Effect of Cultural Condition on the *in vitro* Development of Ovules in *Petunia hybrida* Vilm.

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Synopsis. This experiment was carried out to find a cultural condition for the *in vitro* development of the young ovules of *Petunia hybrida* Vilm.. The ovules excised immediately after fertilization were successfully cultured on the medium supplemented cucumber fruit-juice and adjusted to the favourable osmotic value.

Introduction

Ovule culture is an important means for the physiological studies on the seed development, moreover, it has been found to be a fundamental study for the new technique on plant breeding such as test-tube fertilization (KANTA *et al.* 1963, KAMEYA *et al.* 1970), overcoming self-incompatibility (RANGASWAMY *et al.* 1967) and inducing the division of unfertilized egg (SACHAR *et al.* 1968). These technique, however, have not yet used on plant breeding, because the technique of culturing ovules *in vitro* was not established in many species.

In the first success of ovule culture in *Papaver somniferum*, MAHESHWARI showed that it had been possible to grow the individual ovules excised at the stage of zygote or 2-celled proembryo to maturity (MAHESHWARI 1958, MAHESHWARI *et al.* 1961). In this case, however, the ovules were excised not immediately after fertilization, but 6 days after pollination, when many free endosperm nuclei were produced. Ovules of some other species have been cultured in more recent years by several investigators. An important fact emerging from these reports is that the ovules excised from placenta immediately after fertilization are hardly developed on the artificial medium. Only a successful attempts have been made to obtain mature seeds from young ovules excised from placenta soon after fertilization, i.e. on *Zephyranthes* (KAPOOR 1959), *Trifolium* (NAKAJIMA *et al.*

1969) and *Brassica* (KAMEYA *et al.* 1970). In these success, it was revealed that some plant juices, such as coconut milk in *Zephyranthes* and cucumber fruit-juice in *Trifolium* were effective on the development of young ovules.

In *Petunia* sp., the considerable success has been achieved in culturing young ovules with the whole of central placenta (RANGASWAMY *et al.* 1967, NIIMI 1971). However, the successful development of the young ovules excised separately from placenta soon after fertilization has not been obtained. The present experiment was carried out to find a favourable condition for the *in vitro* development of the excised young ovules of *Petunia hybrida* Vilm.

Materials and Methods

A strain of *Petunia hybrida* Vilm. served as experimental material. In this strain the time at which a pollen tube arrive in a ovule was examined by phase contrast optics (after HERR 1971). The pollen tube did not arrive in the ovule during 24 h after anthesis. And most of embryo sac was penetrated with a pollen tube by 48 h after pollination. Zygote and endosperm nucleus were observed in the ovule excised 3 days after pollination, through paraffin section. At the next day, ovule contained zygote and several endosperm cells. Small globular embryo was observed at 7th day, and mature seeds were obtained in about 30 days after pollination. On the basis of these findings, artificially pollinated flowers were collected 2, 3, 4 and 7 days after pollination. After removing their calyx and corolla ovaries

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were surface sterilized by Ca-hypochlorite solution (100 g/l) for 10 min and rinsed with sterile water. The ovary was then carefully excised without damaging the ovules under aseptic conditions, and ovules excised from placenta with a fine forceps were implanted liberally in test-tube containing 10 ml of sterile medium.

The basic medium comprised solution of minerals (after NITSCH 1951), vitamins and amino acids (after MAHESHWARI *et al.* 1961) and 0.8% agar. Sucrose concentrations were modified. The pH of medium was adjusted to 5.8 before autoclaving. In some experiments the medium was supplemented cucumber fruit-juice prepared from young fruits of *Cucumis sativus* L. (after NAKAJIMA *et al.* 1969). For adjusting the osmotic value of the medium the osmotic value of the cucumber fruit-juice was estimated by examining the freezing point of it (osmometer).

The cultures were kept at $25 \pm 1^\circ\text{C}$ in dark. The cultured ovules were observed after 30 days through dissecting.

Experimental Results

1. Influence of varying concentration of sucrose on the development of ovules.

The ovules of *Petunia hybrida* Vilm. excised 3 days, 4 days and 7 days after pollination were cultured on the media of different concentrations of sucrose. The results were shown in Fig. 1. The ovules excised 7 days after pollination could be developed wherever they had been cultured on the medium containing

Fig. 1. *In vitro* developments of ovules with different concentrations of sucrose in medium.

Twenty ovules excised 4 days after pollination (4 dap) and 60 ovules excised 3 days after pollination (3 dap) were cultured for each treatment and the both were cultured for 30 days.

