IN VITRO CULTURE OF EXCISED OVULES OF WHITE CLOVER, *TRIFOLIUM REPENS* L.

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Synopsis. Many of the ovules excised 4~5 days after anthesis could develop to mature seeds on Nitsch's medium containing yeast extract and NAA. On the other hand, some mature seeds were obtained from the young ovules excised immediately after fertilization, only when they were cultured on the medium supplemented by fruit juice prepared from young fruit of cucumber or water melon.

The ovule culture has been found not only to be an important tool for the fundamental studies on the utilization of "test-tube fertilization" to plant breeding, but also to be a useful method for the physiological studies on the seed formation, dormancy and germination. In recent years, extensive works on the ovule culture have been undertaken mainly by several investigators in Delhi University, India. However, it seems difficult to obtain mature seeds developed from individual young ovules excised separately from placentae soon after fertilization such as one day after pollination by culture *in vitro*.

In consideration with these facts, the present investigation was carried out to find a favorable medium for the development of young ovules of white clover, *Trifolium repens* L. *in vitro*.

Materials and Methods

The growth patterns of ovary, ovule and embryo of white clover var. Ladino *in vivo* are shown in Fig. 1. Ovary and ovule grew to maximum size (5 mm and 1.4 mm in length respectively) in about 12 days after anthesis** and embryo grew to maximum size (1.5 mm) in 16 days after anthesis. Zygote was observed in an ovule excised one day after

anthesis (Fig. 2-A) and 2-celled proembryo could be seen in 2 days-old ovule. A 5 days-old ovule contained an early globular embryo (Fig. 2-B). On the basis of these findings, the ovaries one to five days after anthesis were sterilized by Ca-hypochlorite solution and dissected under binocular for ovule culture. The ovules were transplanted singly in test-tube containing medium and cultured for three weeks.

NITSCH's medium for ovary culture which was composed of minerals, 5% sucrose and 1% agar (NITSCH, 1951) was used as the basic medium. Various concentrations of IAA (Indole-3-acetic acid), NAA (α -naphthalene acetic acid), kinetin (6-furfurylamino purine), gibberellin (GA_3) and yeast extract (Difco's) were added either separately or in various combinations to the basic medium. In some experiments, the media were supplemented

Fig. 1. Growth patterns of ovary, ovule and embryo of white clover *in vivo*.

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** The starting point of the development of ovule is denoted in term of anthesis because artificial pollination is rather difficult in white clover, therefore, the term "days after anthesis" do not strictly mean the days after pollination in this investigation.

Fig. 2. Developmental stages of embryo *in vivo*.

A : zygote (one day after anthesis)

B : globular embryo (5 days after anthesis)

separately with various fruit juices such as cucumber juice (*Cucumis sativus* L.), water melon juice (*Citrullus vulgaris* SCHRAD), tomato juice (*Lycopersicum esculentum* MILL.) and apple juice (*Malus pumila* MILLER var. *domestica* SCHNEIDER). The cucumber juice was obtained by crushing the whole fruit which was harvested about 10 days after anthesis. Then it was sterilized by Milliporefilter and added to the basic medium aseptically. The other fruit juices were also prepared from young fruits in the same manner.

The pH of media was adjusted to 5.8~6.8 before autoclaving in all the experiments.

Experimental Results

1) Development of ovules on various media.

As it had been known that the excised ovule having globular or more developed embryo in many plant species could develop to mature seed on the medium (JOHRI *et al.*, 1963), the ovules of white clover excised 5 days after anthesis which contained globular embryo were cultured on the media supplemented separately by the following substances of various concentration: IAA, NAA, kinetin, gibberellin and yeast extract. On the medium containing yeast extract (1000 mg/l) or NAA (1 mg/l), many ovules could be developed to the size of maturity in length and some of their embryos grew well. A few seeds were also obtained even on the basic medium. On the other hand, the other substances such as IAA, kinetin and gibberellin were not effective.

In the light of these results, the ovules excised 5 days after anthesis were cultured

on the various media containing kinetin and gibberellin together with yeast extract and NAA. The results are shown in Table 1. The size of cultured seeds on all these media was nearly mature-one in an ovary. Well developed embryos were obtained in these seeds and some of them germinated during the course of their culture. Especially, more than 60% of the cultured ovules developed to beconemature seeds, when they had been placed on the medium containing yeast extract and NAA.

