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Studies on the Mechanisms of Pollen Embryogenesis VIII. The Role of Na_2 EDTA in Pollen-plant Formation of *Nicotiana tabacum* L.

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The effects of mineral salts on plantlet formation were investigated in tobacco anther culture paying special attention to Na_2 EDTA, a component of FeEDTA.

Removal of mineral salts other than FeEDTA from the basal medium had little influence on plantlet formation. While, removal of FeEDTA arrested plantlet formation severely, but did not affect the induction of embryogenic pollen grains. Moreover, very few plantlets were obtained from anthers transferred to FeEDTA-free medium after 3 weeks of culture. From these results, it is concluded that FeEDTA plays the most important role in the development from embryoid to green plantlet.

Plantlet formation was almost completely arrested on Na_2EDTA-free medium. In contrast, plantlet formation on ferrous sulphate-free (Na_2EDTA) medium was comparable to that on basal medium. Also, the highest frequency of plantlet formation was obtained at the same concentration (2×10^{-4}M) for both FeEDTA and Na_2 EDTA. These results suggest that the effects of FeEDTA on the plantlet formation in tobacco anther culture refer to the function of Na_2EDTA itself.

Introduction

Pollen-derived plants can be obtained readily now in several crops by employing anther culture. However, no crop has been known to produce haploid embryoids and plantlets as abundantly and quickly as tobacco in anther culture. In order to apply this method to any other recalcitrant crops, detailed analyses of the factors affecting embryogenic mitosis and plantlet formation in tobacco anther culture are required.

Up to now, various factors have been shown to affect androgenesis in tobacco. They are genotype and the physiological state of the material plants, anther wall, physical conditions of anther culture, and culture media[1-4]. The latter factors include saccharides[5-9], activated charcoal[10-13], plant growth regulators[11,14-16], amino acids[14,17], and mineral salts[14,18-22]. Mineral salts, as well as sucrose, are considered to be essential components in the culture medium for obtaining sufficient plantlets[7,14]. Several researchers have reported the necessity of iron ion or FeEDTA for the production of pollen plantlets in tobacco anther culture[14,21]. From these reports, FeEDTA is considered to be one of the most important complements among mineral salts in tobacco anther culture.

In the present paper, the effect of each mineral salt on plantlet formation is investigated in tobacco anther culture paying special attention to Na_2EDTA which is a component of FeEDTA stock solution.

Materials and Methods

Anther donor plants of *Nicotiana tabacum* cv. Bright Yellow ($2n=48$) were cultivated in a greenhouse. Anthers from buds with petal length of 13-18 mm were used for culture, as these anthers have been recognized as containing pollen grains at the late uninucleate and/or early binucleate stages in a previous study[22].

The basal medium used throughout this study was prepared according to the modified Tanaka' s formula (1973)[23] as shown in **Table 1**. This formula was employed because of its simple mineral composition and sufficient ability to form plantlets in tobacco anther culture[22]. Composition and concentration of mineral salts in the basal medium were variously changed in the present study. All the media used in this study were routinely solidified with 0. 8% agar and adjusted pH to 5. 8 with 0. 5 N NaOH or 0. 5 N HCl. Twenty and 30 m*l* of the media were each poured into 50 and 100 m*l* Erlenmeyer flasks, respectively, and these were autoclaved for 15 min. at 120°C.

Fifteen or 20 anthers per flask were aseptically inoculated on the media. About 100 anthers per treatment with a total of 3, 120 anthers were cultured in this study. Cultures were maintained in a 13 hr light/11 hr dark photoperiod, at a light intensity of *ca.* 2, 000 lux, and at 30±1°C.

For the cytological study of pollen grains in cultured anthers, 10 anthers per treatment were fixed in a 3 : 1 mixture of ethanol and acetic acid 25 days after inoculation. After staining with Feulgen reagent according to the methods of our previous study[9], occurrence frequencies of various types of pollen grains as shown in **Table 2**. were assessed by counting more than 1, 500 grains per anther using a light microscope.

