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Influence of Iron, Boron, and Sugars on Viable Seed Production in Excised Ovule Culture in *Petunia hybrida*

Yoshiji NIIMI

Faculty of Agriculture, Niigata University, Niigata, Japan

Summary

The present investigation deals with *in vitro* growth of ovules on a half of the placenta of *Petunia hybrida*, W 166 H×K 146 BH (each clone is self-incompatible), with special reference to the influence of iron, boric acid, and sugars. The culture medium of NITSCH (1951), modified by the addition of 500 mg/*l* KNO_3 and 80 mg/*l* NH_4NO_3, was used as a basal medium.

The optimal initial hydrogen-ion concentration was at pH of 4.5～5.0 in the basal medium and at pH of 6.0 in the basal medium with Fe-EDTA at an iron concentration of ca. 2.5 ppm instead of ferric citrate.

The number of viable seeds per ovary increased with an increasing concentration of ferric citrate from 10 to 40 mg/*l* in the basal medium. Iron complex Fe-EDTA composed of 50 mg/*l* $FeSO_4 \cdot 7H_2O$ and 40 mg/*l* disodium ethylenediaminetetraacetate, dihydrate (Na_2-EDTA) was also an effective compound and was favorable for the viable seed production.

10 mg/*l* or above boric acid should be added to the basal medium containing either ferric citrate or Fe-EDTA. Medium containing 50 mg/*l* boric acid was superior for the viable seed production to those with any other concentration. The efficancy of boron was influenced by the organic forms of iron. The medium with Fe-EDTA at an iron concentration of ca. 2.5 ppm was superior to that with 10 mg/*l* ferric citrate.

Basal medium containing 7.5 per cent sucrose or 5 per cent fructose produced the maximal viable seeds and that containing a mixture of sucrose and fructose or sucrose and glucose was inferior to the basal medium.

From these results, experiments were conducted to clarify the interaction among three components, iron, boric acid, and sugars. About 5～6 normal seedlings with two cotyledons and roots per ovary were obtained in the medium containing ca. 10 ppm Fe as Fe-EDTA, 25 mg/*l* boric acid, and 7.5 per cent sucrose. This was about six times as the number of seedlings produced in the basal medium.

In the basal medium containing fructose instead of sucrose, however, the higher concentrations of boric acid and/or iron failed to bring about the noticeable effect.

Introduction

Previous paper(5) has shown that several seedlings were obtained from *Petunia hybrida*, a self-incompatible plant, by a technique of test-tube fertilization. From this result the author is greatly interested in the culture of ovules soon after fertilization.

To obtain many normal seedlings, it has been investigated whether or not NITSCH's (1951) medium used for the test-tube fertilization was suitable for the growth of ovules. The result(6) showed that when ovules on a half of the placenta excised 3 days after cross-pollination *in vivo* in *Petunia* were cultured on NITSCH's medium supplemented with 500 mg/*l* KNO_3 and 80 mg/*l* NH_4NO_3, many viable seeds were obtained, as compared with NITSCH's medium only.

The purpose of this study is to clarify the influence of concentrations of chelated iron (Fe-EDTA), ferric citrate, boric acid and sugars on the viable seed production *in vitro*, using a mass of ovules on a half of the placenta excised 3 days after cross-pollination in *P. hybrida*.

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Materials and Methods

Clones W 166 H and K 146 BH of *Petunia hybrida*, a self-incompatible plant, were used as the materials. Plants were grown under controlled condition at 23±1°C. Flower buds of clone W 166 H one day before anthesis were emasuculated and bagged to free from open pollination. *In vivo* cross-pollination (W 166 H×K 146 BH) was made immediately or the next day, the corresponding date of anthesis. They were harvested 3 days after pollination. Then the petals, sepals, and style were removed.

15~20 ovaries were used in each experiment. After washing with tap water, ovary with pedicel was surface-sterilized by dipping in 70 per cent ethyl alcohol for a few minutes and then in 10 per cent hypochlorite water for 10 minutes. Ovaries were washed twice with sterile water and transferred to sterile Petri dishes containing two discs of filter paper to absorb the excess water. The ovary was then longitudinally cut into two pieces with a sterile knife with a thin blade. The ovary wall was then cut off by a sterile pincette. Two pieces of excised ovules on a half of the placenta were then placed on nutrient media in a flask.

