A Study on Induction of Plants from Citrus Pollen

CHEN ZHEN-GUANG[1]

Abstract: Since the first haploid plantlet was induced from the pollen of *Citrus madurensis* Loureiro in 1979, the pollen induction experiments have been continued for 3 years in 9 citrus cultivars. All plantlets grew well after transplant to soil. Successful induction of citrus pollen plants appears to be influenced by factors such as the culture materials selected, cold treatments of flower buds, concentrations of phytohormones, as well as incubation temperature. Both cytological and histological investigations show that the developed plantlets come from the pollens.

The anther haploid plant culture in fruit trees has been greatly developed recently. Among them, the most important fruit trees such as citrus (1, 2), grape (7), strawberry (4, 5), and apple (6) have been reported to produce haploid plants. Since the success of the anther culture in 'Calamondin' (*C. madurensis* Loureiro) in 1979, we have continued induction work on citrus pollen plants for 3 years. Results of the investigation are presented in this paper.

Materials and Methods

Nine citrus cultivars, namely 'Calamondin' (*C. madurensis* Loureiro), 'Shekan' (*C. sinensis* (L.) Osbeck), lemon (*C. limon* (L.) Burm.), pummelo (*C. grandis* Osb.), 'Ponkan' (*C. reticulata* Blanco), 'Fujie' (*C. tangerina* Hort. ex. Tanaka), 'Sunkan' (*C. reticulata* x *C. grandis* ?), 'Szipusiang' (*C. sinensis* x *C. reticulata* ?), and 'Wuyi' orange (*C. sinensis*) were used in the experiment. The developmental stage of the pollens in the anther for inoculation was at the middle anaphase period. Flower buds after 0-25 days of 3°C cold treatment were dipped in 0.1% mercuric chloride for 10 minutes, then washed with sterile water 5 times before incubation of the pollens under aseptic conditions.

About 40 anthers were incubated in each medium bottle. N_6 (8) and improved MS (3) culture media together with different concentrations of BA, 2,4-D, NAA, IBA, and LH, etc. were added solely or mixed with the addition of 2-20% sucrose, 0.8% agar, and adjusted to pH 5.8. The media were autoclaved under 15 lb pressure for 20 minutes. The pollen plants were grown in the dark room at 20-30°C. By the time of organ differentiation, the diurnal day and night conditions were 12 h with light intensity of 500-800 lux.

Microspore development of the citrus anther cultures was inspected with Carnoy's fixing solution with ordinary sectioning methods 10, 20, 30, 40, 50, 60, 80, and 100 days before and after incubation.

Results

I. Induction of the embryoids

(1) Differences in the various incubated materials. Citrus anther explants readily produce callus on the surface of the anther or broken end of the filament. Especially when the room temperature and hormone concentration are too high, a large amount of callus is formed on the surface of the anther and the development of pollen is seriously impeded. Since the investigation in 1979, we have found many factors that affected callus production, anther growth, and pollen embryoid development. Among them, the difference between incubation materials seems very important.

Results showed that the 9 cultivars differed in pollen callus formation.

[1]Fujian Agricultural College, Sanming, Fujian, People's Republic of China.

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The induction rate of 'Wuyi' orange ranked the highest at 35.84%, 'Szipusiang' 29.14%, 'Shekan' 27.76%, lemon 27.24%, 'Fujie' 18.16%, 'Ponkan' 14.11%, 'Sukan' 10.64%, 'Calamondin' 6.47%, while the pummelo (originated from pensan variants), being unfertile, ranked the lowest at only 0.71%. From all cultivars investigated, only 'Calamondin' and 'Sukan' produced embryoids (Fig. I-F); but the induction rates were low. 'Calamondin' was 2.21% and 'Sukan' was only 1.10%. Age of flowers influenced embryoid induction during anther culture. For example, the 'Sukan' anthers from the first period of flowering formed embryoid while the second one did not. The growing conditions of the 'Calamondin' tree also affected induction. Pollen gathered from the young, vigorous trees gave a higher embryoid induction rate than pollen from the old, senescent trees. Different genetic constitution of culture materials influenced the production of pollen embryoids.

(2) Effects of the cold treatment of flower buds. Before the explant culture of the 'Calamondin' anther, the flower buds were pretreated at 3°C for 5, 10, 15, 20, or 25 days. The basic medium employed was N_6 plus 2 mg/1 BA and 0.1 mg/1 2,4-D with 15% sucrose and incubated at 20-25°C at pH 5.8. The results, together with the check treatment, i.e. without cold treatment, are shown in Table 1.

