

## Effect of Fe-EDTA on the Development of Isolated Ovules of *Petunia hybrida*

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### Summary

The present investigation dealt with *in vitro* the growth of the excised ovules just after fertilization in *Petunia hybrida*.

Most of these ovules were cultured on a medium composed of inorganic salts, vitamins, glycine, sucrose and agar without any growth regulators or plant extracts. They were able to develop into viable seeds only when cultured on the medium containing Fe-EDTA as an iron source. Some of the seeds germinated *in situ* immediately and developed until flowering. However, ovules cultured on a medium containing ferric citrate failed to develop into viable seeds.

The addition of some growth regulators to the medium containing Fe-EDTA rather inhibited the normal development of ovules. A supply of casamino acids did not also increase the percentage of normal seedlings; however, the number of normal seedlings increased progressively with decreasing the concentration of sucrose.

### Introduction

After the first success in *Papaver somniferum*(11), the aseptic culture of very young ovules after fertilization have been made by many workers for various species(1, 3, 13, 16). When separated from placental tissue, however, the young ovules containing a zygote or 2-celled proembryo could not develop into mature seeds except in *Zephyranthes*(8) and *Trifolium*(13). On the other hand, viable seeds were easily obtained in the culture of entire ovaries(1, 3). GUHA and JOHRI(3) suggested from these results that placental tissue and ovary wall may supply some unknown metabolites which are essential for the early growth of fertilized ovules.

It was shown in previous papers(14, 15) that in *Petunia hybrida* fertilized ovules attached to the placenta, 3 days after pollination, are able to develop easily into viable seeds on a modified Nitsch's medium. Furthermore, the experimental results suggested that an addition of nitrogen of ammonium form and the relatively high concentration of boric acid, iron and sucrose to the Nitsch's medium (1951) are very important for the full development of ovules. The present experiment was carried

out to determine whether the very young ovules, which were isolated from placental tissue immediately after fertilization, can develop into viable seeds on the similar medium.

### Materials and Methods

The two self-incompatible clones of *Petunia hybrida*, W 166 H (incompatibility alleles  $S_2$ ,  $S_3$ ) and K 146 BH ( $S_6$ ,  $S_6$ ), which were originally established by STRAUB (1946), were chosen for the present experiment.

Plants were grown in a phytotron at  $23 \pm 1^\circ\text{C}$ . Flower buds of clone W 166 H were emasculated and bagged one day before an-

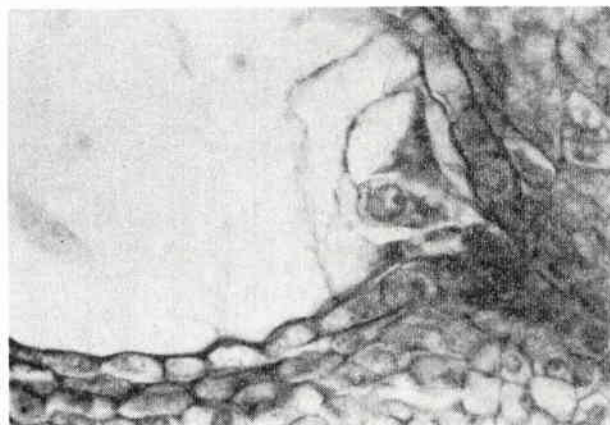


Fig.1. Developmental stage of embryo at the time of inoculation (3 days after pollination).  $\times 100$ .

thesis. The day at anthesis, they were pollinated with fresh pollen grains of K 146 BH. The ovules, 3 days after pollination, were used for culture. The developmental stage of embryo at the time of inoculation is shown in Fig. 1.

The procedures of ovule culture were as follows. The ovary 3 days after pollination was excised and surface-sterilized by dipping them in a 70% ethanol, immersing for 10 minutes in a 10% fresh chlorine water, and finally washing twice with sterile distilled water. Subsequently, the ovary wall was peeled and the placental tissues thus exposed were scraped gently to isolate ovules. The 200~400 isolated ovules per flask were cultured on 20 ml of agar nutrient medium in 50 ml Erlenmeyer flasks.