The basal medium used here was virtually identical with that as described earlier(6). This medium was fortified with macro inorganic nutrient, 500 mg/*l* KNO_3 and 80 mg/*l* NH_4NO_3. The medium was gelled with 0.5 per cent agar. Except where otherwise stated, the basal medium contained 10 mg/*l* ferric citrate as an iron source, 0.5 mg/*l* boric acid as boron, and 5 per cent sucrose as carbon source. The pH was adjusted to a difinite value with 0.1 N NaOH and 0.1 HCl before agar and sugar were added to medium. About 20 m*l* of medium was distributed to a flask and autoclaved at 1.0 kg/cm² pressure for 10 minutes. The flask planted with fertilized ovules was kept under scattered light condition at about 25°C for 30 days. Ovules were then cut off from the placental tissue and sown on a fresh medium of 0.5 per cent agar only. Normal seedlings developed from seeds were counted 20 days thereafter.

Results

Influence of pH and organic forms of iron on the growth of excised ovules.

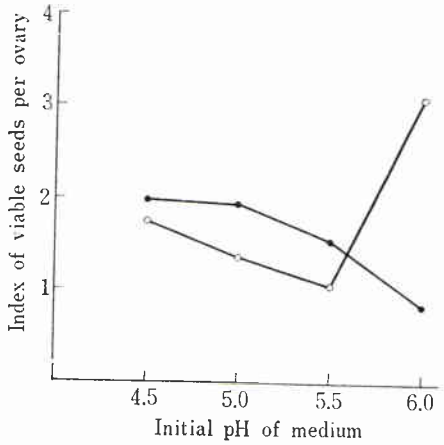

Fig. 1. Influence of initial pH of medium on the growth of excised ovules in *P. hybrida*. Note : Filled circles, the basal medium; open circles, basal medium with 2.5 ppm Fe as Fe-EDTA instead of ferric citrate.

The first experiment was done to determine the optimal pH of the basal medium and of the medium with 25 m*l*/*l* Fe-EDTA (designated BM-Fe-EDTA medium in the text). Fe-EDTA stock-solution consisted of 0.5 grams $FeSO_4 \cdot 7H_2O$ and 0.8 grams disodium ethylenediaminetetraacetate, dihydrate (Na_2-EDTA) per liter of redistilled water.

As shown in Fig. 1 more viable seeds were obtained on the basal medium at pH of 4.5 and 5.0 than 5.5~6.0. On the other hand, the largest number of viable seeds was obtained on the BM-Fe-EDTA medium at pH of 6.0. In addition to normal seedlings, many abnormal ones were observed in the pH range studies on media containing Fe-EDTA (Fig. 2).

Therefore pH was adjusted to 4.6±0.1 in medium with ferric citrate and to 5.9±0.1 in

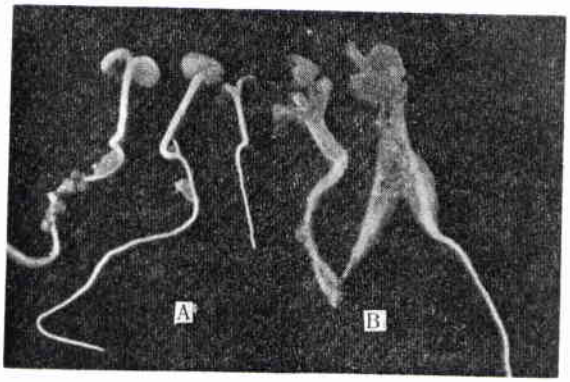

Fig. 2. Normal(A) and abnormal(B) seedlings developed from seeds in *P. hybrida* on the basal medium with Fe-EDTA instead of ferric citrate.