Observation of plantlet formation from anther cultures was carried out at one week intervals, and the average number of plantlets per anther was assessed after 9 weeks of culture.

Results

Removal of each mineral salt from the basal medium had a drastic effect on the plantlet formation (**Fig. 1**). When FeEDTA was removed from the basal medium, only one etiolated

Table 1. Composition of the basal medium used in this study.

Constituent	mg/*l*
Mineral salts (M)	
KNO_3	200
$Ca(NO_3)_2 \cdot 4H_2O$	600
$MgSO_4 \cdot 7H_2O$	100
KH_2PO_4	100
$MnSO_4 \cdot 4H_2O$	10
FeEDTA*[1]	
$FeSO_4 \cdot 7H_2O$	27. 85
Na_2EDTA	37. 35
Sucrose (S)	25, 000
Agar (A)	8, 000

*[1] FeEDTA stock solution was prepared by mixing 2×10^{-2}M concentration of Na_2 EDTA and ferrous sulfate solutions at 50°C.
Na_2EDTA: ethylenediaminetetraacetic acid disodium salt dihydrate.

plantlet was obtained from 96 anthers cultured on this medium, and plantlet formation was inhibited in all the rest of anthers. Anther response (frequency of anthers producing plantlets) on both the calcium nitrate-free and the potassium nitrate-free media were slightly lower than the basal medium. Moreover, anther productivity (average number of plantlets per anther) was considerably lower on the calcium nitrate-free medium (4.3) than on the basal medium (12.7). On the other hand, there was no disadvantageous influence on the plantlet formation in the cases of manganese sulfate-free, potasium sulfate monobasic-free, and magnesium sulfate-free media.

The concentrations of mineral salts were variously changed in the 2nd experiment, though FeEDTA was added at 10^{-4} M to all the media examined (**Fig. 2**). Plantlets were obtained from all the media even in the case of containing no mineral salts other than FeEDTA. Anther response on the mineral-free medium was lower than the basal medium, but 45.6% of the anthers formed plantlets. On the contrary, anther productivity on mineral-free medium showed very poor value (1.8). With the increase in the concentration of mineral salts in media up to 2×the control level, anther productivity rose constantly. Plantlets formed on the media with low mineral concentration had a tendency to develop only slender stems and small leaves.

For clarifying the effect of FeEDTA on the initial embryogenic division of pollen grains in anther culture, microscopic observation was made of the anthers cultured on media with and without FeEDTA, after 25 days of culture (**Table 2**). Frequencies of embryogenic pollen grains were apparently higher on the media containing both sucrose and mineral salts (Treats. 1 and 2) than on the media containing no mineral salts (Treats. 3 and 4) or containing no sucrose (Treats. 5 and 6). None of embryogenic pollen grains with 4 or more nuclei occurred on the sucrose-free media. Moreover, from comparisons of Treat. 1 with Treat. 2, and Treat. 3 with Treat. 4, it was clear that the frequency of embryogenic pollen grains did not increase by the addition of FeEDTA to the

