



Title	Studies on Plant Cell and Tissue Culture : . Production of haploid plants of tobacco by anther culture
Author(s)	NIIZEKI, Minoru
Citation	Journal of the Faculty of Agriculture, Hokkaido University, 57(2), 161-178
Issue Date	1973-03
Doc URL	http://hdl.handle.net/2115/12873
Type	bulletin (article)
File Information	57(2)_p161-178.pdf



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STUDIES ON PLANT CELL AND TISSUE CULTURE

I. Production of Haploid Plants of Tobacco by Anther Culture

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Received June 23, 1972

Introduction

The knowledge of haploid plants dates back to 1922 when Blakeslee and his colleagues first reported the occurrence of a haploid plant in *Datura stramonium*. Since that time, haploid plants have been generally recognized for their usefulness for various approaches in cytogenetics, genetics, and plant breeding research.

Besides the spontaneous occurrence of haploids, various artificial methods have been exploited to induce haploids; such as cross pollination either intervarietal or interspecific, delayed pollination, X-rays or colchicine treatments, and temperature shocks. However, no single way has been found to overcome the difficulties encountered in order to obtain haploids in a high frequency and also to obtain them freely when they are required. Recently, however, the induction of haploid plants by means of anther cultures, gives promise for removing some of these difficulties in haploid production. GUHA and MAHESHWARI (1964, 1966) were the first to report the successful production of haploid plants from anther cultures in *Datura innoxia*. Also, BURGIN and NITSCH (1967) and NAKATA and TANAKA (1968) induced haploid *Nicotiana* plants and NIIZEKI and OONO (1968) succeeded in producing haploid plants of *Oryza sativa* by culturing anthers. Following the reports of these successes, haploid plants of *Brassica* (KAMEYA and HINATA, 1970), *Lolium multiflorum* × *Festuca arundinacea* (NITZSCH, 1970) and *Aegilops* (KIMATA and SAKAMOTO, 1971) have been produced by anther cultures.

In the early study of *Datura innoxia* anther culture, GUHA and MAHESHWARI (1964) found that the induction of embryoids and plantlets were related to the developmental stages of the anther. Precise studies in anther culture of *Nicotiana* species have shown that the stage in which

germ cells within the anthers have the totipotency for embryoid formation is the immature but fully individual uninucleate pollen grain (NITSCH *et al.*, 1968; NITSCH and NITSCH, 1969; SUNDERLAND and WICKS, 1969 a, b). Moreover, SUNDERLAND and WICKS (1969 a, b) considered the critical time whereby embryoid formation may be initiated lays somewhere between the early somewhat immature pollen grain, probably prior to DNA synthesis of the first pollen mitosis, and a later stage when the pollen grain was more mature but was still in the uninucleate condition.

This paper reports experiments aimed at inducing haploid plants of *Nicotiana* species by means of anther culture and obtaining more precise data on the relationship between the time of initiation of embryoid and the developmental stage of the germ cells within the anthers.

Materials and Methods

Three cultivars of *Nicotiana tabacum* (cv. Hicks Broadleaf, cv. Wisconsin 38, and cv. Delhi 34) and *N. affinis* were used. These *Nicotiana* collections were supplied through the courtesy of Dr. B. POVILAITIS of the Canada Department of Agriculture Tobacco Sub-Station, Delhi, Ontario.

Culture media

The basic medium used was the same as medium H described by NITSCH and NITSCH (1969). The growth regulators, indole-3-acetic acid (IAA), kinetin and gibberellic acid (GA) in different combinations and concentrations shown in Table 1. All media were solidified with 8 g/l of Difco Bacto-agar. The pH was adjusted to 6.0 with 1N HCl or 1N NaOH. Cylindrical glass bottles (capacity, 30 ml) with plastic screw cap were used as the container for all cultures.

TABLE 1. The composition of the 5 media used for anther culture showing different combinations and concentrations of growth regulators

Medium type	Growth regulators		
	IAA (mg/l)	Kinetin (mg/l)	GA (mg/l)
A	0.1	—	—
B	0.1	0.1	—
C	4.0	2.0	—
D	0.1	0.1	1.0
E	—	—	—

Culture procedures

The flower buds were classified into three groups which corresponded to three developmental stages of the anthers. The identification of the stages was carried out by a microscopic examination of the meiocytes stained with aceto-carmin. In each species and cultivars, the stages of development of the anthers were found to be closely related with the size of the flower bud and the petal length. Therefore, the size of the flower buds and the length of the flower petals were mainly used as criteria in the selection of anthers prior to cytological examination. The anthers were classified into three developmental stages as follows:

Stage 1. Early prophase I to the quartet stage of meiosis.

Stage 2. Individualized uninucleate pollen grains.

Stage 3. Mature pollen grains with both a vegetative and a generative nucleus.

In preparation for anther culture, the flower buds were dipped into 70% ethanol in a Petri dish for several seconds, and then washed several times with sterilized distilled water to completely remove the ethanol. Subsequently, the flower buds were immersed in a 7% solution of sodium hypochlorite for about 3 minutes and then thoroughly washed several times with sterilized distilled water to remove any traces of the sodium hypochlorite. Then the sterilized flower buds were placed on sterilized filter paper for several minutes to remove the excess water. The excision of the anthers from the flower buds was carried out aseptically with a dissecting knife and needle with the aid of a dissecting microscope. 6 to 12 anthers planted on the surface of agar-solidified medium. The plastic cap of the culture bottles was kept loosened slightly to permit air circulation into the bottles during culture. The culture bottles were illuminated by fluorescent tubes with a light intensity of 4800 lx/m² and maintained at a temperature of 26±0.5°C. After the anthers gave rise to complete plantlets with well developed root-systems on the cultures, they were transplanted directly to pots and grown in a growth chamber until they commenced to flower.