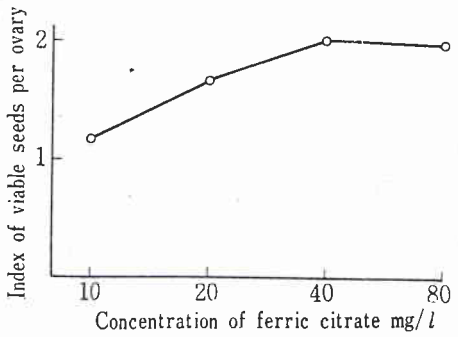

Fig. 3. Effect of concentrations of ferric citrate added to the basal medium on the growth of excised ovules in *P. hybrida*.

that with Fe-EDTA in the following experiments.

Influence of iron and boron concentrations in the basal medium and the BM-Fe-EDTA medium on the growth of excised ovules.

A mass of ovules on a half of the placenta were cultured on the basal medium with the range of 10~80 mg/*l* ferric citrate concentrations. The maximal viable seeds were obtained at a ferric citrate level of 40 mg/*l* and the viable seeds on its medium were about twice as many as those on the basal medium (Fig. 3). Influence of Fe-EDTA prepared by several combinations of $FeSO_4 \cdot 7H_2O$ and Na_2-EDTA was also investigated. Abnormal seedlings similar to those shown in Fig. 2 were also observed in all combinations. The best result was given on the medium containing 50 mg/*l* $FeSO_4 \cdot 7H_2O$ with 40 mg/*l* Na_2-EDTA as Fe-EDTA, and about 2.5 normal seedlings per ovary were produced. Other combinations were proved to be toxic to the growth of ovules except the medium containing Fe-EDTA in the original combination of 0.5 grams $FeSO_4 \cdot 7H_2O$ and 0.8 grams Na_2-EDTA (Fig. 4). If any, there was little difference in viable seed production between the basal medium with 40 mg/*l* ferric citrate as an iron source and that with 10 ppm Fe as Fe-EDTA composed of 50 mg/*l* $FeSO_4 \cdot 7H_2O$ and 40 mg/*l* Na_2-EDTA.

Another experiment was designed to determine the optimal level of boron as boric acid in the basal medium and the BM-Fe-EDTA medium. As shown in Fig. 5 the number of viable seeds per ovary increased linearly with the increase of boric acid from 10 to 50 mg/*l* in the BM-Fe-EDTA medium. The largest number of viable seeds was observed at a boric acid level of 50 mg/*l* which was about six times as many as those on the basal medium. At a boric acid concentration of 100 mg/*l*, however, the productivity of viable seeds fell off sharply on the BM-Fe-EDTA medium. The viable seeds also increased in number on the basal medium when supplemented with boric acid, 10 mg/*l* or above and these on the basal medium with 50 mg/*l* boric acid were about twice as many as those on the basal medium.

From these observations the following deduc-

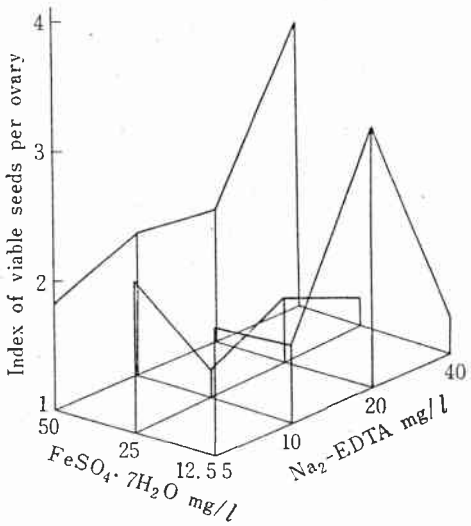

Fig. 4. Effect of combinations of $FeSO_4 \cdot 7H_2O$ and Na_2-EDTA as Fe-EDTA added to the basal medium (without ferric citrate) on the growth of excised ovules in *P. hybrida*.

