J. Japan. Soc. Hort. Sci. 45(2) : 168—172. 1976.

Effect of "Stylar Pollination" on *in vitro* Seed Setting of *Petunia hybrida*

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Summary

The present investigation deals with the effect of pollination on formation of mature seeds on excised placenta cultivated *in vitro*.

Artificial pollination was performed by the two following methods : (i) pollen grains were dusted directly onto the ovules with a placenta (placental pollination) ; (ii) styles were excised aseptically, cut into about 7 to 10 mm in length after applying pollen grains onto the stigma, and then were inoculated on or around ovules with a placenta (stylar pollination).

Placental pollination. In self- and cross-pollination of the clone W166H, 3.1 and 2.9 mature seeds per ovary were obtained, respectively. The mature seeds which were detached from the placenta were sown on a fresh medium containing agar alone, and then some of them germinated.

Germinating pollen grains were observed on both self- and cross-pollination explants of the clone K146BH, but neither of the combinations produced mature seeds.

Stylar pollination. A number of mature seeds were obtained by self- and cross-pollination of clone W166H, that is, 12.2 seeds per ovary in self-pollination and 11.5 seeds in cross-pollination, respectively, were obtained. About one-fourth of these seeds germinated.

In self- and cross-pollination of clone K146BH, 5.2 and 5.9 mature seeds per ovary were obtained, respectively. Some of these seeds which detached from the placenta were germinated normally on a fresh medium containing agar only.

It was concluded that the number of mature seeds per ovary depends upon the technique of pollination, that is, stylar pollination was substantially more successful than the placental pollination.

Introduction

The overcoming of self- and cross-incompatibility has been undertaken by several workers(1, 4, 6, 11, 12, 13) since the technique of test-tube fertilization was developed by Kanta *et al* (1962). Some of these studies have been proved to be successful in several species.

It has been reported in the previous paper(6) that self-incompatibility of *Petunia hybrida* canbe overcome by test-tube fertilization. However, the number of seeds formed after fertilization *in vitro* was relatively small and very variable. This appeared to be caused by various factors, for instance, the technique of pollen application and the composition of nutrient medium on the development of fertilized ovules. Appro- priate composition for the nutrient medium has been investigated through ovary and ovule culture of this species(7, 8, 9).

The inhibition of pollen tube growth due to the self-incompatibility of *P. hybrida*, regularly occurs when pollen tubes elongate about 15 to 18 mm in length(3). Higuchi (1969) reported that, when excised styles of the clone W166H of *P. hybrida* were *in vitro* self- and cross-pollinated and were cultured on a certain medium, many pollen tubes were observed at the cut end of styles(3). The author modified his technique and employed it as a mean of pollen application for the test-tube fertilization. This paper describes an attempt at obtaining viable seeds and for overcoming self-incompatibility by a refined

Received for publication June 16, 1975

technique, "stylar pollination".

Materials and Methods

The two self-incompatible clones of *Petunia hybrida*, W166H (S_2, S_3), K146BH (S_6, S_6) grown in a phytotoron at 23±1°C, were used for the experiments.

The ovary was excised from the flower one day before anthesis. The ovary wall was cut off with a sterile tweezers after being surface-sterilized with 10 % hypochlorite for 10 minutes. After the bare ovary was vertically cut into halves, the two pieces were placed into the same flask containing the culture medium.

Artificial pollination was performed by the following two methods : (i) pollen grains were dusted directly onto the ovules with a placenta (placental pollination); (ii) styles were excised aseptically, cut to about 7 to 10 mm in length after applying pollen grains onto the stigma, and then were inoculated on or a round the ovules with a placenta (stylar pollination).

Aseptic pollen grains were obtained by techniques similar to those described in the previous paper(6). Aseptic styles were obtained as follows : flower buds, one day before anthesis, were surface-sterilized by swabbing with a bsorbent cotton containing 70 % ethyl-alcohol and then the style was excised with a tweezers.

The culture medium was prepared as shown in the preceeding paper(9). The flasks were kept in the culture room of about 25°C under diffuse light for 30 days. The mature seeds thus obtained were then detached from the placenta and were sown on a fresh medium containing 0.5 % agar alone. Normal seedlings with two cotyledons and roots were counted after 10 days of culture.

Results

I. Placental pollination

(1) clone W166H.