Chromosome number determinations of root tips

Roots were pretreated in 0.002 M 8-hydroxyquinoline for 1 hour at room temperature, fixed with alcohol-acetic acid (3:1) for about 12 hours. They were then hydrolyzed with 1N HCl at 60°C in a water bath for 6 minutes and stained with leuco-basic fuchsin (Feulgen technique). Squashes were prepared in the usual manner using 45% acetic acid.

Preparation of anthers for autoradiography

For autoradiography, anthers were classified according to developmental stages as follows.

- Stage 1. Quartets and early individualized uninucleate pollen grains.
- Stage 2. More mature pollen grains but still uninucleate.
- Stage 3. Mature pollen grains with both a vegetative and a generative nucleus.

The identification of developmental stages of the anthers and the sterilization of the material were carried out in the same manner as described previously.

^3H -thymidine (sp. act. 3,000 mc/mM) was added to Medium B and E at a final concentration of 0.5 $\mu\text{C}/\text{ml}$. Excised anthers were planted aseptically on the medium containing the ^3H -thymidine and then incubated under illumination of fluorescent tubes and at a temperature of $26 \pm 0.5^\circ\text{C}$. Samples of anthers for the autoradiographic preparations were removed at 48 hours, 1 week, 2 weeks, 3 weeks and 4 weeks after incubation. Anthers were then washed in cold thymidine (40 mg/l) and in several changes of distilled water. They were then fixed with alcohol-acetic acid solution (3:1) for 12 hours. Acetocarmine was used for staining. A smear method was used for the preparation of slides. However, in order to reduce the risk of losing specimens from the glass slide, a subbing solution containing gelatin (5.0 g/l) and chrom alum (0.5 g/l) was applied to the slides before they were used (GUDE, 1968).

In order to prepare the slides for autoradiography, they were placed on a thermoelectric freezing attachment unit (Komatsu Electronics Inc.) for at least 5 minutes to lower the temperature to -40°C . Then the coverslips were popped off with a razor blade and the slides left to air dry for about 1 hour. The application of liquid emulsion to the slides was carried out in a darkroom under a safe light using a 15 watt bulb and a Wratten Series 2 filter (Eastman Kodak Co.). A Coplin jar containing the liquid emulsion NTB₂ (Eastman Kodak Co.) was placed for approximately 1 hour in a water bath maintained at 42°C in order to melt the emulsion and to allow all the air bubbles to escape. Two slides placed back to back, or individual slides, were immersed vertically into the liquid emulsion three times within a period of 5 seconds. The dipped slides were then dried for 2 hours at room temperature in complete darkness before being placed into slide boxes containing a drying agent (Drierite). The slide boxes containing the dipped slides were then stored at 4°C in a refrigerator for 10 to 20 days to allow the ^3H -thymidine to expose the film. After the period of exposure, devel-

oping was carried out in a darkroom with a Wratten Series 2 filter in a safe light. The slides were immersed in the developer, D-19 (Eastman Kodak Co.) at 20°C for 1 to 3 minutes and then washed in an acid stopper bath (10% of acetic acid) for 5 seconds. They were then transferred to a fixing bath, F-5 (Kodak Co.) for fixation for about 10 minutes. Subsequently, the slides were washed in running water for about 1 hour and then air dried. The slides were made permanent by mounting the coverslips with Euparal and then examined microscopically.

Results

Production of plantlets from the anthers

Three cultivars of *N. tabacum*, cv. Wisconsin 38, cv. Hicks Broadleaf, and cv. Delhi 34, and *N. affinis* were used in this study. Six anthers were planted in each culture bottle on Medium A, B, C, and D.

After 4 to 6 weeks of incubation, plantlets emerged from inside the anthers of all the cultivars on *N. tabacum*, but not from *N. affinis* (Table 2). Some of the regenerated plantlets from cv. Wisconsin 38 showed chlorophyll deficiencies.

The culture media which were found suitable for the production of plantlets from cv. Wisconsin 38 and cv. Delhi 34 were A and B. The former was supplemented with 0.1 mg/l of IAA, and the latter with both 0.1 mg/l of IAA and 0.1 mg/l of kinetin. On the other hand, Medium D which was supplemented with 0.1 mg/l of IAA, 0.1 mg/l of kinetin and 1.0 mg/l of gibberellic acid was found the most suitable for the production of plantlets from anthers of cv. Hicks Broadleaf.

It was found that high concentrations of the growth regulators were inhibitory. Medium C supplemented with 4.0 mg/l of IAA and 2.0 mg/l of kinetin completely inhibited the production of plantlets for all of the cultivars of *N. tabacum*.

The numbers of plantlets which emerged from an anther varied even in the same cultivar, and in some cases, as many as 10 to 20 plantlets arose per anther, in others, only 1 to 4 plantlets developed (Fig. 1 a, b). The anthers from which the earliest plantlets emerged were at developmental stage 2 and contained individual uninucleate pollen grains. In the case of cv. Wisconsin 38, the anthers of developmental stage 1 also produced a few plantlets. However, plantlets from the anthers of developmental stage 1 emerged more than a week later than those from developmental stage 2. None of the anthers of developmental stage 3, which contained matured pollen grains, produced plantlets.