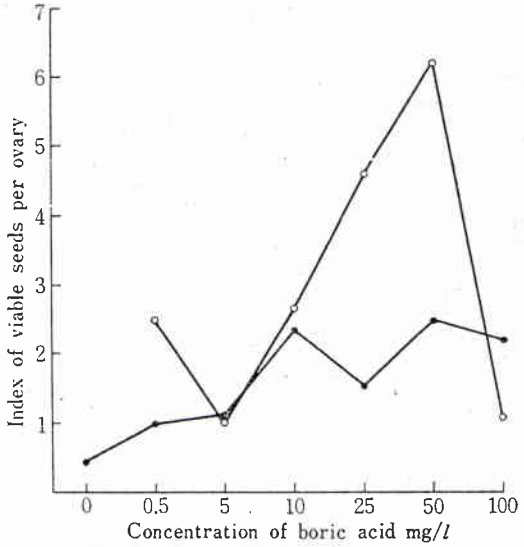

Fig. 5. Effect of boric acid concentration on the growth of excised ovules in *P. hybrida*. Note : Filled circles, the basal medium; open circles, basal medium with 2.5 ppm Fe as Fe-EDTA instead of ferric citrate.

tion may be drown: Boron itself enhance a viable seed production and its efficancy is influenced by the complex organic forms of iron, *e.g.* ferric citrate or Fe-EDTA.

Influence of several kinds of sugars and their concentration on the growth of excised ovules.

In preliminary, experiments were done to ascertain whether or not viable seeds were obtained on the basal medium containing fructose, glucose, maltose, and raffinose singly at the molar concentration comparable to 5 per cent of sucrose instead of sucrose. Fructose and glucose served for the growth of ovules to a limited extent and for the viable seed production. Maltose and raffinose completely failed to support their growth. From these results, experiments were designed to clarify the influence of three sugars, sucrose, fructose, and glucose in more detail.

A mass of ovules on a half of the placenta were cultured on the basal medium containing 0.0, 2.5, 5.0, 7.5, and 10 per cent sucrose. Without sucrose the ovules could never grow. At the concentration of 2.5 per cent, their growth was also poor, but a few viable seeds were formed. The largest number of viable seeds was obtained on the medium containing 7.5 per cent sucrose. When the concentration was raised to 10 per cent, the ovules did not grow at all. On the medium containing 5 per cent fructose, the number of seed-set was almost the same as that with 7.5 per cent sucrose but a seedling-size was small. On the medium containing sucrose and fructose in combination, only a few viable seeds were obtained (Fig. 6). On the other hand, the medium added a mixture of 2.5 per cent sucrose and 2.5 per cent glucose or added 5 per cent glucose only produced approximately the same number of viable seeds as on the basal medium with 5 per cent sucrose. Viable seed production on media supplemented with a mixture of any other combinations such as sucrose and fructose or sucrose and glucose were inferior to that on the basal medium.

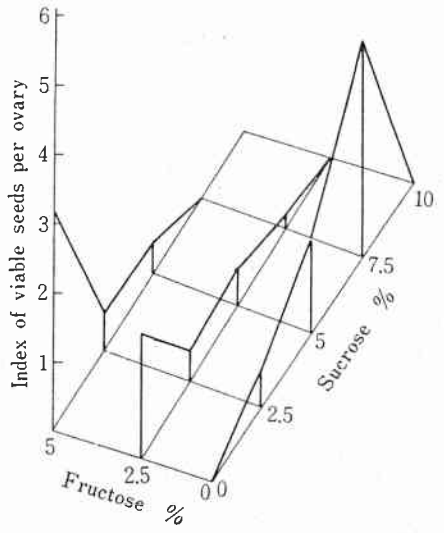

Fig. 6. Effect of concentration of sucrose and fructose separately and in combination on the growth of excised ovules in *P. hybrida* on the basal medium.

Interaction of varied concentrations of Fe-EDTA, ferric citrate, boric acid, sucrose and fructose on the growth of excised ovules.

From the results indicated above, tests were done to determine the influence of concentration and/or organic forms of iron, kinds of sugars, and boron in combinations on growth of excised ovules. Treatments and results are shown in Table 1. The maximal viable seeds were obtained on the medium containing 10

Table 1. Influence of varied concentrations of iron, boric acid, and sugar added to the basal medium* on the growth of excised ovules in *P. hybrida*.