Pollen grains of both the clone W166H and the clone K146BH were dusted directly onto the ovules of W 166 H. Three days after self- and cross-pollination, 2 to 5 ovules began to enlarge, and at the 10 th day these ovules were clearly distinguishable from the atrophied ovules. Post-heart embryo and massive endosperm were shown in sectioned preparations of such ovules on the 15 th day after pollination (Fig. 1). This suggests that double fertilization *in vitro* takes place. Thirty one mature seeds in self- and 23 in cross-pollination were yielded on the 30 th day after pollination. Of these seeds 19 in self- and 13 seeds in cross-pollination germinated normally on a medium containing agar only, and normal seedlings with two cotyledons and roots were obtained (Table 1).

(2) clone K146BH.

Pollen grains of K146BH and W166H, respectively, were dusted directly onto the K146BH ovules. Germinating pollen grains were observed on both self- and cross-pollinated explants, but neither of the combinations produced mature seeds.

II. Stylar pollination

It was investigated in the preliminary experiment whether or not pollen tubes grow sufficiently in self- and cross-pollination of the clone W166H and the clone K146BH. Many pollon tubes were observed at the cut end of each style in self- and cross-pollination of the clone W166H and in cross-pollination of the K146BH, but not in self-pollination of the K146BH. Therefore, styles of the clone W166H were used in self-pollination of the K146BH. In such a combination, many pollen tubes were observed at the cut end of those styles.

(1) clone W166H.

Self- and cross-pollination were made by the use of an *in vitro* cultured style. A style was used for one placenta. The K146BH pollen grains were used to perform cross-

Table 1. Effect of placental pollination on *in vitro* seed setting for clone W166H.

	Self-pollination	Cross-pollination*
Number of pollinated ovaries**	20	20
Number of ovaries with mature seeds	10	8
Mean number of seeds per ovary with mature seeds	3.1	2.9
Total number of germinated seeds	19	10
Mean number of seedlings per ovary with mature seeds	1.9	1.2

* The K146BH pollen grains were used.

** The bare ovary was vertically cut into halves, and the two sections were placed onto the culture medium. The two sections were counted as one ovary.

Table 2. Effect of stylar pollinatin on *in vitro* seed setting for clone W166H and clone K146BH.

	Clone W166H		Clone K146BH	
	Self-pollination	Cross-pollination*	Self-pollination	Cross-pollination**
Number of pollinated ovaries***	60	60	40	40
Number of ovaries with mature seeds	50	42	17	26
Mean number of seeds per ovary with mature seeds	12.2	11.5	5.2	5.9
Total number of germinated seeds	172	130	17	78
Mean number of seedlings per ovary with mature seeds	3.1	3.1	1	3

* K146BH pollen grains were used.
** W166H pollen grains were used.
*** The bare ovary was vertically cut into halves, and the two sections were placed onto the culture medium. The two sections were counted as one ovary.

pollination. After inoculation, many pollen tubes were observed at the cut ends of style in both self- and cross-pollination and then were spread onto the ovule surfaces (Fig. 2). Within 10 days many milky-colored enlarged ovules were observed (Fig. 3), and after 30 days the black mature seeds were obtained by self- and cross-pollination. About one-fourth of the seeds obtained germinated on a medium containing agar alone when detached from the placenta (Fig. 4).

(2) clone K146BH.

Styles of the clone W166H were used in self-pollination of the K146BH. By such a combination pollen tube growth was promoted and many pollen tubes were observed at the cut end of styles. However, several ovules which are only present in the vicinity of the cut end of style developed. The number of mature seeds was, therefore, not so many as that of seeds obtained in the self-pollination of the clone W166H (Table 2).

In the stylar cross-pollination of the K146BH, the development of fertilized ovules wassi milar to that of the W166H. Two days after culture, pollen tubes were visible at the cut end of the style. The ovules which were likely to be fertilized began to enlarge on the placenta and thus were clearly distinguishable from the unfertilized ones. About 6 seeds per ovary grew until maturity (Table 2). They germinated immediately when sown on a fresh medium containing agar alone.

Discussion

To attain a good result in the test-tube fertilization, both pollen germination and development of ovules should be taken place successfully on the identical medium. However, polleng ermination of *P. hybrida* is relatively poor on the nutrient media which facilitate the ovules(6). In the present experiment, pollen germination and pollen tube growth were found to be possible by the use of stylar pollination techniques on the same nutriert medium that ovules develop.