TABLE 2. Organ formation from anthers of *Nicotiana* species

Species	Medium type*	Developmental stage of anthers**	Organ formation from anthers***		
			Shoot	Root	
<i>N. tabacum</i> (cv. Wisconsin 38)	A	1	+	+	
	A	2	+	+	
	A	3	-	-	
	B	1	+	+	
	B	2	+****	-	
	B	3	-	-	
	C	1	-	-	
	C	2	-	-	
	C	3	-	-	
	D	1	-	-	
	D	2	-	-	
	D	3	-	-	
	<i>N. tabacum</i> (cv. Hicks Broadleaf)	A	1	-	-
		A	2	-	-
		A	3	-	-
B		1	-	-	
B		2	-	-	
B		3	-	-	
C		1	-	-	
C		2	-	-	
C		3	-	-	
D		1	-	-	
D		2	+	+	
D		3	-	-	
<i>N. tabacum</i> (cv. Delhi 34)		A	1	-	-
		A	2	+	+
		A	3	-	-
	B	1	-	-	
	B	2	+	+	
	B	3	-	-	
	C	1	-	-	
	C	2	-	-	
	C	3	-	-	
	D	1	-	-	
	D	2	-	-	
	D	3	-	-	
	<i>N. affinis</i>	A	1	-	-
		A	2	-	-
		A	3	-	-
B		1	-	-	
B		2	-	-	
B		3	-	-	
C		1	-	-	
C		2	-	-	
C		3	-	-	
D		1	-	-	
D		2	-	-	
D		3	-	-	

* A, 0.1 mg/l IAA ; B, 0.1 mg/l IAA and 0.1 mg/l kinetin ; C, 4.0 mg/l IAA and 2.0 mg/l kinetin ; D, 0.1 mg/l IAA, 0.1 mg/l kinetin and 1.0 mg/l gibberellic acid.

** 1. Early prophase I to quartet stage of meiosis.
2. Individual uninucleate pollen grains.
3. Mature pollen grains with both a vegetative and a generative nucleus.

*** -, No formation ; +, formation.

**** Shoot which developed was albino.

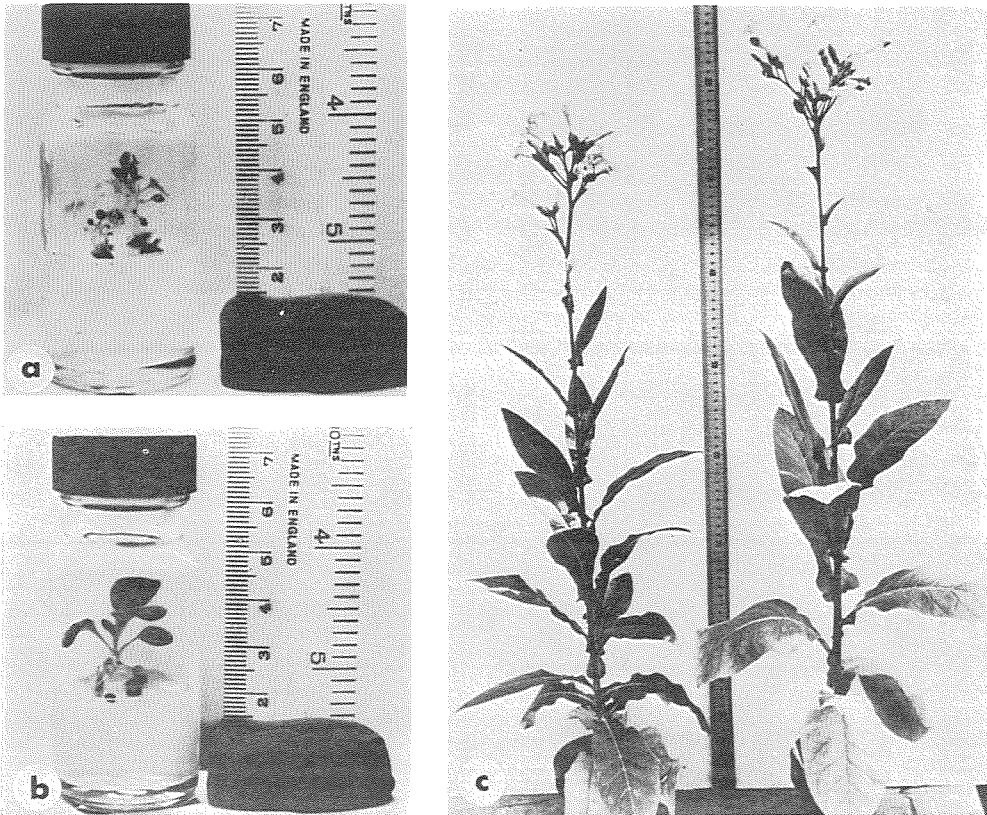


Fig. 1. Production of haploid plants from cultured anthers of *N. tabacum* (cv. Wisconsin 38).

- Proliferation of more than 10 plantlets from a single anther cultured on Medium B after 6 weeks of incubation.
- A single plantlet growing from an anther cultured on Medium A after 6 weeks of incubation.
- An adult haploid plant (left) produced by anther culture and its parent diploid plant (right).