Medium	Sugars	Iron forms	Boric acid mg/l	Index of normal seedlings per ovary
Basal medium	Sucrose 5 %	Ferric citrate 10 mg/l	0.5	1.0
		Ferric citrate 40 mg/l	0.5	1.8
			25	2.5
			50	3.1
		Fe-EDTA 25 ml/l**	0.5	3.3
			25	3.8
			50	2.1
	Sucrose 7.5 %	Ferric citrate 10 mg/l	25	1.3
			50	2.2
		Ferric citrate 40 mg/l	0.5	3.1
			25	4.3
			50	5.6
		Fe-EDTA 25 ml/l**	0.5	2.2
			25	6.6
			50	4.3
	Fructose 5 %	Ferric citrate 10 mg/l	25	3.4
			50	3.4
		Ferric citrate 40 mg/l	0.5	2.0
			25	1.0
			50	0.8
		Fe-EDTA 25 ml/l**	0.5	1.0
			25	1.2
			50	0.8

* Basal medium: Ferric citrate, boric acid and sucrose are excluded from the composition of the medium.

** A stock solution containing 2 grams $FeSO_4 \cdot 7H_2O$ and 1.6 grams Na_2-EDTA per liter of redistilled water. 25 ml/l Fe-EDTA corresponds to about 10 ppm iron.

ppm Fe as 25 m*l*/*l* Fe-EDTA, 25 mg/*l* boric acid, and 7.5 per cent sucrose. The number of viable seeds was about six times as many as those on the basal medium. In these experiments sucrose was superior to fructose as an effective source of carbon and energy. On the medium containing 25 m*l*/*l* Fe-EDTA or 40 mg/*l* ferric citrate and sucrose, the following deduction may be drown : (1) the number of viable seeds increased with the increase of boric acid level regardless of Fe-EDTA or ferric citrate as an iron source, (2) on the medium with Fe-EDTA the optimal concentration of boric acid was at 25 mg/*l* and on that with ferric citrate it was at 50 mg/*l*, (3) the medium with 7.5 per cent sucrose was always superior to that with 5 per cent sucrose. While on a medium containing 5 per cent fructose instead of sucrose, the higher concentrations of boric acid and/or iron such as 25 m*l*/*l* Fe-EDTA or 40 mg/*l* ferric citrate decreased the number of viable seeds.

Discussion

Few reports have been made on the influence of boric acid on the growth of ovules *in vitro*. Its efficancy was proved in the present experiments by the fact that the viable seed production of *Petunia hybrida* increased with an increasing concentration of boric acid. Boron has often been reported to be toxic to an intact plant even at the low concentration of 5~10 ppm(9). In these experiments, ovules tolerate the very high concentration of boron and the best results are given at 25~50 mg/*l* of boric acid.

Boron is recognized as an essential micronutrient for higher plants, but the principal role of this element in plant growth and development is not so clear. The prominent works on the roles of boron are the following : (i) sugar translocation in plant(2), (ii) cell division(11), (iii) cell wall metabolism(12), (iv) phenolic acid metabolism(4), and (v) nucleic acid metabolism(1).

The concept of sugar translocation postulated by GAUCH and DUGGER(2) is more widely accepted than any other physiological roles. WEISER *et al.*(10) has given a different opinion : boron does not enhance sugar (photosynthate) translocation but does enhance the uptake of foliarly applied sugar. O'KELLEY(7) showed that during pollen germination and tube growth in *Tecoma radicans* boron stimulated the absorption of both sucrose and glucose but the absorption of fructose did not increase with addition of boron. The results obtained in the present experiments show that the number of viable seeds of *P. hybrida* increase with an increasing concentration of boric acid in the medium with sucrose, however, in the medium with fructose the viable seeds did not increase in number at a very high level of boric acid, 50 mg/*l*. Thus, the efficancy of boron on the growth of excised ovules is influenced by the kinds of sugars added to the medium.

Relationships between role of boron and metabolism of phenolic acid also should not be overlooked in the growth of excised ovules. LEE and ARONOFF(4) have indicated : boron plays a role in the regulation of phenolic synthesis by interaction with enzyme 6-phosphogulconate dehydrogenase ; in the absence of borate, the inhibition of the enzyme is released, and excess phenolic acids are formed which caused necrosis of plant tissue and eventual death of the plant. This phenomenon may possibly occur during the growth of ovules in *P. hybrida* cultured on the basal medium with a low concentration of boron, however, on the medium with a high concentration of boron the absorption of nutriment and also the uptake or the translocation of sugar must apparently have been kept throughout the long period.

