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STUDIES ON THE MECHANISMS OF POLLEN EMBRYOGENESIS

III. Mitotic Responses of the Pollen to Varied Sucrose Concentrations and the Process of Embryoid Formation in Tobacco Anther Culture

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Abstract

Anthers of tobacco plants (*Nicotiana tabacum* L. cv. Bright Yellow, 2n=48) were cultured on agar media containing sucrose in varied concentrations to assess the effects of sucrose on the mitotic behaviors of pollen grains. Furthermore, a possible process of pollen embryogenesis was discussed on the basis of the data concerning mitotic behavior of embryogenic pollen grains and their occurrence frequency.

The most favorable sucrose concentration was 1/4 M not only for the induction of multinucleate and multicellular grains but for the formation of embryoids and plantlets. Moreover, sucrose was also effective in giving rise to the vegetative nuclear divisions in its high concentration.

On the basis of the present results, the process of pollen embryogenesis was proposed as shown in Fig. 5 of text. Namely, two routes, A and B, are firstly considered in this case. The A route initiates from the G+V type-grains, followed by repeated divisions of the vegetative nuclei only. When the number of nuclei reached 5-8, further three types become distinguished in the pollen grains. One of them is a type that the visible cell wall formation with degeneration of generative nuclei has been induced (A-1 route), and the others are types that the pollen grains containing one or two generative nuclei continue to divide their vegetative nuclei till reaching about 15 nuclei without forming visible cell walls (A-2 and A-3 routes). The B route intiates from the 2V type-grains and subsequently leads to repeated divisions of the vegetative nuclei.

Although every one of these 4 routes may be responsible for pollen embryogenesis, it seems probable to consider that the A route plays a more important role for pollen embryogenesis in tobacco anther culture.

Introduction

In order to produce haploids by means of anther culture in any crop plants, a thorough study has to be made on the mechanisms for changing the developmental pathway of pollen grains in anthers cultured towards embryoid formation. For this, as the first step, it will be necessary to analize in detail not only the factors affecting embryogenic mitosis of pollen grains but also the embryoid and plantlet formation from pollen grains.

In tobacco anther culture, considerably many analyses have already been tried by several workers on the developmental stages of pollen grains and the components of medium[1~4,6,8,9,13,15]. The most suitable stage of pollen grains for inoculation has been pointed out to be from mid-uninucleate to early binucleate[3,6,8,13,15]. With regard to the medium components, however, a critical view is not yet offered up to the present, though various results have so far been presented on the induction of green embryoids on saccharide-free medium[10,12], the plantlet formation on agar medium consisting of sucrose only[1], and the necessity of sucrose and Fe-EDTA for plantlet formation[8]. In previous reports,

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the present authors have found that sucrose was the most effective of all the constituents on the induction of embryogenic mitosis in cultured pollen grains, and the plantlet formation was induced on media with sucrose in concentrations varying from 1/256 to 1/4 M, but not induced not only on medium containing no sucrose but also on medium containing it in 1/2 M[2,4]. Moreover, the same authors have also ascertained that the most favorable sucrose concentration was 1/8 M for "anther response", which denotes the occurrence frequency of anthers from which plantlets emerged, and 1/16 M for "expected productivity", which denotes the expected number of plantlets emerging from one anther[4].

In this report, the sucrose effect is assessed on the mitotic behavior of pollen grains in tobacco anther culture and also the processes are discussed of embryoid and plantlet formation from embryogenic pollen grains on the basis of the data concerning their mitotic behavior and occurrence frequency.

Materials and Methods

Plant material

Seeds of *Nicotiana tabacum* L. cv. Bright Yellow (2n=48) were supplied from Akashi District Office, Japan Tobacco and Salt Public Co-operation.

Medium preparation

The culture media used were prepared on the Murashige & Skoog's formula[5], and grouped into three series by adding sucrose at the concentrations of 1/4 M, 1/16 M and 1/64 M as shown in Table 1. These media all were solidified with 0.8% agar and adjusted to pH 5.8 with 0.2 *N*·NaOH. Eight ml of the media were each poured into 20× 120 mm test-tubes, and after autoclaving (120°C/15 min.) these were settled on the slant at an angle of about 30°.

In vitro culture

Anthers in buds of 10-15 mm lengths were used in this study, for pollen grains in these anthers were recognized as being in the stages from late uninucleate to pollen mitosis in preliminary experiment. Excised buds were sterilized in 70% ethanol for 5 sec. and in 2 % sodium hypochlorite solution for 8 min. After rinsing, anthers were carefully picked out from the buds detaching anther fillaments and 5 ones per test-tube were inoculated on the media. The anthers were kept in a culture box at 30 ± 1°C with 13 hour-illumination of fluorescent light (2,000 Lux) per day.