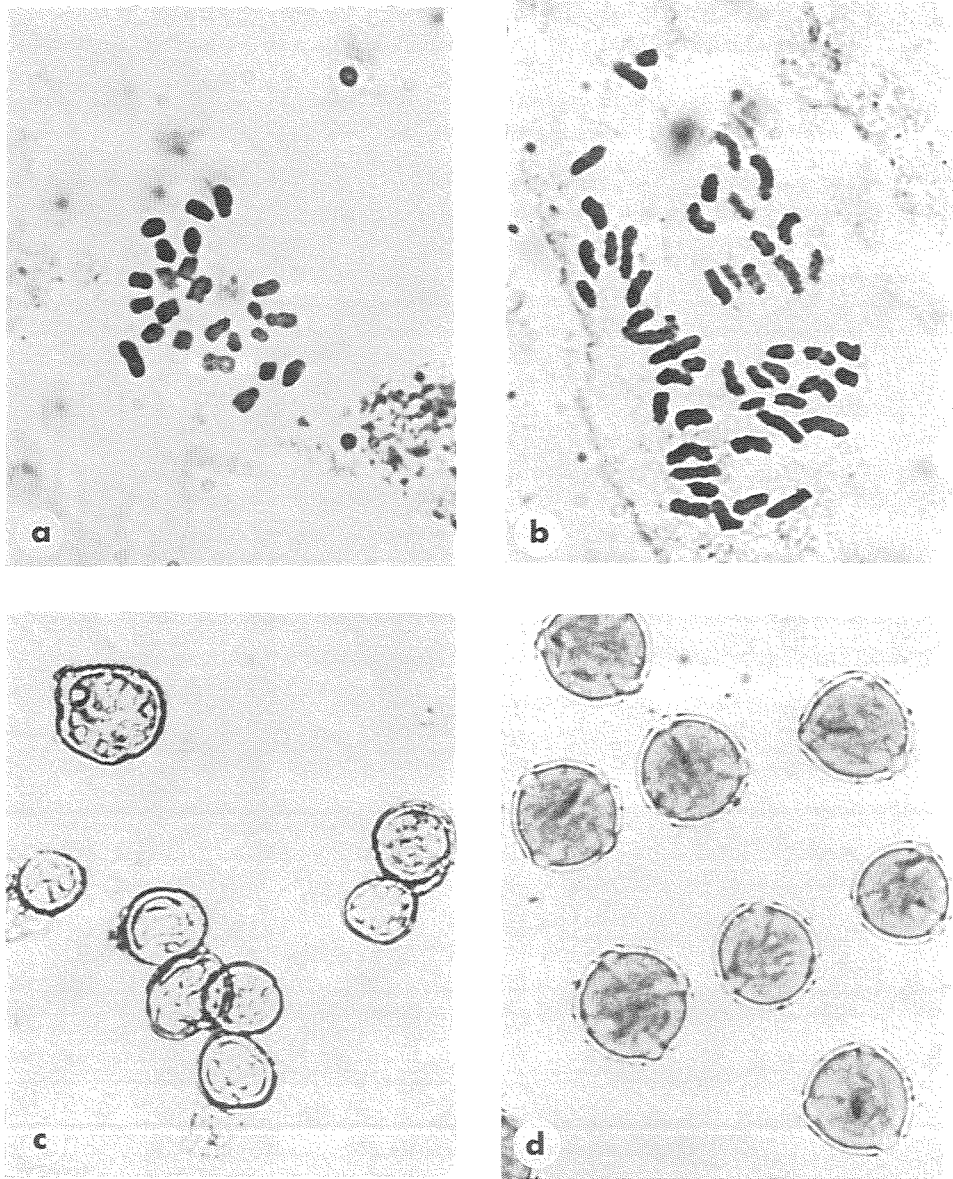


Fig. 2. Chromosome numbers in root tips and pollen fertility of haploid and parental diploid plant of *N. tabacum* (cv. Wisconsin 38).

- a. 24 somatic chromosomes. \times ca. 1600.
- b. 48 somatic chromosomes. \times ca. 1600.
- c. Pollen grains of haploid plant. \times ca. 480.
- d. Pollen grains of diploid plant. \times ca. 480.

Two to three weeks after the proliferation of the plantlets, they produced several leaves and also a well developed root system. A few plantlets which had chlorophyll deficiencies, however, were very slow in development and did not initiate roots even over two months of continued incubation.

Chromosome numbers of regenerated plants and their pollen and seed fertility

Chromosome number determinations made on preparations from root tips excised from regenerated adult plants showed the plants to be haploid with 24 somatic chromosomes (Fig. 2 a). Phenotypic evidence for the haploid condition was the overall smaller stature (about two-thirds the size of the parental plants) and the smaller flowers (Fig. 1 c). The haploid plants were completely sterile in pollen grains (Fig. 2 c) and did not set even a single seed, whereas, the parental plants had 97.5% pollen fertility in the case of *N. tabacum* cv. Wisconsin 38 (Fig. 2 d) and normal seed set.