The nutrient medium containing Fe-ethylenediaminetetraacetic acid as an iron source (designated as Fe-EDTA medium in the text) was prepared upon the basis of preceding results(15) obtained from the culture of ovules attached to the placenta (Table 1). Unless otherwise described, 7.5% sucrose and 0.5% agar were incorporated into the medium. The

Table 1. Composition of the Fe-EDTA medium for cultures of isolated ovules containing a zygote in *Petunia hybrida*.

a) Major elements	mg/l
KNO <sub>3</sub>	625
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4 H <sub>2</sub> O	500
NH <sub>4</sub> NO <sub>3</sub>	80
KH <sub>2</sub> PO <sub>4</sub>	125
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	125
b) Minor elements	mg/l
H <sub>3</sub> BO <sub>3</sub>	25
ZnSO <sub>4</sub> ·7 H <sub>2</sub> O	0.5
MnSO <sub>4</sub> ·4~6 H <sub>2</sub> O	3
CuSO <sub>4</sub> ·5 H <sub>2</sub> O	0.025
Na <sub>2</sub> MoO <sub>4</sub> ·2 H <sub>2</sub> O	0.025
c) Fe-EDTA*	10 ml/l
d) Glycine	7.5 mg/l
e) A mixture of vitamins	mg/l
Calcium pantothenate	0.25
Pyridoxine-HCl	0.25
Thiamine-HCl	0.25
Nicotinic acid	1.25

\* A stock solution containing 5 grams FeSO<sub>4</sub>·7 H<sub>2</sub>O and 4 grams disodium ethylenediaminetetraacetate, dihydrate (Na<sub>2</sub>-EDTA) per liter of redistilled water. 10 ml/l Fe-EDTA corresponds to 10 mg/l iron

pH of the Fe-EDTA medium was adjusted to 5.7 using 0.1 N NaOH and 0.1 N HCl before autoclaving. A medium containing 40 mg/l ferric citrate instead of Fe-EDTA (designated as ferric citrate medium in the text) was also used and adjusted to pH 4.5. Difco Bacto vitamin-free casamino acids and growth regulators such as indole-3-acetic acid (IAA), gibberellic acid (GA<sub>3</sub>) and kinetin were in some instances added to the Fe-EDTA medium.

All cultures were maintained in the room at about 25°C. Some cultures were used for embryonic studies and the sectioned preparations were stained by Delafield's hematoxylin.

## Results

### *Development of ovules in vitro under the presence or absence of Fe-EDTA.*

After 27 days of culture on the Fe-EDTA medium, a well-developed embryo with normal cotyledons, massive endosperm tissue and testa were observed (Fig. 2 A). At the 30~50th day some of them germinated *in situ* without

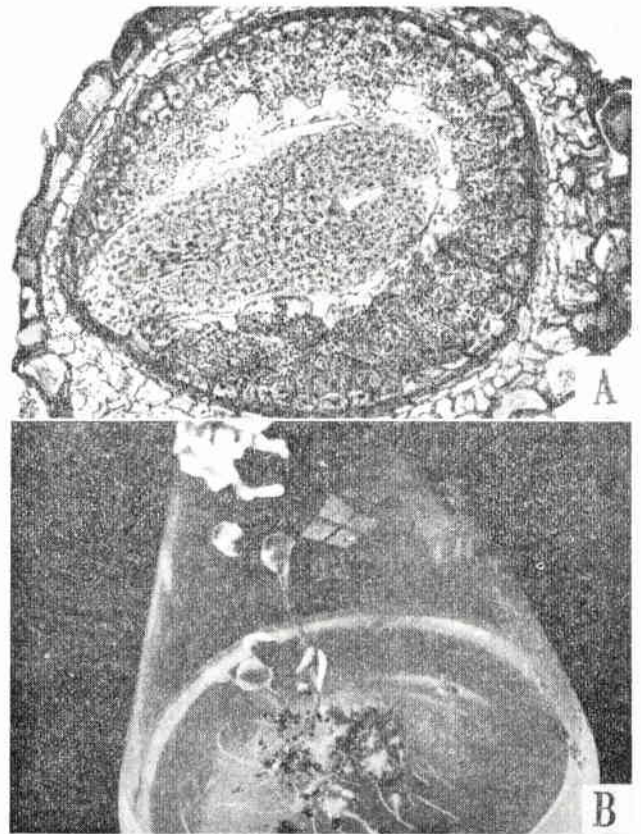


Fig. 2. Growth of excised ovules on the Fe-EDTA medium. A, 27 days-old embryo and endosperm. ×100. B, seedlings formed *in situ* in the 40th day.