Observations

For assessing the pollen development in cultured anthers, 10 anthers per treetment were collected, 10, 20 and 30 days after inoculation. They were fixed in a 3 : 1 mixture of ethanol and acetic acid. The fixed anthers were hydrolysed for 8 min. in *N*·HCl at 60°C and after staining with Feulgen reagent for 3 hours they were rinsed for about 30 min. with running water, and then squashed in a drop of 45% acetic acid on a slide glass. These slides were obsereved with light microscope and the occurrence frequency of pollen grains with such various mitotic features as shown in Table 1 was assessed by counting more than 1,500 grains per anther. In this assessment, only the pollen grains inside which cell walls were clearly visible were regarded as multicellular grains.

The cultured anthers were morphologically obsereved at 5 days interval for anther color, loculi bursted and plantlet formation.

Table 1. Effects of sucrose

Days after inoculation	Sucrose concentrations (M)	2N		
		2G	2V	G+V
10	1/4	0.20	1.47	82.76
	1/16	0.36	1.05	69.75
	1/64	1.17	1.22	67.20
20	1/4	0.28	1.04	22.65
	1/16	0.20	0.74	9.85
	1/64	0.73	0.65	21.56
30	1/4	0.03	0.60	0.84
	1/16	0.10	0.19	0.54
	1/64	0.53	0.56	13.03

Results

I. Embryogenic Response of the Pollen to Sucrose Concentrations

The results of microscopic observations on the pollen development in the cultured anthers are given in Table 1 and Fig. 1. These results are described in the course of dates at which observations were made; that is as follows:

After 10 days of culture

Binucleate grains: At this stage, as seen from Table 1, binucleate pollen grains containing one generative and one vegetative nuclei were of most frequent occurrence, their frequency in 1/4 M sucrose medium being 82.8%. Such a instance was referred to as G+V type in this paper. According to the detailed observations, the G+V type-grains were divided into two groups by the shape of generative nucleus. One of them was composed of the grains containing two nuclei with such a globular shape as in binucleate grains *in vivo*, and the other comprised the grains in which generative nuclei changed into flat shaped ones. Pollen grains in the latter group tended to occur frequently in the 1/64 M sucrose medium. In regard to the grains containing two equal sized vegetative nuclei, referred to as 2V type, differences were not found both in their morphological features and in their occurrence frequency among the 1/4, 1/16 and 1/64 M media.

Multinucleate grains: At this stage, the multinucleate grains containing more than 5 nuclei were not found at all. Most of the multinucleate grains were of G+2V type consisting of one generative and two vegetative nuclei. Such grains were more frequently observed in the 1/4 and 1/16 M sucrose media than in the 1/64 M. Grains containing only generative nuclei (nG type) or ones containing 2-5 generative and one vegetative nuclei (nG+V type) had a tendency to increase the frequency of their occurrence with decreasing sucrose concentration in medium. On the contrary, the grains containing 3-6 vegetative nuclei only, referred to as nV type, were more frequently observed in media with sucrose in higher concentrations, though their frequency was actually low, showing 0.39, 0.21 and 0.17% in 1/4 M, 1/16 M and 1/64 M media, respectively.

After 20 days of culture

Binucleate grains: The frequency of binucleate grains, especially of the G+V type, decreased remarkably from the 10th day after inoculation onwards. On the 1/4 M sucrose medium, mitosis of vegetative nuclei was often observed in the G+V type-grains, but not in the 2G and 2V types.

Multinucleate grains: At this stage, mul-

concentrations on the mitotic behaviors of pollen grains in tobacco anther culture.

	Frequency (%) of pollen grains with											
2C	3–6N			3–6C		7N≤	7C≤	GE	HE	TE	S	D
	nG+V	nV	G+nV	G+nC	nC							
—	0.45	0.39	3.30	—	—	—	—	—	—	—	0.52	10.98
—	0.67	0.21	4.78	—	—	—	—	—	—	—	0.42	22.77
—	1.92	0.17	1.18	—	—	—	—	—	—	—	0.02	27.03
—	0.97	0.93	10.00	—	—	0.72	0.01	—	—	—	1.87	61.51
—	0.39	0.54	3.68	0.01	0.01	0.31	0.11	—	—	—	0.83	83.37
—	1.74	0.35	2.18	—	0.02	0.15	0.07	—	—	—	0.02	72.54
0.48	0.15	0.49	1.82	0.37	1.76	0.90	2.06	0.29	0.02	0.01	3.08	87.02
0.30	0.25	0.21	1.34	0.50	1.46	0.36	1.27	0.04	—	—	0.85	92.46
0.02	0.04	0.54	2.63	0.01	0.26	0.15	0.26	—	—	—	0.07	80.89