Table 2 shows the results of experiments where the ovules excised 4 days and 3 days after anthesis were cultured on various media. On the medium holding both NAA and yeast extract, more than 50% of the young ovules excised 4 days after anthesis could develop to mature seeds. On the other media, some of the ovules also developed to seeds. In the case of ovules excised 3 days after anthesis, a few of them developed to complete seeds, when they were cultured only on the media containing yeast extract or both yeast extract and NAA, but none of them could develop to seeds on the other media.

On the contrary, when the younger ovules such as two days or one day after anthesis were put on the same media as above mentioned, the mature seeds could not be obtained.

These findings evidently indicated that yeast extract and NAA are effective to the development of excised ovules of white clover, such as ovules excised five or four days after anthesis and that NAA stimulates the development of embryo in the cultured ovules.

Table 1. In vitro development of ovules excised 5 days after anthesis on various media

Media	No. of ovules cultured	Average length of seeds (mm)	No. of mature seeds* obtained	No. of germinated seeds
BM	10	1.10±0.08	4 (40 %)	0
+YE	30	1.26±0.10	10 (33.3%)	2
+NAA	30	1.37±0.09	16 (53.3%)	5
+YE, NAA	30	1.30±0.08	19 (63.3%)	2
+YE, GA	31	1.24±0.09	14 (45.1%)	1
+YE, NAA, GA	23	1.24±0.11	4 (17.4%)	0
+YE, NAA, K	30	1.37±0.11	11 (36.7%)	3
+YE, NAA, GA, K	31	1.32±0.11	17 (54.8%)	0

BM : Nitsch's medium (minerals, 5% sucrose, 1% agar) ; YE : yeast extract 1000 mg/l ; NAA : α -naphthalenacetic acid 1 mg/l ; GA : gibberellic acid 10 mg/l ; K : kinetin 0.01 mg/l

* mature seed : having developed embryo.

Table 2. In vitro development of young ovules on various media

Media	4 days after anthesis		3 days after anthesis	
	Average length of seeds (mm)	% of mature seeds obtained	Average length of seeds (mm)	% of mature seeds obtained
BM+YE	1.11±0.09	25.8	0.85±0.12	20.0
+NAA	0.99±0.14	18.4	1.00±0.13	0
+YE, NAA	1.27±0.10	55.6	0.95±0.11	18.0
+YE, NAA, GA	1.15±0.09	12.9	0.91±0.08	0
+YE, NAA, K	1.18±0.08	15.4	0.93±0.08	8.1
+YE, NAA, GA, K	1.15±0.10	25.0	0.94±0.16	0

Explanation of table : see table 1.

2) Effect of various fruit juices on the development of excised ovules.

For the culture of very young ovules which could not be developed on the above mentioned media, various fruit juices such as cucumber juice, water melon juice, tomato juice and apple juice were separately added to the basic medium. Fig. 3 shows the effect of various fruit juices on the development of ovules excised three days after anthesis. On the medium supplemented by 20% cucumber juice or 10% water melon juice, more than 50% of the cultured ovules developed to mature seeds. Therefore, it can be said that cucumber juice and water melon juice are effective for the *in vitro* development of young ovule. On the other hand, tomato juice and apple juice were not effective.

The effects of cucumber juice and

water melon juice on the development of ovules excised in younger stage is shown in Fig. 4. This figure depicts that even in the

Fig. 3. Effects of various fruit juices on the development of cultured ovules excised 3 days after anthesis.

Note : degree of the development of ovule is shown by the percentage of mature seeds having developed embryo.

Fig. 4. Effect of cucumber juice and water melon juice on the development of excised ovule.

Note : see Fig. 3.

Fig. 5. Effect of cucumber juice and gibberellic acid on the development of excised ovules.

CJ : Nitsch's medium + cucumber juice 10%
CJ+GA : Nitsch's medium + cucumber juice 10% + GA₃ 10 mg/l

case of ovules excised one day after anthesis, some of the cultured ovules on these media could develop to mature seeds, especially more than 10% of cultured ovules had well developed embryos on the cucumber juice-medium. From these experiments, it was clear that both of the fruit juices were effective for the development of cultured ovules excised in very young stage. Furthermore,

cucumber juice was more effective than water melon juice.

The effects of cucumber juice combined with some growth substances were examined on the development of ovules excised one day to five days after anthesis. The results obtained in the case of GA₃ are shown Fig. 5. From this figure, it is clear that gibberellic acid accelerated the growth of cultured ovules. Especially, mature seeds obtained in high percentage such as 90% of cultured ovules in the case of 5 days-old ovules.

The other growth substances, namely NAA and kinetin were also examined in the same manner. But these substances rather inhibited the effect of cucumber juice on the growth of cultured ovules.