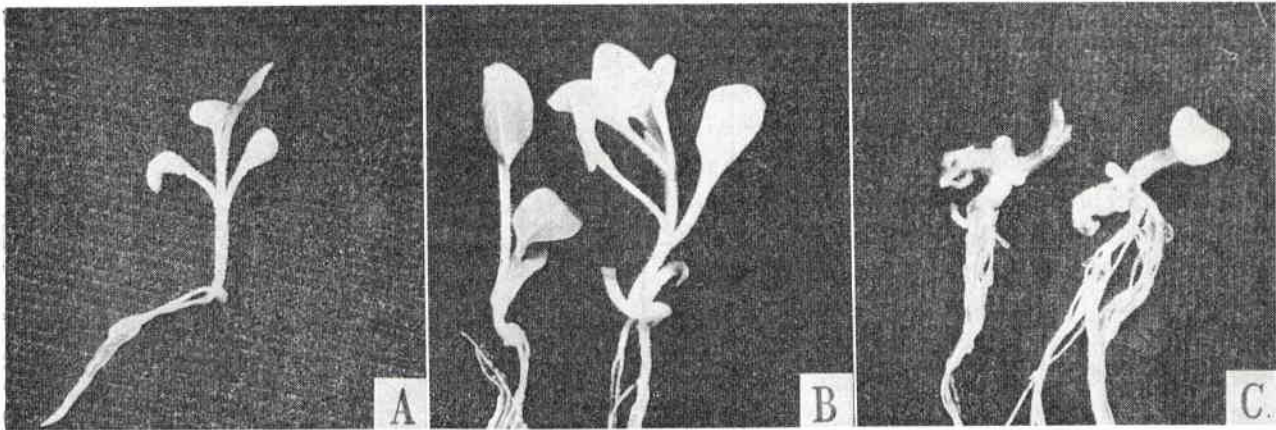


Fig. 3. Seven weeks old seedlings obtained through isolated ovules on the Fe-EDTA medium. (A) normal seedling with two cotyledons and roots. (B) seedling with one cotyledon and roots at the germination stage. Later it differentiated leaves and developed into normal mature plant. (C) a callus-like mass with leaves and roots.

undergoing dormancy (Fig. 2 B). Seedlings from this medium could be classified into three different types (Fig. 3); (i) normal seedling with two cotyledons and roots (designated as type-A. Fig. 3 A), (ii) slightly malformed seedling with only one cotyledon and roots, which eventually differentiated normal shoot (designated as type-B. Fig. 3 B) and (iii) a callus-like mass with leaves and roots, which fails to develop into normal seedling (type-C. Fig. 3 C).

In the ferric citrate medium the seeds did not germinate at all. After 27 days the embryo reached to the globular stage, but further development did not proceed normally (Fig. 4 A). Even after 47 days almost all the embryos still remained in the globular shape, although the size of seed became to be comparable to that of seed on the Fe-EDTA medium (Fig. 4 B).

These findings indicate evidently that Fe-EDTA is effective for the development of ovules excised soon after fertilization and stimulates strongly the development of inner embryo.

*Influence of decreased level of sucrose on seedling development.*

The preceding experiments showed that Fe-EDTA was essential for the development of ovules *in vitro*. However, the development of cotyledon retarded frequently (type-B and -C). The experiment was carried out to investigate whether the concentrations of sucrose was influenced on the differentiation of embryo, especially the development of

cotyledons.