N: Nucleus; C: Cell; G: Generative nucleus; V: Vegetative nucleus; GE: Globular embryoid; HE: Heart-shaped embryoid; TE: Torpedo-shaped embryoid; S: Starch-filled pollen grain; D: Degenerated pollen grain; n: the numbers of nuclei or cells ranging from 2 to 6.

tinucleate grains occurred extensively and the maximum number of nuclei in such grains was G+18V, G+16V and G+13V in 1/4 M, 1/16 M and 1/64 M sucrose media, respectively. The occurrence frequency of grains containing 3-6 nuclei was highest in the 1/4 M sucrose medium, showing 11.9%. From the detailed observations of nuclear components, it was found that vigorous multinucleate grains did almost always contain one or two generative nuclei in addition to vegetative nuclei (Fig. 2-d, e and f). Like multinucleate grains with only generative nuclei, grains containing only vegetative nuclei were of very rare occurrence and in this case there were not encountered the grains containing only vegetative nuclei more than 10. In these multinucleate grains, those containing generative nuclei, the sizes of which are 2-4 times larger than those of normal haploid nuclei, were rarely observed though their actual frequency of occurrence was not assessed (Fig. 2-c).

Multicellular grains: A few multicellular grains were encountered at this stage. These grains were commonly composed of cells with vegetative nuclei though with such a few exceptions as shown in Fig. 2-f.

After 30 days of culture

Binucleate and bicellular grains: Binucleate grains occurred in markedly decreased frequency at this stage. These grains appeared to degenerate gradually with the lapse of time. Bicellular grains were observed firstly after 30 days of culture, but their occurrence was low in frequency (Fig. 2-g). These grains also appeared to be degenerating ones.

Multinucleate and multicellular grains: Multinucleate grains comprising 3-6 nuclei were in general found in slightly decreased frequency. Instead of these grains, multicellular grains increased rapidly in the 1/4 M and 1/16 M sucrose media, their frequencies being 2.13 and 1.96%, respectively. By observing carefully, two types of multicellular grains could be distinguished, namely, one type comprising grains with vegetative cells only and the other consisting of grains both vegetative cells and generative nuclei. Some of grains belonging to the former type did burst their exine at the 10 or so celled stage (Fig. 2-i). Grains of the latter type kept themselves in globular shape and were fully