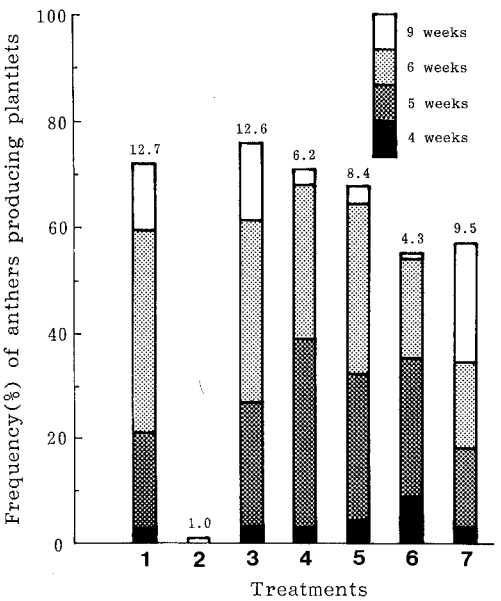

Fig. 1 Differential effects of mineral salts in the basal medium on plantlet formation in tobacco anther culture.
Treat. 1: Basal medium (control); Treat. 2: FeEDTA-free medium; Treat. 3: Manganese sulfate-free medium; Treat. 4: Potassium phosphate monobasic-free medium; Treat 5: Magnesium sulfate-free medium; Treat 6: Calcium nitrate-free medium; Treat. 7: Potassium nitrate-free medium. Values on the top of bars indicate the average number of plantlets per anther after 9 weeks of culture.

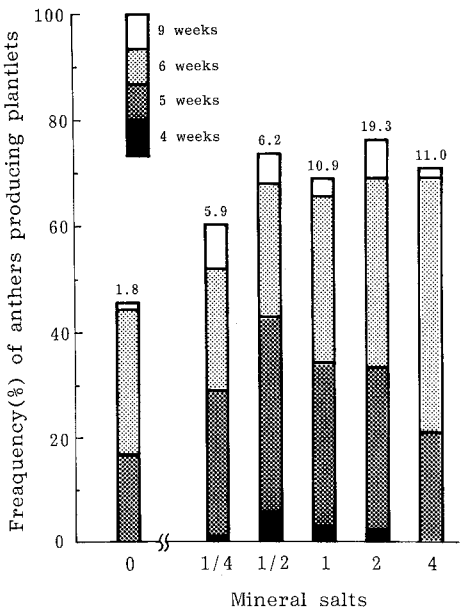

Fig. 2 Effects of varied concentrations of mineral salts on plantlet formation in tobacco anther culture.
All the media used in this experiment containd FeEDTA at 10^{-4}M. Each medium containing mineral salts with varied concentrations of 0, ×1/4, ×1/2, ×1, ×2 and ×4 levels of the basal medium. Values on the top of bars indicate the average number of plantlets per anther after 9 weeks of culture.

Table 2. Mitotic behaviors of pollen grains in tobacco anther cultures as affected by medium composition (25 days after inoculation).

Treat. no.	Medium composition*1	Freq. (%) of non-embryogenic pollen*2			Freq. (%) of embryogenic pollen*3			Maximum development of embryoid*4
		D	S	U+Bi	3 C≦	4 C≦	7 C≦	
1	A+S+M+FeEDTA	82.8	12.9	3.8	6.0	2.5	0.5	GE
2	A+S+M	84.0	3.3	11.3	14.6	3.5	1.6	18 V
3	A+S +FeEDTA	88.3	9.2	2.3	1.9	1.0	0.5	12 V
4	A+S	89.7	1.3	8.8	1.9	0.8	0.4	G+18 V
5	A +FeEDTA	97.6	0.0	2.4	0.0	0.0	0.0	−
6	A	91.8	0.0	8.2	0.2	0.0	0.0	G+2 V

*1 For the explanation of each constituent, see **Table 1**. Treat. No. 1 is the basal medium.
*2 D: Degenerated grains; S: Starch-filled grains; U+Bi: Uninucleate and binucleate grains.
*3 3 C≦, 4 C≦, and 7 C≦: Grains with 3 or more cells, 4 or more cells, and 7 or more cells, respectively.
*4 G: Generative cell; V: Vegetative cell; GE: Globular embryoid.

medium. Anthers cultured on the medium containing both sucrose and FeEDTA (Treats. 1 and 3) had a tendency to produce starch-filled grains.

In the next experiment, anthers were transferred between the basal medium and FeEDTA-free medium reciprocally after 3 weeks of first culture to assertain the effective culture period on FeEDTA medium (**Table 3**). Only 2 plantlets were formed in Treatment 2 where FeEDTA-free medium was used as second medium. On the other hand, plantlet formation was not greatly inhibited when FeEDTA-free medium was used as first medium.