Microscopic observations to determine the site of origin of embryoid formation

After six weeks of incubation of the cultured anthers, microscopic observations were carried out to determine if embryoid formation had initiated from the germ cells. These observations were carried out for the three cultivars of *N. tabacum*. It was observed that multicellular masses had developed from the pollen grains in all three cultivars. The most active formations occurred in the pollen grains from anthers of developmental stage 2. Indeed, some of the anthers cultures of this stage had multicellular masses forming from as high as 12% of the anthers. However, there was considerable variation in the formation of multicellular masses between anthers. Also, in one anther, various stages in the development of embryoids were observed from initial cell division of the pollen grains to the cotyledon stage (Fig. 3 a, b, c, d, e, f). The development of the embryoids was observed to be proceeding normally, and several stages of embryogenesis could be clearly defined, such as globular masses which establish polarity, followed by the "heart shape" stage and the "torpedo" stage. Subsequently, the embryos germinated and gave rise to complete plantlets with cotyledons. Also, anthers in developmental stage 1 of cv. Wisconsin 38 formed multicellular masses from pollen grains and developed into embryoids. The frequency of the latter, however, was lower than from anthers in developmental stage 2. On the other hand, anthers of cv. Hicks Broadleaf and Delhi 34 at developmental stage 1 did not form any multicellular masses and meiocytes in the quartet stage remained in this same stage even after six weeks of incubation.

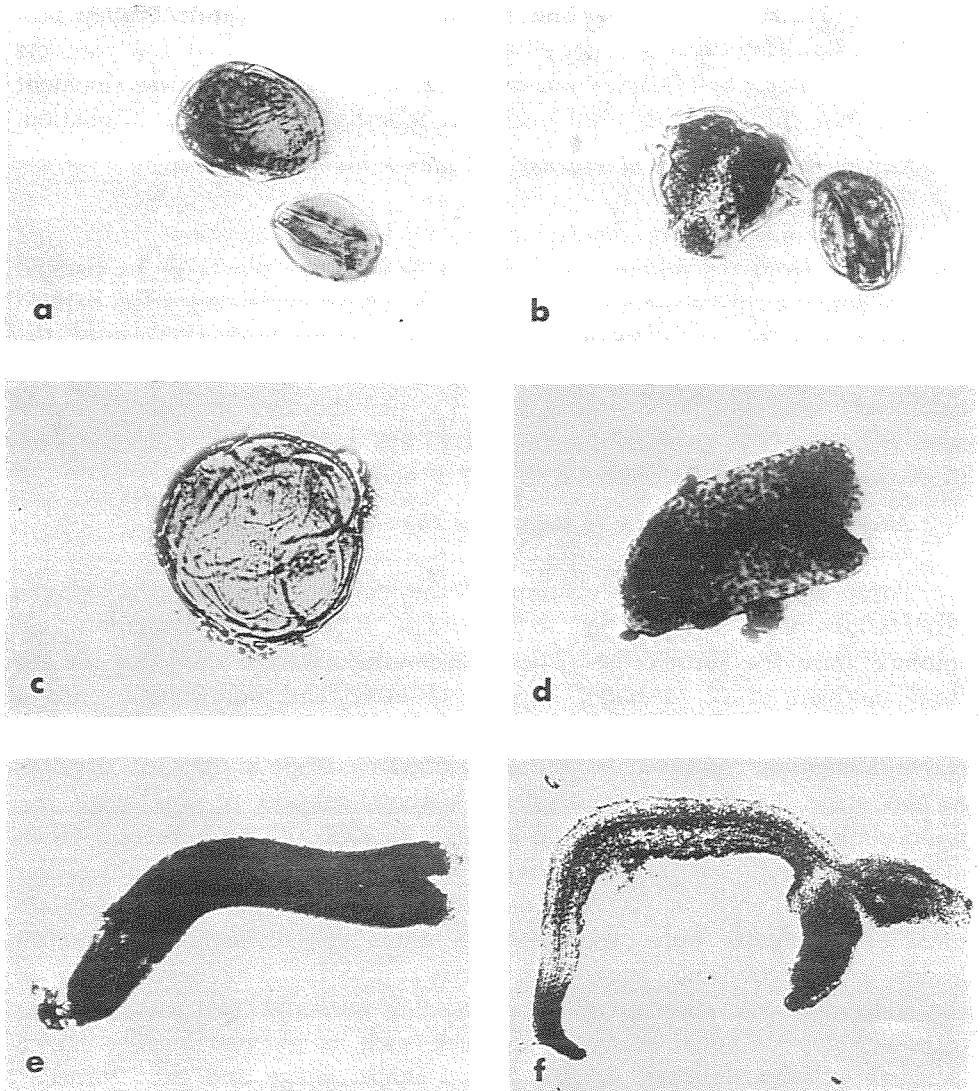


Fig. 3. Various stages in the formation of multicellular masses and embryoids in organ development from pollen grains in cultured anthers of *N. tabacum* (cv. Wisconsin 38).

- a. Initiation of cell division within a pollen grain. \times ca. 520.
- b. A cluster of several cells. \times ca. 520.
- c. A spherical mass with more than 10 cells. \times ca. 520.
- d. Heart shape stage. \times ca. 95.
- e. Torpedo stage. \times ca. 60.
- f. Cotyledonary stage. \times ca. 30.

Further observations on the anthers of developmental stage 1 of cv. Wisconsin 38 revealed that they contained pollen grains in a more advanced stage than the quartet stage, up to the early stage of individual pollen grains. It was considered that these pollen grains gave rise to the multicellular masses and embryoids, and not the cells of the quartet stage, or earlier stages.

A morphological change which occurred in the pollen grains was the accumulation of numerous starch grains. This was frequently observed in pollen grains within anthers of developmental stage 2. These pollen grains increased several times in size and the cell walls were prominently thicker than those of normal pollen grains.