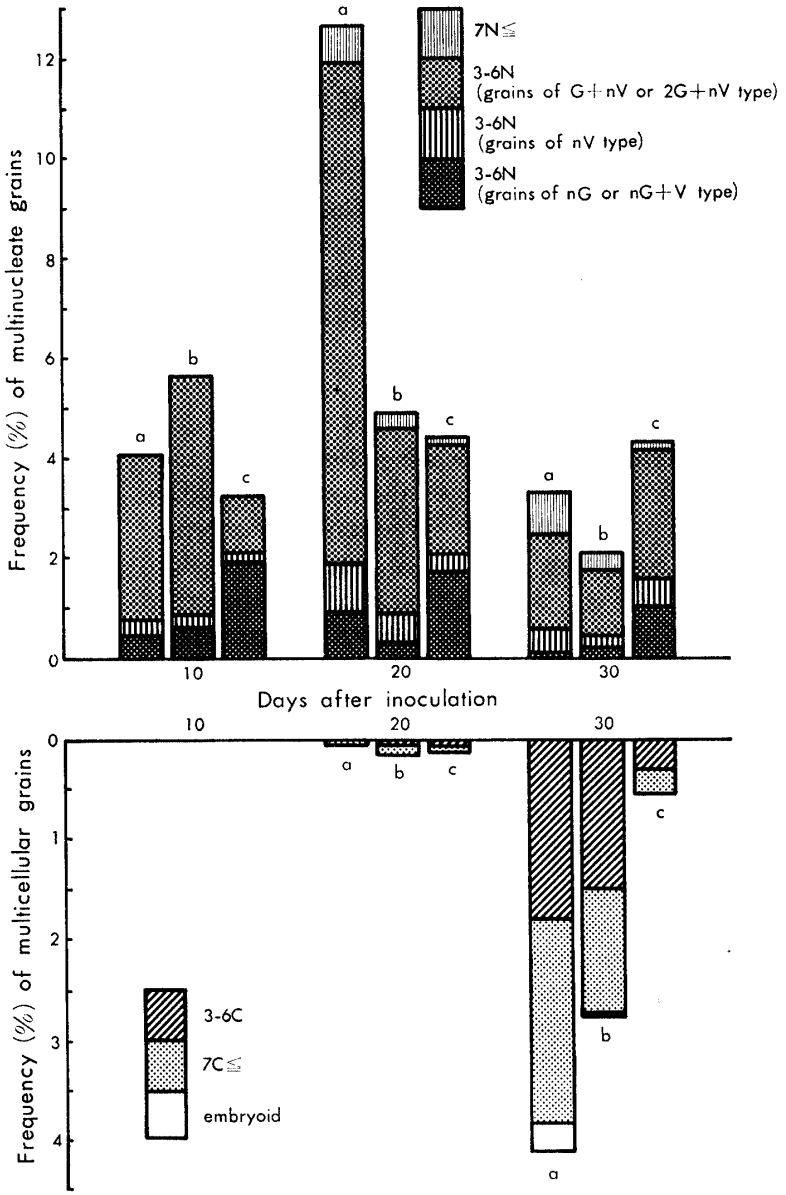

Fig. 1. Frequency of dividing pollen grains as affected by three sucrose concentrations in tobacco anther culture. (For the explanation of the mark, see Table 1.)
a: 1/4 M sucrose concentration;
b: 1/16 M sucrose concentration;
c: 1/64 M sucrose concentration.