In order to elucidate the function of FeEDTA in detail, ferrous sulfate and Na_2EDTA were separately removed from the basal medium as shown in **Fig. 3**. Few plantlets formed on the media

Table 3. Comparison of culture period on FeEDTA-free medium for the plantlet formation in tobacco anther culture (after 8 weeks of culture).

Treat. no.	Culture period*1 (week)		No. of anthers cultured	Plantlet formation		
	1st-3rd	4th-8th		No. of anthers	Frequency (%)	Ave. no. per anther
1	Basal	Basal	135	94	69.6	10.6
2	Basal	−FeEDTA	135	2	1.5	4.0
3	−FeEDTA	Basal	135	77	57.0	13.4

*1 Basal: Basal medium; −FeEDTA: FeEDTA-free medium; For the composition of medium, see **Table 1**.

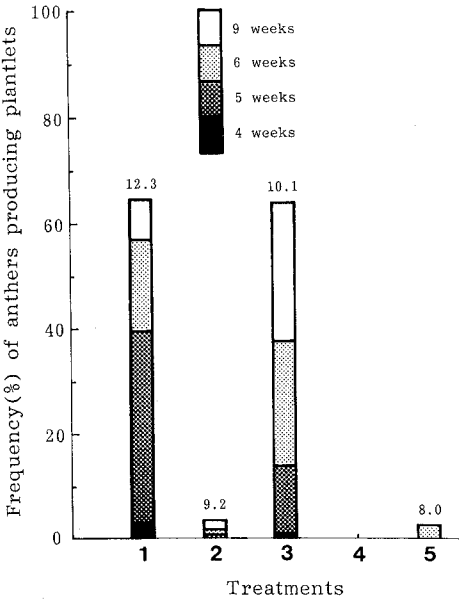

Fig. 3 Effects of ferrous sulfate and Na_2EDTA, components of FeEDTA, on plantlet formation in tobacco anther culture.
Treat. 1: Basal medium (control); Treat. 2: Na_2EDTA-free medium; Treat. 3: Ferrous sulfate - free medium; Treat. 4: FeEDTA - free medium; Treat. 5: Na_2EDTA - free medium containing ferrous sulfate at 5× level of control. Values on the top of bars indicate the average number of plantlets per anther after 9 weeks of culture.

containing no Na_2EDTA, irrespective of the existence of ferrous sulfate (**Figs. 3** and **4**). On the contrary, more than 60% of the anthers formed plantlets on the media containing Na_2EDTA at 10^{-4} M. Anther response on the ferrous sulfate-free (Na_2EDTA) medium was lower than that on the besal medium until the 6th week of culture, but the rates became nearly the same after 9 weeks of culture (**Fig. 3**). Plantlets developed on this medium, however, tended to form pale green leaves.

The effects of Na_2EDTA on plantlet formation were compared with FeEDTA by changing their concentrations in the media as shown in **Fig. 5**. Effective concentrations on the anther response were limited from 5×10^{-5}M to 4×10^{-4}M in both components, and plantlets were scarcely observed on the media containing more than 8×10^{-4}M or less than 10^{-5}M of either. Anther response was highest on the medium containing them at 2×10^{-4}M in both series. Anther response on the media containing Na_2EDTA at the same concentration in the two series were similar to each other except in the case of 5×10^{-5}M level where anther response was lower on the Na_2EDTA medium than on the FeEDTA one. Although the plantlet formation on the FeEDTA medium was most frequent