Fig. 5 shows the influence of sucrose concentrations on the development of cotyledons and roots. With 6.5% sucrose the number of type-A seedlings increased slightly as compared with those obtained on the media containing 7.0 or 7.5% sucrose. Although many mature seeds were formed even in the

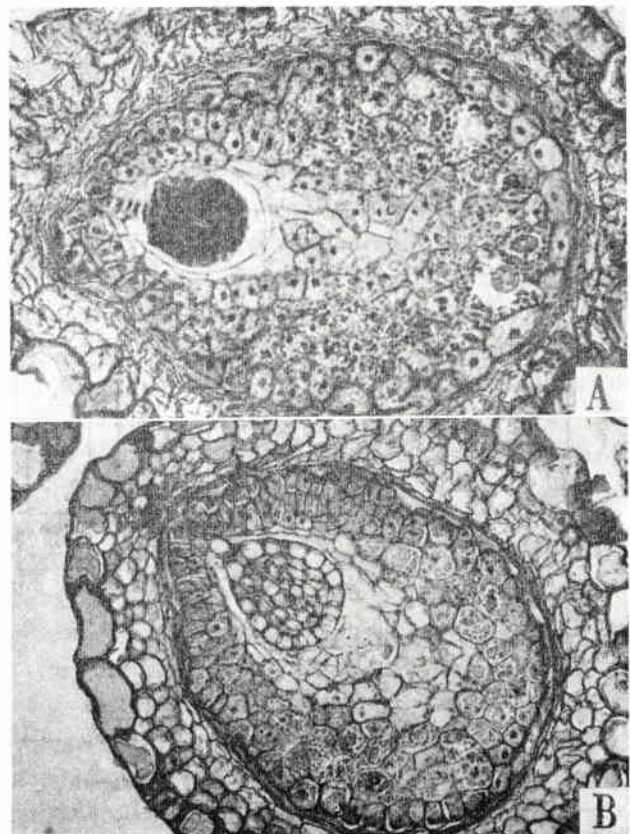


Fig. 4. Growth of excised ovules on the ferric citrate medium. A, 27 days-old after culture. B, 47 days-old.  $\times 100$ .

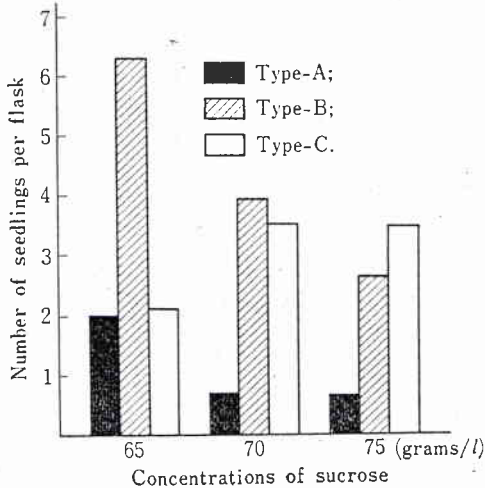


Fig. 5. Influence of sucrose concentrations.

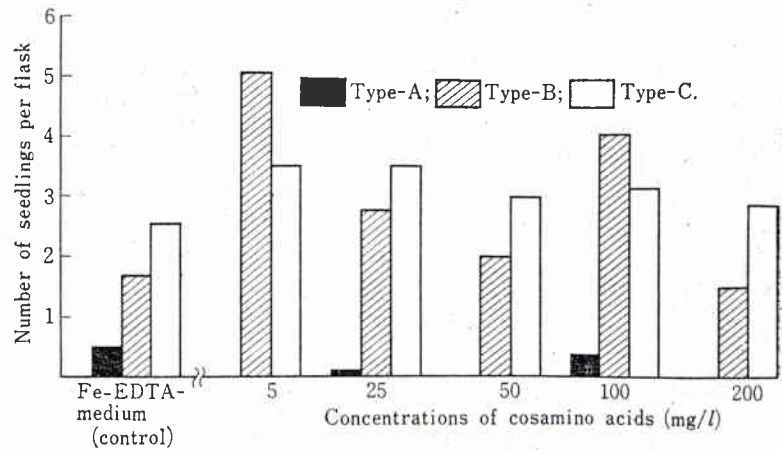


Fig. 6. Influence of varying concentrations of casamino acids on the production of seedlings.

7.0 and 7.5% sucrose media, which could eventually germinate, the development of cotyledons was considerably poor. As a result, the number of type-A decreased and the seedlings of type-B and -C increased.