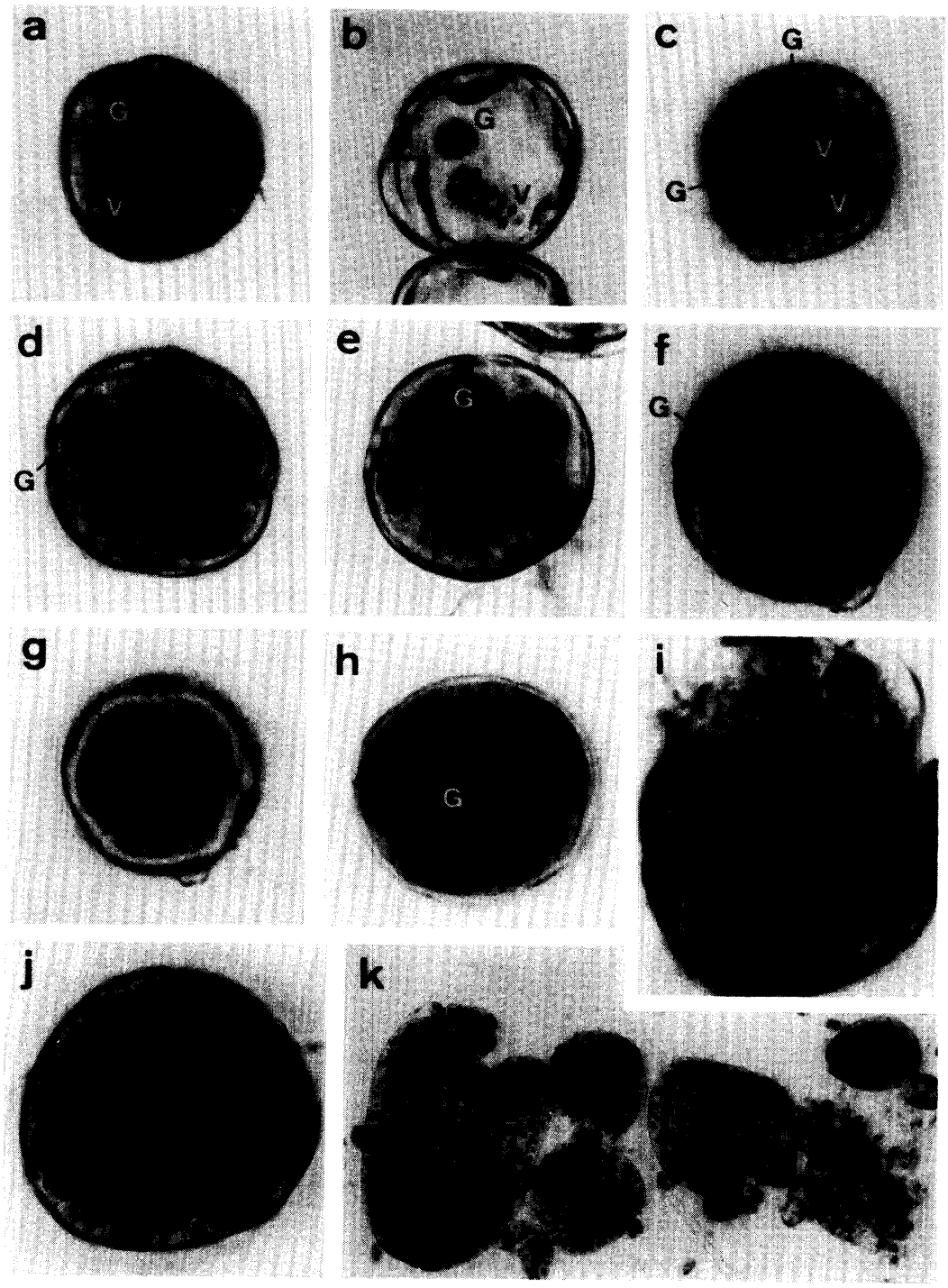

Fig. 2. Pollen embryogenesis in tobacco anther culture. (a–i : *ca.* × 1,000 ; j : *ca.* × 300 ; k : *ca.* × 80. a–f : after 20 days of culture ; g–k : after 30 days of culture)
The letters G and V indicate the generative and vegetative nuclei, respectively.
a and b : Binucleate grains with G + V, vegetative nucleus being under mitosis ; c : Tetranucleate grain with 2G + 2V. The size of the generative nuclei are 2–4 times larger than that of normal haploid nuclei. d : Multinucleate grain with G + 5V. Two of the vegetative nuclei are present in piles ; e : Multinucleate grain with G + 15V. One of the vegetative nuclei is under mitosis (indicated by arrow) ; f : Multicellular grain with G + 9C. Generative nucleus is adhering to the inside of the intine ; g : Bicellular grain with equal sized nuclei ; h : Multinucleate grain with G + 21V ; i : Multicellular grain, getting out after bursting the exine ; j : Globular embryoid ; k : Various embryoids occurring on the medium containing sucrose in 1/4M.

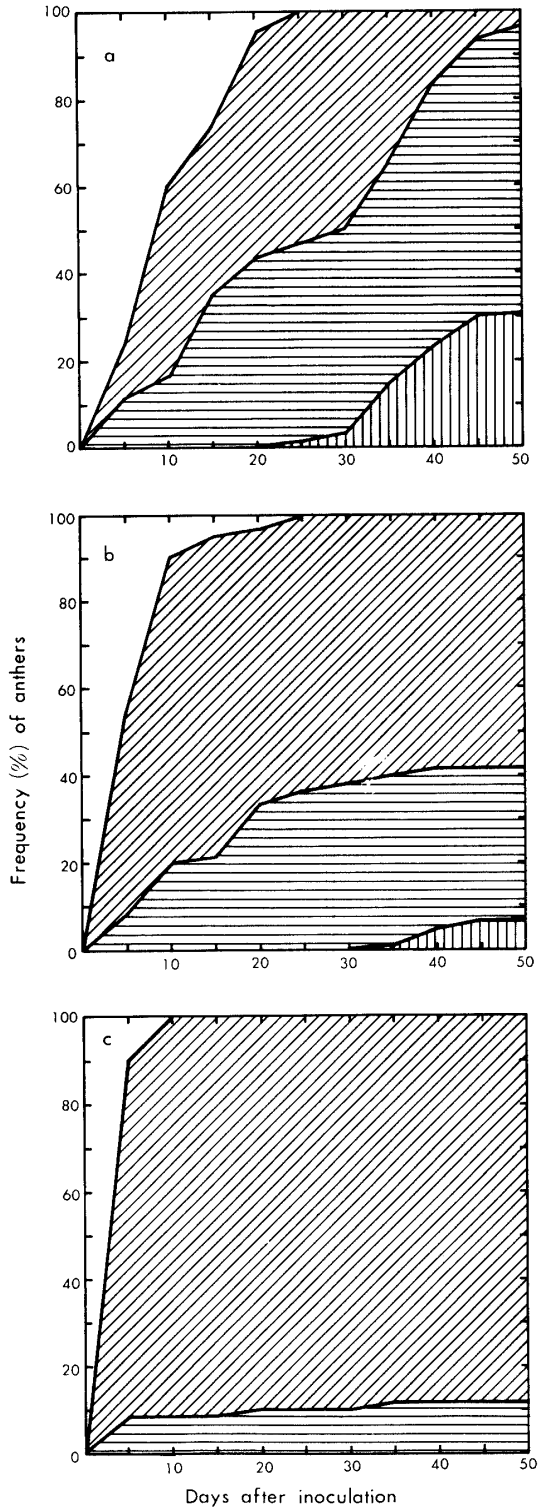

Fig. 3. Morphological changes of tobacco anthers as affected by varied sucrose concentrations in media.
a: 1/4 M sucrose concentration;
b: 1/16 M sucrose concentration;
c: 1/64 M sucrose concentration.
Green-yellowish green anthers.
Light brown or dark green – dark brown anthers.
Anthers bursting open their loculi.
Anthers from which plantlets occurred.

filled with compact cells. In these instances, there were often found generative nuclei changing their globular shape to flat and adhering to the inside of the intine, these nuclei having the same feature as shown in Fig. 2-f.