Data in Table 1 show that proper treatments of cold temperature enhanced the formation of callus as well as embryoids, especially as 5-10 days' treatment induced rates as high as 1.66% in comparision with the non-treated check, which was only 0.94%. But when the cold treatment period was extended to 15 days or more, the embryoid induction rate appeared lower than the check. Thus, the cold treatment of 3°C to the citrus flower buds should not be too long, and the optimum limit is about 5 to 10 days.

Table 1. The effects of 3°C cold treatment on callus formation and embryoid production.

Days of treatment	Anthers incubated	Somatic callus		Production of pollen embryoid	
		(No.)	(%)	(No.)	(%)
0 (check)	848	5	0.59	8	0.94
5	823	8	1.11	12	1.66
10	902	9	1.00	15	1.66
15	846	18	2.13	5	0.59
20	470	5	1.06	2	0.43
25	434	3	0.69	3	0.69

(3) The effects of culture media. Previous reports on this aspect were numerous. We employed different combinations of phytohormones for embryoid differentiation. Starting in 1981, we used N_6 basic medium added to various concentrations of cytokinin and auxins and results are shown in Table 2.

We found that embryoids produced following addition of 2 to 4 mg/1 of BA, but increased concentrations of 2,4-D distinctly affected their differentiation. When 2,4-D concentrations were increased, the rate of callus formation was also increased, but the rate of embryoid induction is decreased to zero with the increased concentration of 2,4-D. This shows that a hormone combination favorable to callus production inhibited the development of pollen embryoids. With regard to the appearance of citrus embryoids, the determining factor is not the ratio of BA to 2,4-D, but the absolute quantity of 2,4-D in the medium. It seems that 0.05-0.1 mg/1 of 2,4-D is favorable to the production of embryoids. Concentration above 1 mg/1 produces callus, but not embryoids. The best combination of hormones for 'Calamondin' anther culture to produce embryoids in this experiment was 1 mg/1 of BA and 0.1 mg/1 of 2,4-D.

Sucrose is essential as the carbon source for plant tissue culture. Fur-

Table 2. Effects of different hormone concentrations on the formation of anther callus and embryoids in 'Calamondin.'[z]

Hormone combinations mg/l of BA	mg/l of 2,4-D	No. of anthers incubated	Somatic callus (No.)	Somatic callus (%)	Pollen embryoid (No.)	Pollen embryoid (%)
	0.05	536	1	0.19	4	0.75
	0.1	700	1	0.14	4	0.57
	0.5	575	7	0.22	1	0.17
4	1.0	722	23	3.19	1	0.14
	2.0	590	21	3.56	0	0.0
	4.0	624	22	3.56	0	0.0
	0.05	481	0	0.0	4	0.83
	0.1	612	1	0.17	2	0.33
	0.5	678	10	1.48	0	0.0
2	1.0	569	8	1.41	0	0.0
	2.0	667	18	2.70	0	0.0
	4.0	622	34	5.47	0	0.0
	0.05	524	12	2.29	6	1.15
	0.1	751	9	1.20	10	1.33
	0.5	607	9	1.48	1	0.17
1	1.0	477	3	0.63	1	0.21
	2.0	562	16	2.85	0	0.0
	4.0	570	20	3.51	0	0.0

thermore, sucrose maintains the proper osmotic potential of the medium. N_6 culture medium was used with the addition of 2 mg/liter of BA and 2 mg/liter 2,4-D and adjusted with sucrose contents of 5, 10, 15 and 20% to study sucrose effects on the formation of anther cell wall callus of 'Calamondin' (Table 3).

With high concentrations of hormones, 10% sucrose gave the maximum production of anther callus, with an induction rate of 11.59%, while 5 and 15% sucrose gave 6.75 and 5.85% induction, respectively. High sucrose concentrations of 15 and 20% showed distinct inhibition.

(4) The effect of cultural condition. In the explant culture of anthers, the development of microspores was comparatively sensitive to external conditions. We tried 4 environmental combinations for citrus anther culture; namely 1) photoperiods of 12 h, with light intensity of 500-800 lux and 20-25°C; 2) photoperiods of 12 h with light intensity of 500-800 lux and 26-30°C; 3) dark conditions and 20-25°C; and 4) dark conditions and 26-30°C (Table 4).

