some diploid plants originated from somatic cells of nucellus.

Our results on *N. rustica* showed that the addition of kinetin was effective on the induction of gynogenesis. Further studies on the culture of unpollinated ovules will yield valuable information about the mechanism by which embryogenesis starts after fertilization.

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《和文要約》

ルスチカタバコ（*N. rustica*）の未受精胚珠からの
半数体植物作出について

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ルスチカタバコの未受精胚珠培養により，不定胚またはカルス経由の半数体植物が高頻度で得られた．不定胚及びカルス作出のための培養条件を検討した結果，カイネチンの添加が重要であることが明らかとなった．また，培養胚珠の組織学的観察により，得られた半数体植物の起源が雌性配偶体であることが確認された．