4~10% sucrose. And the ovules excised 3 days after pollination grew slightly but a few ovules on the medium containing 8% sucrose were developed to mature seeds. The ovules excised 4 days after pollination, however, were remarkably affected with sucrose concentration. The most mature seeds were obtained, when they were cultured on the medium with 6% sucrose. In this case, more than 20% of those young ovules could developed to maturity on the medium. On the other hand, on the media containing 4%, 9% and 10% sucrose, a few of them developed to mature seeds.

2. Development of ovules on the medium added cucumber fruit-juice.

As the high osmotic value of the cucumber fruit-juice seems to affect the development of cultured ovules, the osmotic value of the juice prepared from cucumber fruits at different stages were investigated. The freezing point of the the fruit-juice was compared with of sucrose solution established concentration (Fig. 2). Then it was observed that the osmotic value of the juice prepared from younger fruits was higher than that from older ones.

The cucumber fruit-juice used through the present experiment was prepared from about 10 day-old fruits. Fig. 2 shows that its osmotic value is equivalent to that of 8% sucrose solution. On the basis of this, the osmotic value of the medium containing cucumber fruit-juice was adjusted to be equivalent to that of the medium without the juice, through decreasing sucrose concentration.

On the media containing different concentrations of cucumber fruit-juice (15, 20 and 25% v/v), the ovule excised 4 days after pollination

Fig. 2. Changes in the osmotic value of cucumber juice prepared from fruits at different stages of development.

were cultured. The osmotic value of each medium was adjusted to be equivalent to that of 6% sucrose solution by above-mentioned means. The results of the experiment are shown in Fig. 3. Although the medium added 25% cucumber fruit-juice had contained only 4% sucrose, the ovules could develop as well as the ovules on the medium containing 6% sucrose. And in this experiment that the media were adjusted to the favourable osmotic value, the concentrations of sucrose and cucumber fruit-juice did not bring into relation with the percentage of mature seeds obtained.

By the way, on the medium added cucumber fruit-juice the development of the ovules was abnormal (Fig. 4). A dehiscence of integument was observed in about 10 days after inoculation. The endosperm protruding from the integument onto the surface of medium grew rapidly and vigorously. This was followed by the rapid growth and germination of the torpedo stage embryo parting with the endosperm. As the poor development of integument was recognized in microtome preparations of cultured ovules, it was revealed that the dehiscence of integument followed the

Fig. 3. The developments of ovules excised 4 days after pollination on the media supplemented cucumber juice.

Each medium was constant in osmotic value (equivalent to NITSCH's medium containing 6% sucrose). Eighty-five ovules were cultured for each treatment and cultured for 30 days. The open bar represents ovules producing endosperm, the closed bar shows ovules having a mature embryo.

The osmotic value of medium in this and Fig. 5 was checked up with osmometer.

Fig. 4. Abnormal growth of ovules on the medium containing cucumber fruit-juice.

Ovules excised 4 days after pollination were cultured for 20 days. E; Embryo, En; Endosperm, In; Integument.

rapid and vigorous growth of endosperm.

3. Development of the young ovules excised immediately after fertilization.

As the above-mentioned result, when the ovules excised 3 days after pollination were cultured on the basal medium containing 8% sucrose, a few of them developed to mature seeds. To obtain more seeds, those ovules were inoculated on the media added cucumber fruit-juice (5%) and different concentrations of sucrose. The osmotic value of each medium was adjusted to be equivalent to that of established sucrose solution through decreasing sucrose concentration. When the osmotic value of the medium had been adjusted to be same as 8% sucrose solution, more than 40% of the ovules inoculated developed to maturity (Fig. 5). In this case, the development of endosperm was observed in 55% of the ovules inoculated. Also, the abnormality on the development of the ovules such as mentioned before was recognized. And endosperm and embryo grew more rapidly than one cultured on the medium without cucumber fruit-juice or one of *in vivo*. Some seedlings could be observed 20 days after inoculation. On the other hand, on the media of which osmotic value were equivalent to 7% or 9% sucrose solution the number of developing ovules sharply decreased.

Fig. 5. The influence of osmotic value of medium on developments of ovules collected 3 days after pollination and cultured for 30 days. Thirty ovules were cultured for each treatment. Each medium contains cucumber juice (5%). Osmotic value of medium expressed by its equivalent in sucrose.
 ●—●; Ovules producing endosperm and embryo (mature seed).
 ○—○; Ovules producing endosperm.