From these results, it was assumed that growth factor/s for the development of young ovule including embryogenesis of white clover are contained in cucumber juice and water melon juice and that gibberellic acid accelerates the action of such factor/s.

Discussion

Since the first success of ovule culture in *Papaver somniferum* (N. MAHESHWARI, 1958; N. MAHESHWARI *et al.*, 1961), many reports have appeared also on *Cooperia pedunculata* (SACHAR *et al.*, 1958), *Citrus microcarpa* (RANGASWAMY,

1959), *Zephyranthes* sp. (SACHAR *et al.*, 1959; KAPOOR, 1959), *Gossypium herbaceum* (JOSHI, 1962), *Gynandropsis gynandra* and *Impatiens balsamina* (CHOPRA *et al.* 1963), *Citrus reticulata* (SABHARWAL, 1963), *Nicotiana tabacum* (DULIEU, 1966) and *Allium cepa* (GUHA *et al.*, 1966). But except for *Papaver* and *Zephyranthes* in which the ovules containing zygote or 2-celled embryo could develop to mature seeds (N. MAHESHWARI *et al.*, 1961; KAPOOR, 1959), none of the cultured ovules could be developed to maturity when they were excised separately from placentae immediately after fertilization. In many cases, it seems that the ovule having globular embryo or a more developed one can comparatively easily develop to seed, but younger ovules are hard to develop under the *in vitro* culture. Also in the present experiment with *Trifolium*, some of the ovules containing globular embryo could mature into seeds even when they were planted on Nitsch's medium only. The mature seeds were obtained from the very young ovules excised one day or two days after anthesis only on the medium supplemented by fruit juice prepared from young fruit of cucumber or water melon. This fact suggest that the nutritional and hormonal requirements of ovules vary with their developmental stages, especially very young ovules are sensitive to these requirements.

On the other hand, in the case of culturing the ovules with placentae such as "test-tube fertilization" (KANTA *et al.*, 1963; RANGAWAMY *et al.*, 1967) and in many cases of ovary culture (JOHRI *et al.*, 1963; P. MAHESHWARI *et al.*, 1966), some viable seeds were obtained from very young ovules. These findings show that the placenta and other parts of ovary play an important role in the stimulation to the development of the just fertilized ovules. From this point of view, it can be said that the interest is focussed on the remarkable effect of coconut milk for *Zephyranthes* (KAPOOR, 1959), of ovule extract for *Gossypium* (JOSHI, 1962) and of fruit juice for *Trifolium* (this experiment).

In this experiment, it was recognized that gibberellic acid accelerated the action of fruit juice on the development of very young ovules. S. MAHESHWARI *et al.* (1966 a, b) showed

that fruit juice of water melon contains gibberellin-like factors and also kinin-like factor/s. On the other hand, changes in the amount of gibberellin-like substances during the course of seed development were reported in several plants (CORCORAN *et al.*, 1962; OGAWA, 1963 a, b; S. MAHESHWARI *et al.*, 1965). Especially, S. MAHESHWARI *et al.* clearly showed that the contents of gibberellin-like substances in seed became to maximum in the early stage of embryo development. From these findings, it is supposed that gibberellin plays an important role in ovule development, and that the above mentioned factor/s in fruit juice for the development of cultured ovules are related with gibberellin. However, in culturing very young ovules, no mature seeds could be obtained on the medium added separately gibberellin and also kinetin.

It is considered that analysis of these factor/s in fruit juice is needed in *in vitro* culture of excised ovules and also to the physiological analysis on seed development.

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白クローバーの胚珠培養

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“試験管内受精法”を育種に利用するための基礎として、白クローバーの胚珠培養を試みた。

開花後 4~5 日を経過した胚珠には、初期球状胚が認められるが、このような胚珠を胎座から切り取って培養すると、NITSCH の培地だけでも若干の成熟種子が得られたが、イーストエキスおよび NAA を添加した培地では、よく発育して多数の発芽可能の種子が得られた。これに反し受精直後の胚珠の培養では、上述の培地でも、またさらにジベレリン、カイネチンなどを組合せても、全く成熟種子が得られなかった。

受精直後の胚珠を培養するために、種々な植物の未熟果実から得た果汁を培地に加えたところ、キュウリおよびスイカの果汁は顕著な効果を示し、開花翌日の胚珠も、わずかではあるが発育して、発芽可能の種子となった。また、キュウリ果汁にジベレリンを組合せた培地では、開花後 5 日目の胚珠は、実にその 90% が成熟種子に発育した。

このような実験から、キュウリあるいはスイカの未熟果実中に、胚珠の発育に有効な物質が存在すると考えられた。