In anthers of developmental stage 3, pollen tubes were observed arising from almost 60% of the pollen grains in all three varieties. Some of the pollen grains in anthers of developmental stage 2 continued their development and also initiated pollen tubes.

The relationship between developmental stages of the anthers and the initiation of embryoids

The medium used in this study was Medium B (containing 0.1 mg/l of IAA and 0.1 mg/l of kinetin) which was the most successful in the previous experiment for the induction of haploid plants of *N. tabacum*. Medium E (without supplemental growth regulators) was used as a control. Both media were supplemented with ³H-thymidine at a final concentration of 0.5 µc/ml. Only *N. tabacum* (cv. Wisconsin 38) was used in this study.

After 4 to 6 weeks of incubation, plantlets had initiated from the anthers. The results are summarized in Table 3. On Medium B, 33.3%

TABLE 3. Plantlet formation from anthers of *Nicotiana tabacum* (cv. Wisconsin 38)

Medium type*	Developmental stage of anthers**	Number of anthers used	Number of anthers forming plantlets	Percentage of anthers forming plantlets
B	1	59	10	16.9
	2	24	8	33.3
	3	22	0	0
E	1	18	0	0
	2	13	2	15.4
	3	8	0	0

* B, 0.1 mg/l IAA and 0.1 mg/l kinetin; E, no growth regulator.

** 1. Quartets and early individual uninucleate pollen grains.

2. More mature pollen grains but still uninucleate.

3. Mature pollen grains with both a vegetative and a generative nucleus.

of anthers of developmental stage 2 produced plantlets. On the Medium E, 15.4% of the anthers of this same stage also produced plantlets. These results indicated that the growth regulators, IAA and kinetin, apparently promoted the production of plantlets from anthers of developmental stage 2, containing semi-mature uninucleate pollen grains, but that they were not absolutely essential factors. On the other hand, IAA and kinetin appeared to be essential for the anthers of developmental stage 1 (containing quartets and immature individual uninucleate pollen grains), because 16.9% of anthers of this stage produced plantlets on Medium B, but none of them produced plantlets on Medium E.

Samples of anthers from each of the developmental stages were removed at 48 hours, 1 week, 2 weeks, 3 weeks, and 4 weeks after incubation for autoradiographic preparation and also for determining the degree of embryoid formation. From 4 to 6 slides were examined at 10 to 20 days after exposure. The observations are as follows.

(1) *Germ cells in the anthers of developmental stage 1 :*

During the first week of incubation, germ cells of this stage did not change morphologically and none of them were labelled with ^3H -thymidine (Fig. 4 a). By the second week of incubation, however, the individual uninucleate pollen grains increased in size to several times the size of normal pollen grains and appeared to be vacuolated (Fig. 4 b). At this time a single nucleus was clearly visible in the pollen grain. Some of these

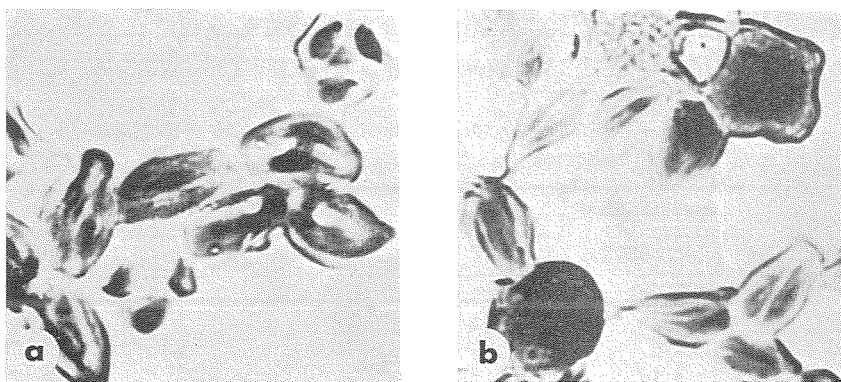
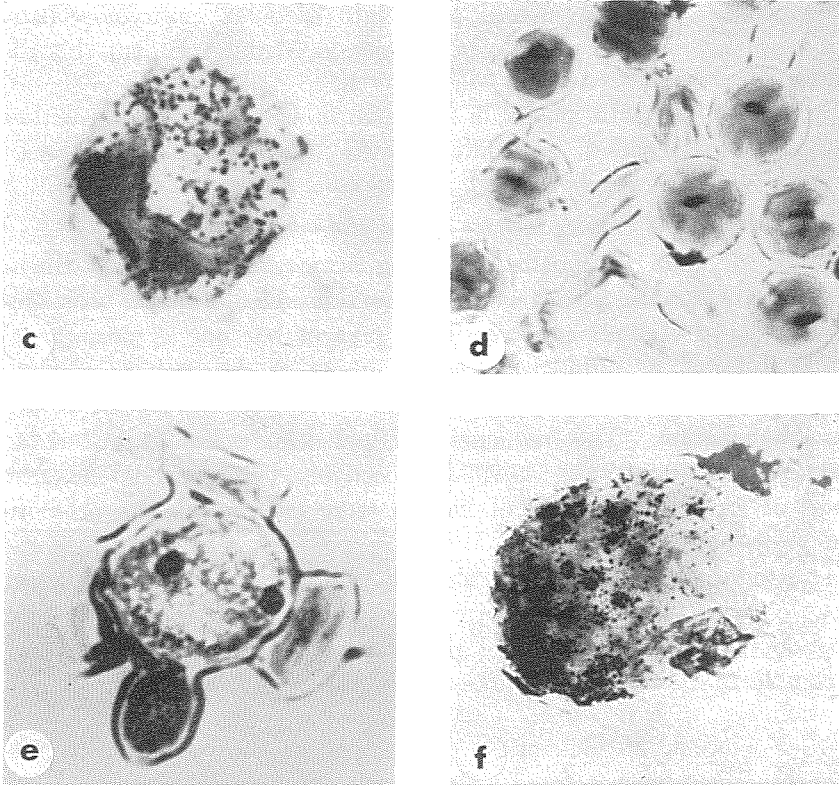


Fig. 4. Autoradiographs of cultured anthers of *N. tabacum* (cv. Wisconsin 38) after ^3H -thymidine application.