Some ovules excised immediately after fertilization (48 h after pollination) were also cultured on similar media and developments of ovules were investigated. In this case, endosperm in some of the ovules developed on the basal medium containing 8% and 9% sucrose (Table 1). Moreover, on the medium added cucumber fruit-juice (5%) a few embryo developed, and two seedlings were obtained on the same medium.

Discussion

In this experiment, it observed that the range of favourable concentration of sucrose for culturing young ovules was very limited. The medium containing 6% sucrose was most favourable for development of the free ovules excised 4 days after pollination. And also, when osmotic value of the medium were adjusted to equivalent to 6% sucrose solution by adding cucumber fruit-juice, the ovules were developed at same rate of the former. Therefore, it is recognized that high osmotic value of the medium act upon the development of young

Table 1. *In vitro* developments of ovules excised 2 days after pollination. The ovules were cultured for 30 days

Medium	Ovules per treatment No. (%)	Ovules developed* No. (%)	Remark
BM + Sucrose (%)			
5	65	0 (0)	
6	66	3 (4.5)	
7	64	3 (4.7)	
8	49	8 (16.3)	
9	92	14 (15.2)	
BM + CJ 5% + Sucrose (%)			
5	116	1 (0.9)	
6	96	2 (2.1)	
7	71	6 (8.5)	2 seedlings
8	116	1 (0.9)	
9	126	8 (6.3)	2 torpedo-embryos

* Ovules producing endosperm, including ovules producing endosperm-like tissue recognized through dissection.

BM; Basic medium, CJ; Cucumber fruit-juice.

ovules. Recently, AMMIRATO *et al.* (1971) also reported that a development of embryoid from cultured cell took place under the influence of a high osmotic value of medium. And also RYCZKOWSKI (1960) and AMMIRATO *et al.* (1971) showed that the osmotic value was very high in the central vacuole of a embryo sac.

On the other hand, when the ovules excised prior to the appearing of endosperm consisting of several cells (3 days after pollination) were inoculated, the most favourable osmotic value of the medium was equivalent to 8% sucrose solution which was higher than one for culturing the ovules having several celled endosperm (4 days after pollination). KAMEYA *et al.* (1970) also reported the same fact; younger ovules of *Brassica* sp. required a higher sucrose concentration than older one. On the studies of embryo culture, results showing that young embryo required a high sucrose concentration have appesred (RIETSEMA *et al.* 1953, SANDERS *et al.* 1963). The change of osmotic value of the juice prepeared from cucumber fruits at different stages showed also same pettern. Moreover, it was observed that the range of osmotic value for culturing younger ovules was narrow than that for older one. Therefore, it is recognized that the osmotic value of medium affects more remarkably the development

of younger ovules. So for culturing young ovules such as one excised soon after fertilization, the osmotic value of a medium seems to be one of the important factors.

In the experiment of culturing ovules excised soon after fertilization, even though the osmotic value of the medium was adjusted favourably, the cucumber fruit-juice was effective. And if the excised ovules had produced endosperm consisting of several cells, the development of the ovules carried out on the medium without those juice. These findings show the factor/s in the fruit-juice assist in the initial growth of embryo and endosperm of *Petunia* as recently reported on ovule culture of *Trifolium repens* (NAKAJIMA *et al.* 1969). In addition to that, it was observed that cucumber fruit-juice accelerated the growth of endosperm and embryo, but not of integument. On ovule culture, therefore, it is considered that the conditions for developments of embryo and endosperm differ from one for the growth of integument.

In conclusion, it appears that the cultural conditions, i. e. osmotic value as an environmental factor and substance/s such as one in cucumber fruit-juice play a role for the *in vitro* development of young ovules.

Summary

The successful development of the young ovules excised separately from placenta soon after fertilization has not been obtained in *Petunia* sp.. This experiment was carried out to find a favourable condition for the *in vitro* development of the excised young ovules of *Petunia hybrida* Vilm. The results are as follow :

(1) The medium containing 6% sucrose was most favourable for development of the free ovules excised 4 days after pollination. And also when osmotic value of the medium were adjusted to equivalent to 6% sucrose solution by adding cucumber fruit-juice, the ovules were developed at same rate of the former. Therefore, it is recognized that high osmotic value of the medium act upon the development of young ovules.

(2) When the ovules excised 3 days after pollination were inoculated, the most favourable osmotic value of a medium was equivalent to 8% sucrose solution which was higher than

one for culturing the ovules excised 4 days after pollination. Moreover, it was observed that the range of osmotic value for culturing younger ovules was narrow than that for older one. Therefore, it is recognized the osmotic value of medium affects more remarkably the development of younger ovules.