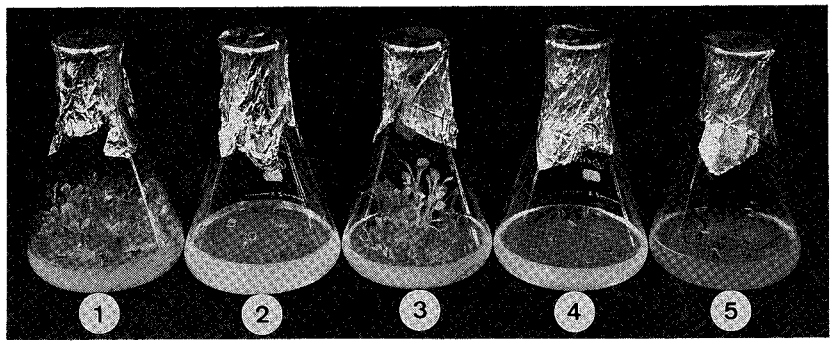

Fig. 4 Plantlet formation in tobacco anther cultures as affected by Na_2EDTA and FeEDTA. Plantlets were formed in ferrous sulfate-free medium (Treat. 3) as well as in basal medium (Treat 1). For the mineral composition of each medium, see **Fig. 3.**

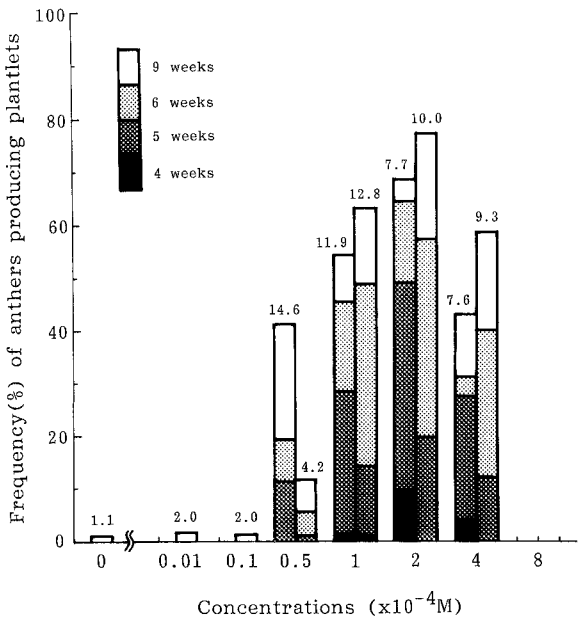

Fig. 5 Effects of various concentrations of FeEDTA and Na_2EDTA on plantlet formation in tobacco anther culture.
Two bars at the same concentration denote the anther response on the FeEDTA (left) and Na_2EDTA (right) media, respectively. Values on the top of bars indicate the average number of plantlets per anther after 9 weeks of culture.

during the 5th week of culture, the greater parts of plantlets were formed during the 6th week of culture on the Na_2EDTA medium. Anther productivity was highest at 5×10^{-5}M level on the FeEDTA medium and at 10^{-4}M on the Na_2EDTA medium.

Discussion

In the present study, as it has been known, FeEDTA was proved to be the essential component in the basal medium for plantlet formation in tobacco anther culture (**Fig. 1**). Nitsch (1969)[14] has reported that the embryoid stopped its development at the globular stage on iron-free medium. Havranek and Vagera (1979)[21] observed the accumulation of viable embryoids at the globular stage in anthers cultured on the iron-free medium, and obtained haploid plants by adding chelated iron solution to the medium. Also, it has been found that a supply of iron during the 3rd week of culture gave a higher percentage of anthers yielding haploid plantlets than supply during 2nd or 1st

week[24]. Moreover, in the present study, few plantlets were obtained from anthers which were transferred to FeEDTA-free medium after 3 weeks of culture on the basal medium (**Table 3**). It has been reported that the development of early embryoids was retarded on iron-free medium[21]. However, removal of FeEDTA from basal medium did not influence the frequency of embryogenic pollen grains at all in the present study (**Table 2**). From these facts, it is concluded that FeEDTA plays the most important role in development from globular embryoid to green plantlet.