- a. No labelling of quartets and individual pollen grains in the anthers of developmental stage 1 after 48 hours of incubation. \times ca. 540.
- b. Increased size of pollen grains in anthers of developmental stage 1 after 2 weeks of incubation. \times ca. 540.



- c. Pollen grain with a labelled nucleus in an anther of developmental stage 1 after 2 weeks of incubation. \times ca. 610.
- d. Binucleate pollen grains from an anther of developmental stage 2 after 48 hours of incubation. \times ca. 540.
- e. Increased size of a pollen grain with an unlabelled binucleus in an anther of developmental stage 2 after 1 week of incubation. \times ca. 610.
- f. A globular mass with labelled nuclei in the anther of developmental stage 2 after 2 weeks of incubation. \times ca. 480.

pollen grains were labelled with ^3H -thymidine, indicating that DNA synthesis had taken place (Fig. 4 c). These labelled pollen grains were observed primarily on Medium B which contained the growth regulators, IAA and kinetin. On the other hand, morphological changes and DNA synthesis were not observed in germ cells of the quartet stage.

By the third week, the pollen grains, which had undergone DNA synthesis gave rise to globular masses with 5 to 12 cells in a frequency of 1.6% on Medium B, and 0.6% on Medium E, of the individual pollen grains

on the media. The cell masses grew into globular embryoids displaying polarity by the fourth week of incubation on Medium B, but this did not occur on Medium E.

Germ cells on both media in the quartet stage, however, neither matured into normal pollen, nor differentiated into multicellular masses, even after four weeks of incubation.

(2) *Germ cells in the anthers of developmental stage 2 :*

After 48 hours of incubation, using acetocarmine to stain the nuclei, the generative nucleus, with its characteristic spindle shape, was observed clearly, and the vegetative nucleus was present but not as prominent (Fig. 4 d). Therefore, the uninucleate pollen grains in the anthers of this stage had carried out the first pollen mitosis and had become binucleate after 48 hours of incubation. The frequencies of binucleate pollen grains on Medium B and E were 70.5% and 49.7%, respectively. This would suggest that 0.1 mg/l of IAA and 0.1 mg/l of kinetin were effective in the promotion of pollen grain mitosis in culture.

The autoradiographic study showed that pollen either uninucleate, or binucleate, were not labelled with ^3H -thymidine during the 48 hours of incubation. This would indicate that the pollen in anthers of this stage had completed their DNA synthesis for the first pollen mitosis prior to incubation.

After 1 week of incubation, the pollen grains increased in size and became prominently vacuolated. Both nuclei were clearly observed in these pollen grains (Fig. 4 e). At the same time, a few pollen grains which still remained in the uninucleate stage were observed and these were also vacuolated. These vacuolated binucleate and uninucleate pollen grains continued to undergo successive mitoses.

By the second week, globular masses of various numbers of cells were observed on both Media B and E and their frequencies were 4.4% and 2.6%, respectively. Their nuclei were densely labelled with ^3H -thymidine (Fig. 4 f). By the fourth week, different cell masses had developed into embryoids, cotyledons, and plantlets.

After three weeks of incubation, a few pollen grains had formed pollen tubes. This suggested that culturing anthers on these media promoted the pollen grains not only to differentiate into embryoids but also in their normal development.

(3) *Germ cells in the anthers of developmental stage 3 :*

By the end of the first week, pollen grains in this stage had germinated and pollen tubes into which the generative nucleus had migrated were

frequently observed. However, not a single observation was made to indicate any further morphological development such as the formation of embryoids. There was no difference in pollen germination on Medium B and E.

Discussion

The induction of haploid tobacco plants [*Nicotiana tabacum* (cv. Wisconsin 38, Delhi 34, and Hicks Broadleaf)] from cultured anthers was successful on the basic medium described by NITSCH and NITSCH (1969). The growth regulators, 0.1 mg/l of IAA alone, 0.1 mg/l of IAA and 0.1 mg/l of kinetin in combination, and 0.1 mg/l of IAA, 0.1 mg/l of kinetin and 1.0 mg/l of gibberellic acid in combination, were suitable for the induction of plantlets from the cultured anthers. High concentrations of growth regulators, 4.0 mg/l of IAA and 2.0 mg/l of kinetin, were inhibitory. NITSCH and NITSCH (1969) reported that they found the optimal concentration of IAA was 0.1 mg/l, and that 0.2 mg/l of kinetin was generally inhibitory. However, it was found that high concentrations of growth regulators which were supplied to basic media other than that of NITSCH and NITSCH (1969) was not inhibitory (NIIZEKI, 1972). When 4.0 mg/l of IAA and 2.0 mg/l of cytokinin (6-benzylaminopurine) were supplied in the basic medium described by MILLER (1963), the cultured anthers of *N. tabacum* (cv. Wisconsin 38) produced not only a number of plantlets but also calluses. NAKATA and TANAKA (1968) also succeeded in producing plantlets from anthers of *N. tabacum* (cv. Bright Yellow) on the modified RM-1964 medium of LINSMAIER and SKOOG (1965) containing a rather high concentration of growth regulators such as 2.0 mg/l of IAA and 4.0 mg/l of kinetin. Therefore, these results suggest that the concentration of exogenous growth regulators supplied to the medium is not the only decisive factor for the production of plantlets from cultured anthers of *N. tabacum*. The interaction between growth regulators and different types of basic media also appeared to be an important role for the production of plantlets.