Globular, heart- and torpedo-shaped embryoids: As shown in Table 1, embryoids at the globular stage were found both in the 1/4 M and 1/16 M sucrose media (Fig. 2-j). Moreover, heart- and torpedo-shaped embryoids were found only in the 1/4 M sucrose medium (Fig. 2-k). In the 1/64 M sucrose medium, however, no embryoids could be observed.

II. Morphological Changes of Anthers and Formation of Plantlet

In Fig. 1 are shown the morphological changes of cultured anthers and the process of plantlet formation from the anthers. Anthers cultured on media containing sucrose in low concentration were browned at an early time of culture, for example in the case of 1/64 M concentration, 90% of the

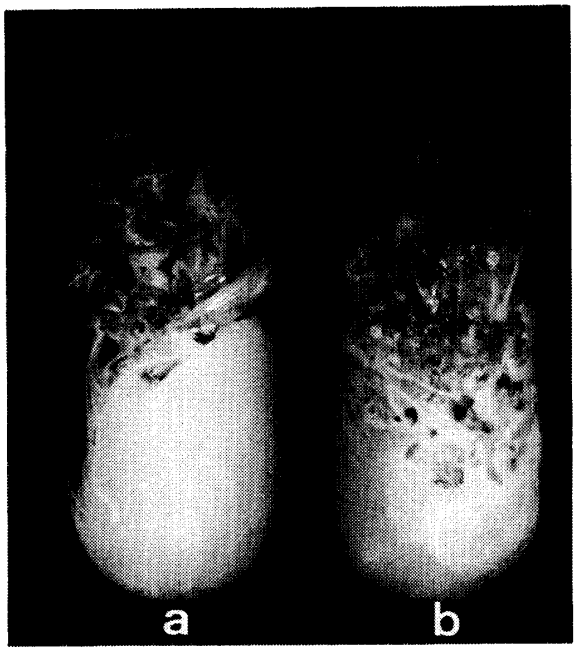

Fig. 4. Plantlet formation from tobacco anther cultures as affected by sucrose concentrations after 70 days of culture.
a: 1/4 M sucrose concentration;
b: 1/16 M sucrose concentration.
Note the morphological differences between both.

anthers changed their color to brown after 15 days of culture. On the other hand, anthers cultured on the 1/4 M sucrose medium didn't change their color, and 26.6% of the anthers showed green or yellowish-green color till 15 days after inoculation. The frequency of anthers bursting open their loculi was apparently high in the 1/4 M sucrose concentration (45 %), and low in the 1/64 M (11.7 %).

Plantlet formation was found on the media containing sucrose in 1/4 M and 1/16 M concentrations, respectively, with the frequency of 30.9 and 6.7%, but not in the 1/64 M sucrose medium. As can be seen in Fig. 4, plantlets formed on the 1/4 M sucrose medium not only had thick, roundish and green leaves but also put forth vigorously roots. On the other hand, the plantlets in the case of 1/16 M sucrose concentration had thin, slender and light colored leaves, and formed poorly roots.

Discussion

I. Effects of Varied Sucrose Concentrations on the Pollen Embryogenesis

In anther culture, sucrose has generally been used in concentrations of 2-3%. It has been found in previous work[2] that the occurrence frequency of multinucleate pollen grains decreases remarkably on medium containing no sucrose. Moreover, one of the present author, MASTUBAYASHI and his coworker[1] have suggested that 2-5% sucrose concentrations are effective in giving rise to the formation of multinucleate pollen grains. With regard to the effect of sucrose concentration on plantlet formation, 2% or 3.4 -6.8% have been recommended by some workers for tobacco[8,12]. The present authors have also assessed how much concentration of sucrose is effective for plantlet formation in previous report[4] and found that 1/8 M (*ca.* 4.3%) and 1/16 M (*ca.* 2.1%) concentrations were suitable for "anther response" and "expected productivity", respectively. In the present study, the most favorable sucrose concentration for induction of multinucleate grains and multicellular grains was ascertained to be 1/4 M (*ca.* 8.5 %), a high plantlet formation being found in this concentration. The reason why a difference occurred between the previous and the present results may be attributed to the difference of mineral composition in the media used, for MURASHIGE & SKOOG's formula[5] was employed here instead of TANAKA's formula[15]. If so, it is considered that the concentration ratio of sucrose to mineral salts may be affect in any way pollen embryogenesis. That is, in the case of a high level of mineral salts, a high sucrose concentration will be needed for pollen embryogenesis, and the converse will also be true.

As has been already seen, in a higher sucrose concentration the G+nV, 2G+nV and nV type-grains occurred more frequently than in lower sucrose concentrations, but nG and nG+V type-grains, on the contrary, occurred more frequently in media of lower sucrose concentrations. These facts suggest that sucrose is effective in inducing divisions of the vegetative nuclei in its high concentration. The vegetative and generative nuclei often changed their shapes from globular to slender or flat, especially in lower concentrations of sucrose, and the pollen grains degenerated from an early day of culture in such sucrose concentrations. This fact also suggest that sucrose has an effect in keeping the nuclei vigor.