(3) In the experiment of culturing ovules excised immediately after fertilization (2 days and 3 days after pollination), even though the osmotic value of the medium was adjusted favourably, the cucumber fruit-juice was effective for development of embryo and endosperm. Through the experiment, the ovules excised immediately after fertilization were successfully cultured on the medium supplemented cucumber fruit-juice and adjusted to the favourable osmotic value.

(4) In conclusion, it appears that the cultural conditions, i. e. osmotic value of medium as an environmental factor and substance/s such as one in cucumber fruit-juice play a role for the *in vitro* development of young ovules.

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ペチュニア胚珠の培養条件について

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胚珠培養法は種子形成の機構を明らかにするための有効な手段である。また試験管内受精など育種技術の基礎研究にとっても重要であると考えられている。しかしながら現在のところ、胚珠の培養法が確立されているとは言いがたい状態である。そこで、本実験では胚珠培養法を確立するための基礎研究として、ペチュニア (*Petunia hybrida* Vilm.) の胚珠培養を行い、培養胚珠の発育に影響する培地条件について検討した。

受粉後7日目の胚珠には球状胚が認められる。この程度にまで発育した胚珠は無機塩類、ビタミン類および蔗糖を含む簡単な培地でよく発育することが認められた。とくに、培地の蔗糖濃度を種々(4~10%)に変えてみたが、いずれの濃度でも置床した胚珠のほとんど大部分が発育して完熟種子となった。

受粉後4日目の胚珠には、接合子と数細胞からなる胚乳が認められる。このような胚珠は培地の蔗糖濃度の影響を受け、蔗糖6%の培地でもっともよく発育したのに対し、蔗糖濃度がそれより高い場合も低い場合も完熟する種子は減少した。とくに蔗糖4%、9%の培地では、ほとんど完熟種子が得られなかった。

幼胚珠の培養にキュウリ果汁が有効であることが知られているので、キュウリ果汁の浸透価を調べてみた。若い果実からの果汁の方がより高い浸透価を示し、胚珠培養に用いられている開花後10日目程度の果実からの果汁は蔗糖8%に相当する浸透価を持っていることが明らかになった。この事実にもとづき、培地に種々な濃度のキュウリ果汁を加え、培地の浸透価が蔗糖6%相当になるように加える蔗糖の濃度をおとした培地を調整した。たとえばキュウリ果汁25%を含む培地では蔗糖は4%しか加えていないのであるが、このような培地で受粉後4日目の胚珠を培養したところ、いずれの培地でもほぼ同程度の率で完熟種子が得られた。すなわち、受粉後4

日目の胚珠では浸透価が適当ならば、蔗糖濃度やキュウリ果汁の濃度は胚珠の発育に影響をほとんどおよぼさないことが明らかになった。

受粉後3日目の胚珠には胚乳の発育もほとんどみられない。このような胚珠は蔗糖濃度(浸透価)を種々かえても、キュウリ果汁を含まない培地では完熟種子はほとんど得られなかった。キュウリ果汁5%と種々な濃度の蔗糖を加えることによって、種々な浸透価を示す培地を調整して受粉後3日目の胚珠を培養したところ、蔗糖8%相当の浸透価を示す培地で、もっとも多くの完熟種子(40%)が得られた。培地の浸透価が蔗糖8%相当よりも、わずかに増減しても、発育する胚珠は著しく低下した。

位相差顕微鏡による観察から真に受精直後と認められた受粉後2日目の胚珠も、キュウリ果汁を添加した高い浸透価を示す培地上で、極めてわずかではあるが完熟種子となった。

以上の結果から、受粉後日数の少ない胚珠ほど、より高い培地の浸透価を要求すること、また培地の浸透価により影響を受けやすいことが明らかになった。

一方、受粉後3日目および4日目の胚珠について共通して認められたのであるが、キュウリ果汁を含む培地では胚乳および胚の急速な発育が認められた。すなわち急速に発育した胚乳は珠皮を破って培地上に露出し、その後胚乳から離れた幼胚も培地に直接接して急速に発育することが観察された。このような急速な発育の結果、子房内では完熟種子にいたるに約30日間を要するのに対し、培地上では約20日後に発芽個体が得られたのであった。したがって、上述の培地の浸透価のみならず、受粉後日数の少ない胚珠の培養には、このようなキュウリ果汁中の胚乳、胚の発育を促進する要因もまた重要な条件の一つと考えられる。