Two pollination techniques, stylar pollination and placental pollination, were employed. The former is a more useful technique for obtaining mature seeds than the latter. The latter has been applied by Rangaswamy and Shivanna (1967), and they succeeded in overcoming the self-incompatibility of *Petunia axillaris*(10, 11). According to their experimental results, they dusted pollen grains directly onto the ovules of *P. axillaris*, because among the media there were some differences in the composition of nutrients which were favorable for pollen germination, pollen tube growth and/or development of fertilized ovules. Balatkóva and Túpy (1972) have reported that the number of mature seeds formed per culture was relatively small when the placental pollination technique was applied, and that better results were obtained in case of maintaining certain distance between pollen grains and ovules. They concluded that a great many pollen tubes formed after dusting pollen over the entire placental surface unfavorably affected the development of fertilized ovules. The present author (1970) previously suggested that, when pollen grains were dusted directly onto the ovules, certain substances which diffuse from pollen grains interfere with fertilization and the development of fertilized ovules(6). Due to these factors, it would be reasonable

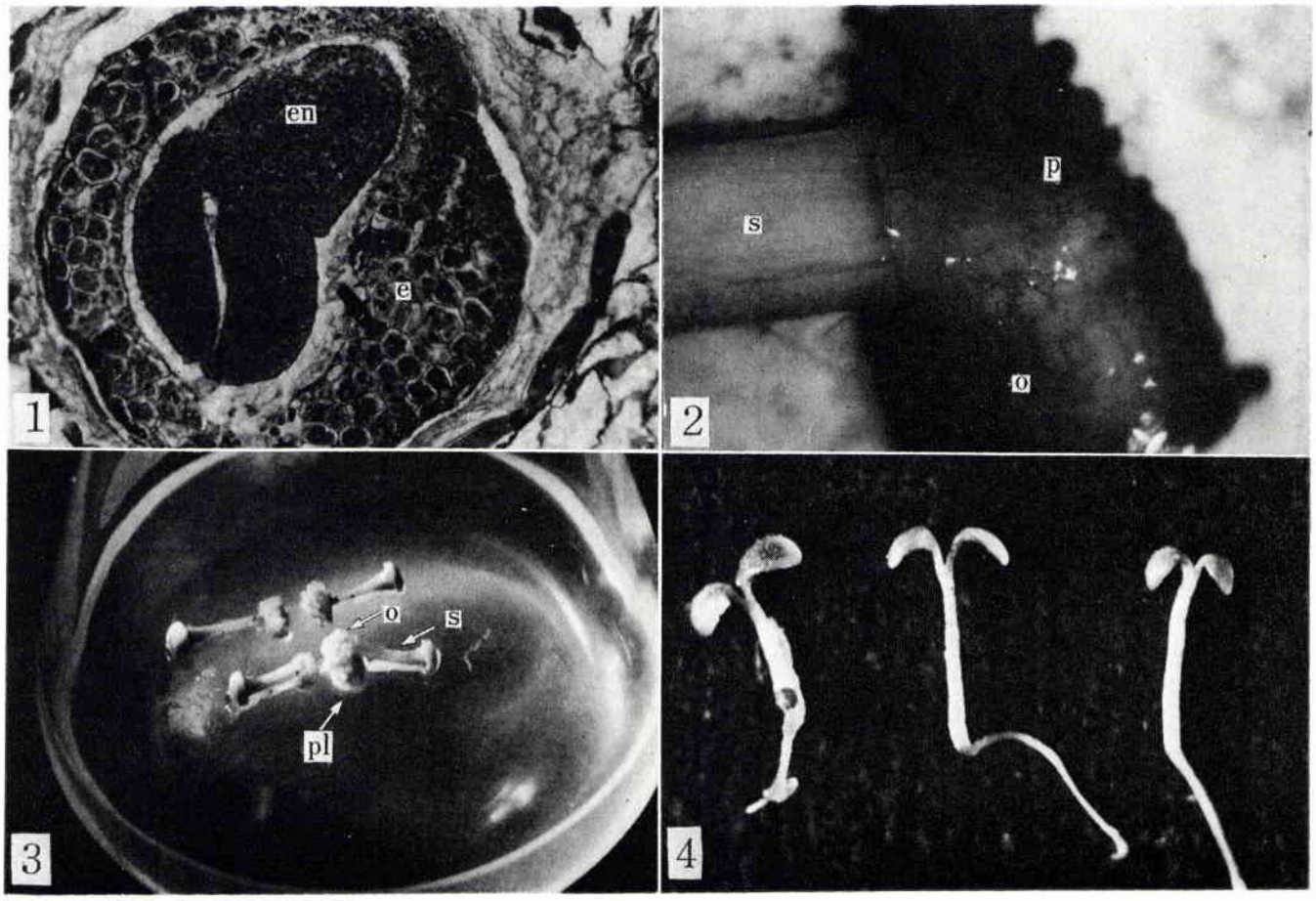

Fig. 1-4. Test-tube fertilization in *Petunia hybrida*, clone W 166 H.

Fig. 1. Longitudinal section of young seed formed 15 days after culture by means of the placental pollination technique, showing post-heart embryo (e) and massive endosperm (en). Fig. 2-4. Pollen tube growth, seed development and normal seedlings in the stylar self-pollination technique : Fig. 2, pollen tubes on the ovules 2 days after inoculation (s ; style, p ; pollen tubes, o ; ovules) ; Fig. 3, ovaries which were cut in half, 10 days after stylar self-pollination, showing enlarged milky-colored ovules (s ; style, o ; ovules, pl ; placenta); Fig. 4, normal seedlings obtained by self-pollination.