II. Process of Pollen Embryogenesis

In regard to the embryogenic process of pollen grains, several routes have been described by earlier workers[3,7,11,14], and it has been pointed out that they were common in the point that the embyogenic pollen grains are always composed of cells with vegetative nuclei. SUNDERLAND[14] has reviewed that *Datura* and *Nicotiana* take different routes to embryogenesis, that is, the former is a type that the embryoid formation occurs from the 2V type-grains and the latter is a type that almost all embryoids develop from the normally quiescent vegetative cells in the G+V type-grains and generative nuciei degenerate at an early time of culture.

On the contrary, NITSCH[7] has proposed that 2V type-pollen grains participate in embryogenesis. RASHID and STREET[11] have

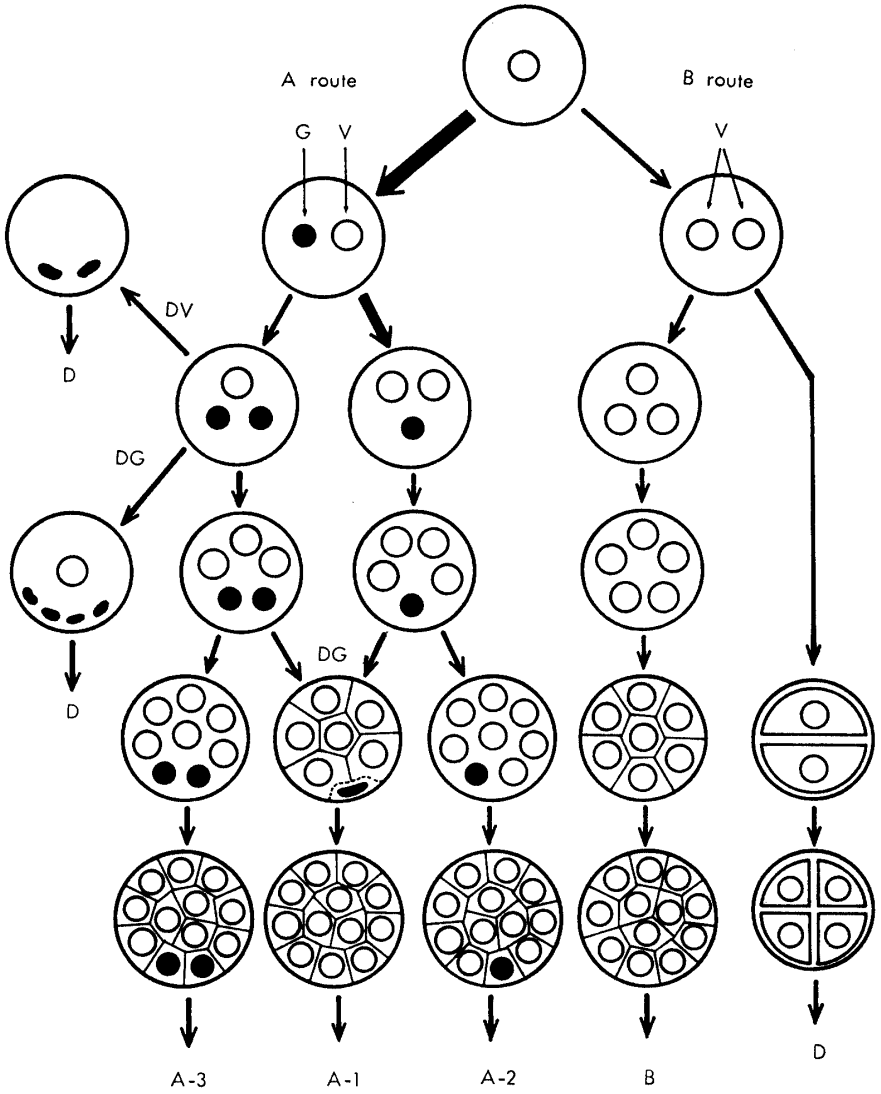

Fig. 5. Diagram illustrating the embryogenic process of pollen grains in tobacco anther culture.
G: Generative nucleus;
V: Vegetative nucleus;
DG: Degeneration of generative nucleus;
DV: Degeneration of vegetative nucleus;
D: Degeneration of pollen grains.

also reported such a route in *N. tabacum* and *N. sylvestris*.