An efficancy of boron is also influenced by the organic forms of iron, Fe-EDTA or ferric citrate added to a medium. There was a great difference in number of viable seeds between two media with Fe-EDTA and with 10 mg/*l* ferric citrate. With the increase of concentration of ferric citrate, however, the difference disappeared. There must be a certain relationship between iron availability and boron action.

Each medium with ferric citrate or Fe-EDTA has its own optimal pH. This may be attributed to a difference of iron availabiliy due to pH in media. STREET *et al.*(8) have reported that ferric and ferrous salts are readily soluble at the acid pH but their solubility

decreases with the increase of hydroxyl ion concentration, and little of the iron will be retained in solution at pH of 5.0 or above. At a higher concentration of ferric citrate, however, the pH range may extend to 5.8 to 6.0(8). On the other hand, the solubility of Fe-EDTA is proved to be extremely stable even at alkaline pH(3). STREET *et al.*(8) suggested that media containing this iron complex showed a more pronounced acid drift on autoclaving than the other iron source, and at initial pH of 5.5 or above, this drift was of the order of one pH unit. The similar phenomenon may also occurred in media used in this experiment. Some suggestions about it are as follows: both the basal medium and the medium containing 40 mg/*l* ferric citrate support a similar rate of growth during the first period of culture, and iron availability in the basal medium decreases with the alkaline drift as time goes by. However, the medium with 40 g/*l* ferric citrate is considered to show a less-marked reduction of its activity at high pH. Moreover, in the medium with Fe-EDTA the widening of pH range for iron availability occurred. Thus, media with Fe-EDTA or 40 mg/*l* ferric citrate will maintain an iron availability over a long period of culture.

The author concludes on the basis of the present findings that under the condition of boron sufficiency and iron deficiency, and *uice versa*, the uptake or the translocation of sucrose for the growth of excised ovules of *P. hybrida* will not proceed normally.

Acknowledgment

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Petunia hybrida のはい珠培養における鉄，ホウ酸，および糖が発芽種子数におよぼす影響

新　美　芳　二
（新潟大学農学部）

摘　要

自家不和合性 *Petunia hybrida* の 2 系統，W 116 H と K 146 BH の交雑受粉 3 日後すなわち受精直後の胎座つきはい珠の培養を行ない，鉄，ホウ酸，および糖の種類ならびに濃度の発芽種子数におよぼす影響について検討した．

基本培地が鉄源としてクエン酸第二鉄を含む場合は pH 4.5～5.0，Fe-EDTA を含む場合は pH 6.0 付近が他の pH 値よりもよかつた．また鉄の濃度を従来より高めると発芽種子数が増加することもわかつた．

基本培地がクエン酸第二鉄 10 mg/*l* または Fe-EDTA（鉄として約 2.5 ppm）を含有した場合，ホウ酸の濃度を高めるとともに発芽種子数は増加し 50 mg/*l* が最適濃度であつた．また鉄の種類が違うことによりホウ酸の効果に差が生じ，Fe-EDTA を含有する培地のほうがクエン酸第二鉄を含む培地よりもその効果が大きかつた．

基本培地においてはい珠の発達に効果のあつた糖はしよ糖と果糖であつた．しよ糖 7.5% と果糖 5% を含有した培地間では実生数において差はなかつたが，実生の大きさに関してしよ糖を含む培地から得られた実生は果糖を含む培地から得られたものより大きかつた．

以上の実験結果にもとづき，各要素の相互効果を検討したところ，Fe·EDTA（$FeSO_4$-7 H_2O 50 mg/*l* + Na_2-EDTA 40 mg/*l*），ホウ酸 25 mg/*l*，しよ糖 7.5% を含む培地でもつとも多くの発芽種子がえられ，子房あたり約 5 から 6 本であつた．それは基本培地から得られたものと比較して約 6 倍であつた．また培地がしよ糖の代わりに果糖を含有する場合，ホウ素および鉄の濃度を高めても発芽種子数は増加しなかつた．