to expect that more favorable results might be obtained by the use of the stylar pollination technique. It was shown in the present experiment that stylar pollination overcame certain faults in the placental pollination technique which have been observed by Balatkóva and Túpy (1972) and Niimi (1970). Furthermore, stylar pollination was superior to placental pollination in a few respects : (i) pollen tubes elongate greatly on a relatively complex medium ; (ii) the division of generative nucleus occurs normally during pollen tube growth in the style.

According to the experiments using stylar pollination (Table 2), there is a little difference in the number of mature seeds between self- and cross-pollination for both clone W166H and clone K146BH, while there is a great difference between clone W166H and clone K146BH. In addition, the *in vitro* setting of mature seeds was very low even for cross-pollination in comparison with *in vivo* setting. This may be due to various factors, for instance, the lack of nutrient elements necessary for development of fertilized ovules. Therefore, the growth factors of *in vivo* fertilized ovules must be clarified from a physiological point of view to attain the most suitable composition for the nutrient medium.

Acknowledgement

The author is grateful to Profs. Y. Kato and T. Takano for a critical reading of the manuscript.

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Petunia hybrida の自家不和合性打破に関する新法

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摘　要

自家不和性植物 *Petunia hybrida*, 系統 W 166 H (S_2S_3) と系統 K 146 BH (S_6S_6) を用いて試験管内受精を行なつた．受粉法として次の2方法を用いた：(i) Rangaswamy と Shivanna (1967) が使った，花粉を未受精はい珠に直接散布する方法(Placental pollination)；(ii)無菌的に摘出した花柱に，あらかじめ用意した花粉を自家受粉または他家受粉したあと，花柱を 7～10 mm の長さに切り，それらの花柱の切り口をはい珠にできる限り近づけ置床する．この新しい受粉法を Stylar pollination と呼ぶ．

Placental pollination：W 166 H の自家受粉および他家受粉（K 146 BH の花粉を使用）で，それぞれ子房あたり 3.1 粒，2.9 粒の種子が得られた．それらの種子を胎座から切り離し，新しく用意した寒天培地には種したところ多数の実生が得られた．一方 K 146 BH のはい珠に K 146 BH および W 166 H の花粉を散布したが，はい珠表面での花粉管伸長は良好であるにもかかわらず，完熟種子は自家受粉および他家受粉区とも全く得られなかつた．

Stylar pollination：W 166 H の自家受粉および他家受粉区で子房あたり 12.2 粒と 11.5 粒の完熟種子が得られた．また K 146 BH でも自家受粉区で 5.2 粒，他家受粉区で 5.9 粒の完熟種子が得られた．Stylar pollination で得られたこれらの種子を寒天培地上には種したところ多数の正常実生が得られた．

以上のことから試験管内受精で自家不和合性の打破を試みるさいの受粉法として，Stylar pollination が placental pollination よりも有効であり，多くの種子が確実に得られることが明らかとなつた．