#### *Influence of the concentration of casamino acids.*

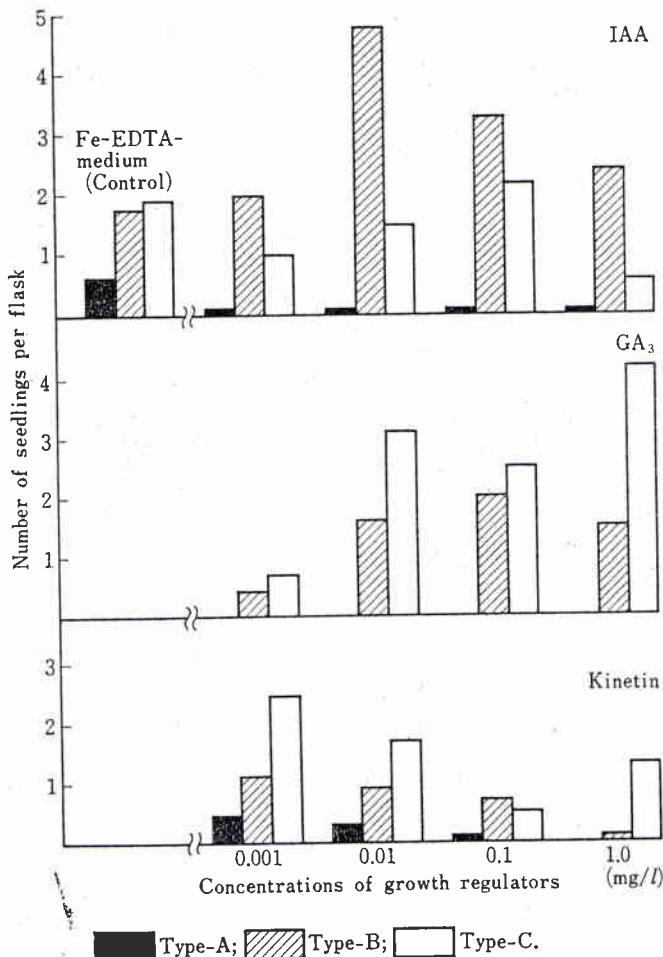
When the excised ovules were cultured on the Fe-EDTA medium supplemented with various concentration of casamino acids, any increase in number of type-A seedlings was not seen (Fig. 6). On the other hand, the rate of the appearance of the type-B was much higher than that on the control medium. It seems from these results that an addition of casamino acids to the Fe-EDTA medium is ineffective for the normal differentiation of embryo, especially for the development of cotyledon.

#### *Influence of some growth regulators.*

The number of the type-B seedlings increased when IAA was added to the Fe-EDTA medium, as shown in Fig. 7. After 30 days of culture the seeds contained a large embryo but not endosperm (Fig. 8 A). The length of cotyledons were shorter than that on the Fe-EDTA medium.

An increase in number of type-A and -B seedlings was not obtained by  $GA_3$ . On the contrary, the type-C seedlings increased as the concentrations of  $GA_3$  increased. 0.001 mg/l  $GA_3$ , inhibited strongly the development of embryo and endosperm (Fig. 8 B).

Fig. 7 also shows the frequency of seedling formation under various concentrations of kinetin (0.001, 0.01, 0.1 and 1.0 mg/l). After 30 days of culture on 0.001 mg/l kinetin an abnormal embryo and a massive endosperm were seen (Fig. 8 C). The number of type-A and -B seedlings decreased with

Fig. 7. Influence of IAA,  $GA_3$  and kinetin added singly to the Fe-EDTA medium on the production of seedlings.