NAKATA and TANAKA (1968) showed that the earliest developmental stage at which the anthers of *N. tabacum* could be cultured in order to ensure the induction of haploid plantlets was the quartet stage. In the present study, however, the earliest stage from which plantlets arose was the fully individual uninucleate pollen grain stage. Germ cells at earlier stages failed to differentiate into embryoids. The results of this study agree with those, also on cultured anthers of *Nicotiana* species, of NITSCH *et al.* (1968), NITSCH and NITSCH (1969), and SUNDERLAND and WICKS (1969 a, b).

More precise investigations carried out with the aid of autoradiography using ^3H -thymidine showed that differentiation from normal pollen to the embryoid stage could be initiated from individual pollen grains either at the early immature uninucleate stage (before DNA synthesis occurred in the nucleus) or at a later uninucleate stage (after the nucleus had undergone DNA synthesis). Differentiation was initially accompanied by an increase in pollen grain size and vacuolization. SUNDERLAND and WICKS (1969 a, b) also reported the occurrence of vacuolization of the pollen grains after the first microspore mitosis and before multicellular formation.

Although the growth regulators, 0.1 mg/l of IAA and 0.1 mg/l of kinetin when present in the medium are considered to have promoted the initial increase in pollen grain size, the subsequent multicellular condition and embryoid formation, they were not absolutely essential since individual pollen grains at the late uninucleate stage could give rise to complete plantlets on media lacking growth regulators. However, pollen grains at the early uninucleate stage could not form entire embryoids. These results would indicate that the exogenous growth regulators themselves do not function as a trigger of differentiation from the pollen but as a promotor of the multicellular condition and of embryoid development. In anther cultures of *Nicotiana*, therefore, some complexity can be assumed to exist in the triggering mechanism for differentiation which may be regulated by the alteration or the synergism of endogenous growth regulators, or by the exogenous supply of some unknown elements in the anther culture—the precise factors being totally unknown.

Summary

The anthers of *Nicotiana tabacum* (cv. Wisconsin 38, Delhi 34, Hicks Broadleaf) and *N. affinis* were cultured on media containing the basic medium of NITSCH and NITSCH (1969), plus the growth regulators, IAA, kinetin, and gibberellic acid, in various combinations. After 4 to 6 weeks of incubation, plantlets emerged from inside the anthers of *N. tabacum*, but not from *N. affinis*. Plantlets which developed good root-systems were then transplanted to pots and grown in a growth chamber. Chromosome number determinations on regenerated adult plants showed them to be haploid with 24 somatic chromosomes.

After six weeks of incubation, microscopic observations on the cultured anthers showed that the most active multicellular formations developed from pollen grains which were in the individual uninucleate stage. In one anther, different stages in the development of embryoids from the pollen grains

were observed from a few initial cell divisions up to the cotyledon stage. The development of the embryoids occurred as in normal embryogenesis. Globular masses formed in which polarity was established, and the "heart shape" and the "torpedo" stages followed. Germ cells in the quartet stage did not give rise to any multicellular masses or embryoids. Likewise, germ cells which were in the mature pollen grain stage germinated in a high percentage, but did not give rise to multicellular masses or embryoid formations.

The autoradiographic study with ^3H -thymidine on cultured anthers of *N. tabacum* revealed the critical stage at which the germ cells within the anthers have the totipotency for embryoid formation and also the effect that growth regulators (0.1 mg/l of IAA and 0.1 mg/l of kinetin) have on DNA synthesis and embryoid formation. Pollen differentiation into multicellular masses occurred both before and after DNA synthesis in the first mitosis of the early individual uninucleate pollen grains, but not in the quartet stage nor in the mature binucleate pollen grain stage. The initial morphological changes observed in differentiation were an increase in pollen grain size and vacuolization. In pollen grains in which the nucleus was in the post-DNA synthesis stage, differentiation occurred one week earlier than for those pollen grains in which the nucleus was in the pre-DNA synthesis stage. The exogenous growth regulators, IAA and kinetin, appeared to be responsible for multicellular development and subsequent embryoid formation, although they were not absolutely essential factors for their initiation.

Acknowledgments

The author wishes to extend his sincere appreciation to Dr. W. F. GRANT, Professor of Genetics, Macdonald Campus of McGill University, for the excellent guidance on this study. The author is also indebted to Dr. G. O. HENNEBERRY, of the Department of Agriculture Chemistry, Macdonald College, for advice and facilities for carrying out the autoradiographic part of this study. The author also wishes to express his gratitude to Dr. M. TAKAHASHI and Dr. F. KITA, Professors of Faculty of Agriculture, Hokkaido University, for encouragement throughout the course of this study.

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