The effect of Na_2EDTA on plantlet formation is considered worth noting. In order to know the actual concentration of iron in the medium, a preliminary analysis was carried out using an atomic absorption spectrophotometer (unpublished). From this analysis, 6. 1 ppm iron was detected in the basal medium to which 10^{-4}M FeEDTA (5. 6 ppm as ferrous ion) was added. While, only 0. 3 ppm of iron was included in ferrous sulfate-free medium, and was considered as impurities in the medium. On the other hand, anther walls and pollen grains themselves are considered to contain various kinds of organic and inorganic substances including iron, but this analysis was not carried out on the culture materials used in this study.

Anther response on ferrous sulfate-free (Na_2EDTA) medium was comparable to that on basal medium in this study (**Fig. 3**). Furthermore, concentration effects of Na_2EDTA on the anther response were very similar to those of FeEDTA (**Fig. 5**). These results indicate that Na_2EDTA by itself affects plantlet formation. In anther culture of *Datura*, 26% anther response has been obtained by adding ferrous sulfate at 10^{-5}M to the medium without chelating agents[25]. In an earlier study on tobacco anther culture, Na_2EDTA by itself was considered to have only a small effect in the medium[14]. Also, the addition of EDTA alone has been known to be toxic for growth of the habituated tissue of *Daucus carota*[26]. On the contrary, the effect of chelating agents without iron ion on the vegetative bud formation in tobacco callus cultures has been demonstrated[27]. Thus, the effects of chelating agents are different from one another according to the culture materials and methods used.

In a preliminary experiment on tobacco anther culture, several kinds of chelating agents such as diethylenetriamine pentaacetic acid (DTPA) and ethylenediamine-di-*o*-hydroxyphenyl acetic acid (EDDHA) containing no sodium ion were compared with Na_2EDTA in the basal medium. In this experiment, plantlets were formed sufficiently on such media as well as on the Na_2EDTA medium (unpublished). From this result, it is suggested that sodium ion in Na_2EDTA is not essential but chelating ability itself is important for plantlet formation.

Effects of Na_2EDTA on the anther response was highest at 2×10^{-4}M in this study (**Fig. 5**). In such medium, almost all of Na_2EDTA are considered to be free from chelation with iron ion in the medium. These facts indicate that the effects of Na_2EDTA on plantlet formation can not be explained only by chelation of Na_2EDTA and iron ion in the medium. It is likely that these effects must be caused by the chelating ability of Na_2EDTA not only with iron ion but also with various other minor mineral ions in the medium and culture materials themselves, at least in the case of tobacco anther culture.

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《和文要約》

花粉粒の胚形成機構に関する研究
第8報　タバコの花粉起源植物体の形成における Na_2EDTA の役割

三十尾修司

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タバコの葯培養における培地中の無機成分，とりわけ Na_2EDTA が花粉起源植物体の形成に与える効果について解析した．培地中の無機塩類を1成分ずつ除去したところ，FeEDTA の除去により植物体形成が強く阻止された．これに対し，他の無機塩類の除去は葯当たり形成植物体数が若干減少するものの，FeEDTA の存在下では他の無機塩類が皆無の場合でも 45.6% の葯で植物体形成が認められた．一方，培養葯内花粉粒の顕微鏡観察から，FeEDTA の有無は培養初期の花粉粒の分裂には影響しないことが判った．

また，培養3週間目にFeEDTA除去培地に移植した葯では植物体が形成されなかった．これらの結果から，FeEDTAは胚様体が植物体に発育する段階で重要な役割を演じていると考えられる．

FeEDTA貯蔵液の構成成分である硫酸鉄とNa_2EDTAを分けて培地に添加したところ，Na_2EDTAを除去した培地では植物体形成が阻止されたのに対し，Na_2EDTAのみを加えた培地では完全培地と同様の形成率を示した．また，FeEDTAあるいはNa_2EDTAの添加濃度の変更に対して，植物体形成率は両者で同様の変化を示し，いずれも2×10^{-4}M濃度で最高値が得られた．これらのことから，少なくともタバコの葯培養におけるFeEDTAの効果は，Na_2EDTA自身の機能によりもたらされているものと示唆される．