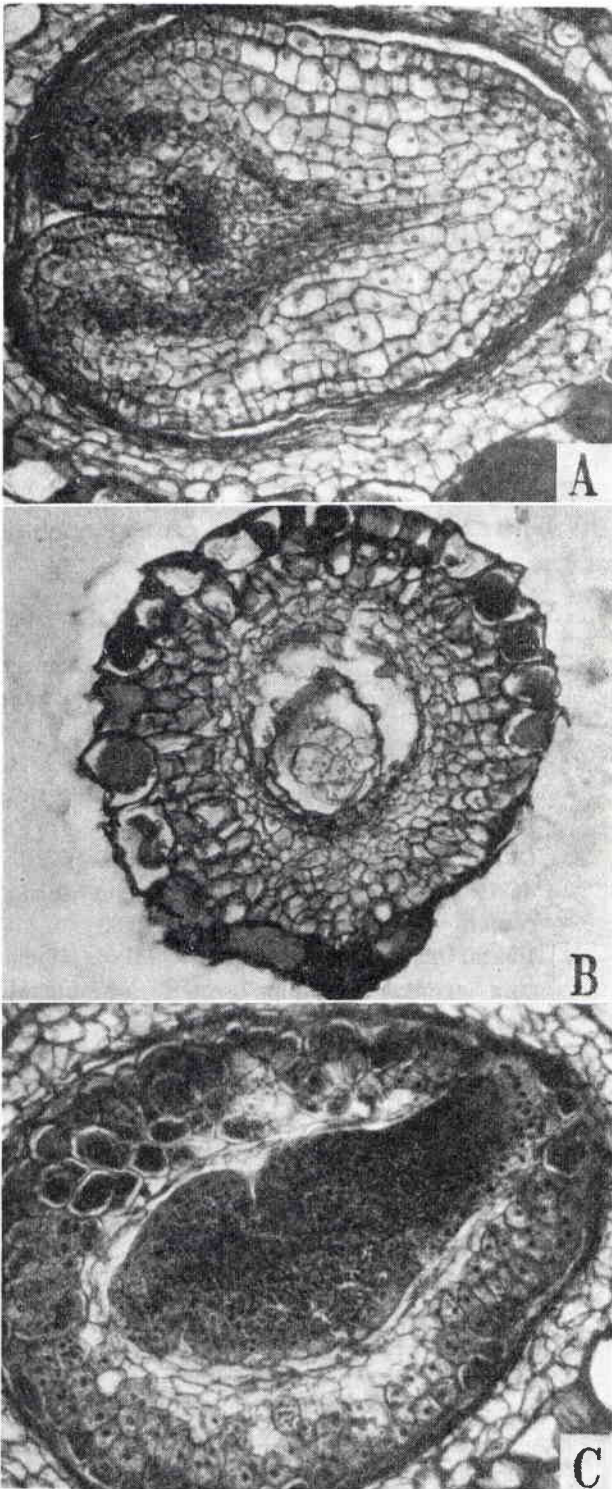


Fig.8. Growth of excised ovules on the Fe-EDTA medium supplemented with growth regulators, IAA,  $GA_3$  or kinetin. A, B and C: embryos and endosperms from the Fe-EDTA medium plus 0.001 mg/l IAA (A), 0.001 mg/l  $GA_3$  (B) and 0.001 mg/l kinetin (C), respectively, 30 days after culture.  $\times 100$ .

increasing the concentrations of kinetin.

### Discussion

The present investigation clearly showed

that ovules containing the zygote separated from the placenta can develop into the viable seeds. They germinated *in situ* on the medium composed of inorganic salts, Fe-EDTA, vitamins, glycine, sucrose and agar without any growth regulators or plant extracts. When cultured on a medium containing ferric citrate as an iron source, however, they failed to develop into mature seeds.

Extensive works on ovule and ovary cultures have been made by the use of NITSCH's (1951) inorganic and vitamins solution supplemented with growth regulators, casamino acids and/or plant extracts (3, 8, 11, 12, 13). The standard Nitsch's medium contains 10 mg/l ferric citrate as an iron source. As growth proceeds in plant tissue culture, the pH drifts towards neutrality (6) and alteration in pH toward neutrality would result in the limited availability of the iron and the other metal ions (7). STREET (16) found that the value of pH at which a low concentration of iron citrate or tartrate becomes unavailable is about 6.0. As the ferric citrate medium used in the present investigation is adjusted to pH 4.5 and also contains a high concentration of about 8 mg/l iron, the availability of Fe to the isolated ovules may be left out of consideration.