On the basis of the results obtained in this study, the present authors will propose for tobacco such processes of pollen embryogenesis as illustrated in Fig. 5. First, two routes, A and B, are set up as has already been suggested by SUNDERLAND[14], and the A route is further differentiated into three routes, A-1, A-2, and A-3. The A route initiates from the G+V type-grains where successive divisions take place in the vegetative nuclei. When the number of nuclei reached 5-8, three types of grains emerge. First of them is a type in which the visible cell wall formation occurs within the pollen grains, being attended by the degeneration of generative nuclei (A-1 route); the second is a type in which one generative nucleus is held in each of the pollen grains and the vegetative nuclei only divide repeatedly till reaching about 15 nuclei, without forming visible cell walls (A-2 route); and the third is a type in which the pollen grains take the same course to embryogenesis as the above second type, differing only in that they

possess two generative nuclei in each of them (A-3 route).

The B route, on the other hand, initiates from the 2V type-grains and subsequently leads to repeated divisions of the vegetative nuclei. These grains have a tendency to form cell walls in an early stage of nuclear division such as 2-5 nucleate.

The problem lies in whether embryogenesis proceeds through the A or B route. The present authors tend to interpret the results obtained here from such a point of view as described above. At first, it should be pointed out that the frequency of G+V type-grains after 10 and 20 days of culture, as given in Table 1, is remarkably high as compared with that of 2V and nV type-grains, especially this being marked in the 1/4 M sucrose concentration where the plantlet formation was frequent. Moreover, the frequencies of multinucleate grains, multicellular grains and embryoids were 3.28% in a total on the 1/4 M sucrose medium after 30 days of culture, whereas the frequency of 2V and nV type-grains was only 1.97% on the same medium after 20 days of culture. Compared with the former, the latter is too low. This fact indicates that the nV type-grains, even though they develop into multicellular grains and embryoids, cannot cover all of the embryogenic pollen grains.

The grains which form visible cell walls at a considerably late stage of nuclear divisions seem to be favorable for embryogenesis, because such grains have a tendency to form compact cell groupes whose cell nuclei are highly stainable with FEULGEN reagent. It is impossible, however, to set up the only one route for pollen embryogenesis, because, as pointed out by earlier workers[11], in this study also there was not certified the existance of generative nuclei in highly dividing grains reaching more than 30 cells. Every one of the 4 routes, therefore, may be responsible for pollen embryogenesis. However, considering the occurrence frequency of embryogenic pollen grains, it will be probable to conclude that the A route plays a more important role for pollen embryogenesis in anther culture at least so far as the present material concerns.

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花粉粒の胚形成機構に関する研究

第3報　異なる蔗糖濃度に対するタバコ葯培養花粉粒の分裂反応性及び胚状体形成過程

三十尾　修　司・吉　田　健　一・松　林　元　一

要　　約

培養葯内花粉粒の核分裂に対する糖の効果を明らかにするために，蔗糖を 1/4M，1/16M 及び 1/64M 含む培地でタバコ（*Nicotiana tabacum* L. cv. Bright Yellow, 2n＝48）の葯を培養し，生殖核と栄養核の数によって類別された各型の花粉粒の出現頻度を調査した。さらにそれらの出現頻度に基づいて，従来から異論のあった花粉粒起源胚状体の形成過程について若干の考察を試みた。

多核化花粉粒，多細胞化花粉粒及び胚状体のいずれの形成に対しても 1/4M 糖濃度培地が最も良好な結果を示した。加えて，この培地では他の濃度培地に比べて，培養初期における花粉粒の枯死率が低下し，栄養核の多核化及び多細胞化を示す花粉粒の出現頻度が高まる傾向がみられた。

一方，上記各型の分裂花粉粒の出現頻度に基づいて，本文中の Fig. 5 に示すような花粉粒の胚状体形成過程を推定した。この過程は生殖核と栄養核をそれぞれ1個有する花粉粒から開始されるA経路と，栄養核2個を有する花粉粒から開始されるB経路に分けられる。B経路では栄養核が多核化と多細胞化をくり返して胚状体の形成を導くが，A経路にはさらに次の3つの場合がある。すなわち，栄養核の反復分裂によって多核化が5～8核に達したときに生殖核の退化と細胞膜の形成がみられるA－1経路，1個の生殖核を保ちながら栄養核がさらに多核化を続けて14～17核前後ではっきりとした細胞膜を形成するA－2経路，及び2個の生殖核を保ちながらA－2経路と同様の発育経過をたどるA－3経路に分けられる。

これらの4経路は，いずれも胚状体形成の可能性をもつものと考えられるが，生殖核を保ちながら栄養核の分裂を続ける花粉粒の出現頻度が高いこと等から，タバコの葯培養では花粉粒起源の胚状体の形成が主としてこの経路によって起こると考えるのが妥当であろう。