The results described above lead to a consideration of the mechanism by which chelating agent such as EDTA influence the development of embryo. This may undoubtedly be the most important question raised by the present study. Although several reports have been presented about the action of chelating agents on the growth of intact plant, there is no report about the effect of EDTA on the growth of embryo and endosperm in ovule culture. HEATH and CLARK (4, 5) reported the chelating agents such as EDTA or 8-hydroxyquinoline were, in fact, auxins as operationally defined. They observed that EDTA, like indole-3-acetic acid, suppressed the elongation of wheat roots. BURSTRÖM and TULLIN (2) found, as reported by HEATH and CLARK (4), the EDTA suppressed root growth. This suppression, however, was due to a deficiency in cell multiplication, whereas auxin suppressed only cell elongation. On the other hand, THIMANN and TAKAHASHI (18) have indicated that the effect of EDTA and auxin are clearly

different. KLEIN and MANOS(9) also reported that Fe-EDTA increased the growth of crown gall tissue of both *Parthenocissus* and *Helianthus*, but its effect is not that of an auxin. They found that these tissue cultures are protrophic for auxins and are, in fact, inhibited by these growth regulators. KOCHCHAR *et al* (10) reported that inclusion of chelated metal ions to the nutrient medium influences the differentiation of a callus of *Nicotiana tabacum* in culture. They observed that vegetative buds could be induced on the callus obtained from the haploid plant by the chelating agents and pointed out that chelating agents, besides stimulating growth, might also regulate morphogenesis. Furthermore, they emphasized that the differential effects of the chelating agents indicate the involvement of some specific action of the chelated metal ions which might activate some specific enzyme system involved in organogenesis.

In *Petunia*, the presence of Fe-EDTA is essential for the development of ovules containing a zygote, although Fe-EDTA does not always function as a substitute of the placental tissue. SACHAR and KAPOOR(16) reported that the addition of IAA, GA, 2,4-D, IBA and kinetin to the Nitsch's medium (1951) fails to induce growth of *Zephyranthes* if isolated prior to the formation of globular stage. Moreover, these substances inhibit the development of endosperm. The results reported by other workers(1,3,12,13) are in general agreement with those of SACHAR and KAPOOR(16). In the present investigation, when excised at zygote stage and cultured on the Fe-EDTA medium, the ovules grew up to the mature seeds which germinated *in situ*, but when some growth regulators were added to the Fe-EDTA medium the normal development of embryo and endosperm was inhibited. Further, any viable seed was not formed even when IAA was added to a medium containing ferric citrate as an iron source (unpublished). Thus, it is supposed that Fe-EDTA plays a role different from growth regulators for the development of ovules.

On the basis of these observations and other results(15), we are inclined to think that some of the positive effect of Fe-EDTA on the development of isolated ovules may be

due to both the buffering of iron by EDTA and the complexing of EDTA and other metals, for example, in some specific enzyme system. Further, without evidence, we presumed that the placental tissue supplies some substances which are essential for the normal development of ovules; besides, it also plays some important roles in the regulation of utilization of nutrient elements including growth substances which are produced by itself and the developing ovules.

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### *Petunia hybrida* のはい珠培養における Fe-EDTA の効果

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#### 摘 要

受精後まもない接合子をもつたはい珠を胎座から切り離し培養したところ、植物生長調節物質や植物抽出物を全く含まない無機塩やビタミン類を中心とする完全合成培地で多くの健全な実生を得ることができた。

若いはい珠を完熟種子に育て、健全な実生を得ることができるかどうかは、培地に添加する鉄の種類によつて大きく左右されることが明らかとなつた。すなわち、Fe-EDTA (Fe として 10 mg/l) を含有する培地では多くの実生が得られた。しかし Fe-EDAT のかわりにクエン酸第二鉄を添加した培地からは実生は全く得られなかつた。

Fe-EDTA を含む培地から得られる実生には、形態のうえからおおよそ三種類あり、健全な実生とともに子葉が十分に分化していない奇形の実生があつた。

以上の結果に基づき、しよ糖の濃度、植物生長調節物質およびカザミノ酸がはい珠の発達、とくにはいの分化に影響するかどうかを Fe-EDTA を含む培地を用いて検討した。しよ糖濃度を 7.5% から 6.5% に減らすと健全な実生数が増加した。しかし植物生長調節物質やカザミノ酸の添加はい珠の正常な発達に効果がなかつた。