No embryoids developed under cultural temperatures of 26-30°C, in either light or dark treatment. But embryoids appeared in 20-25°C, especially under dark conditions where the induction rate reached as high as 2.21%. This showed that temperature is more important than light in the induction of citrus pollen embryoids.

II. Differentiation of plant

Table 3. Effects of sucrose concentrations on the formation of anther callus.[z]

Concentrations (%)	No. of anthers incubated	Somatic callus (No.)	Somatic callus (%)
5	385	26	6.75
10	302	35	11.59
15	342	20	5.85
20	355	4	1.13

[z]Cold treatments 3°C for 10 days before anther incubation.

Table 4. Effects of photoperiod and temperature on the development of anther callus and embryoids.[z]

Cultural conditions		No. of anthers incubated	Somatic callus (No.)	Somatic callus (%)	Pollen-embryoids (No.)	Pollen-embryoids (%)
20-25°C	Light	583	12	2.09	22	0.34
	Dark	680	44	6.47	15	2.21
26-30°C	Light	490	0	0.0	0	0.0
	Dark	661	38	5.75	0	0.0

[z]Culture media: N6 + BA 2 mg/1 + 2,4-D 0.5 mg/1 + sugar 10%, pH 5.8, 3°C cold treatment for 10 days before anther incubation.

In order to make further differentiation of plant organs, we transferred the green embryoid to improved MS media, added different concentrations of cytokinin and auxin, and cultured under light or dark condition at 20-25°C. Because of the difference in added substances, the development of the embryoids varied. Large numbers of embryoids did not develop into seedlings or form false bulbils, trumpet-shaped and fasciated embryoids. Such abnormal embryoids occurred mostly in the culture with high BA concentration. Large numbers of trials showed that when the embryoids were transferred to 0.25 mg/1 BA, 0.1 mg/1 IAA, and 500 mg/1 LH hormone combinations, plantlets were easily differentiated (plate I-G). Citrus pollen plants developed roots slowly and scantily, and sometimes even retained buds that were without roots. Experimental results showed that transferring plantlets to media containing concentrations of NAA greater than 2 mg/1 caused the plantlets to develop great patches of callus. They did not produce roots and even died at the aboveground parts. When the concentration of NAA was decreased to 1 mg/1, small quantities of roots formed. Root development of the test-tube plants could be enhanced by using the basic medium and adding 0.1-0.2 mg/1 IBA, or soaking the plantlets 2 h, in 50 mg/1 IBA solution (Fig. I-H).

III. Transplanting of the test-tube seedlings

The citrus pollen plants grew weakly and usually died during the abrupt change of environmental conditions when transplanted from the test tubes to the soil. Through many trials and failures, we succeeded in transplanting living seedlings and they grew normally (Fig. I-I). But, the art of transplanting is dexterous and some important factors must be overcome.

(1) Vitality of the seedlings. Vigorous seedlings were essential to the success of transplanting of the test-tube plants. Weak seedlings usually died after transplanting. We dipped the plantlets in 50 mg/1 IBA for 2 h, transferred them to improved MS medium in big, wide-mouth bottles, and grew the plantlets under high light intensities until the plants became vigorous with well-developed root systems before transplanting.

(2) Protection of the plantlets against rotting. The transplanted plantlets were not resistant to diseases and usually were attacked by rotting microbes, causing decay and rotting of the roots. This was the main cause of seedling fatality. Thus, measures should be taken, such as washing of media sticking on the roots, shearing off the old, degenerated rots, and applying liquid disinfectants, etc.

(3) Soils for transplanting. The soil should be a mixture of 2 parts peat,

1 part sand, and 1 part garden soil. Such mixture of soils was ideal for the continuous growth of the test plants.

(4) Humidity control. Newly transplanted plantlets should be covered with a glass coverlet to ensure humidity for growth, and they should be watered every 1 or 2 days. After a fortnight, the seedling should be aerated once in a while and the glass coverlet should be removed after 50-60 days. This was the time when the seedlings were well adapted to the natural environments.

When the plantlets were growing well, they were fertilized with diluted nutrient solution to promote growth. Crown roots should be inspected often for disease. When plantlets suffered seriously by the disease, the above-ground portion was cut off and cuttings made from them. Normal growth temperature and proper light intensity should be maintained after transplanting.

IV. Cytological investigation of the microspore development in the anther culture

During the culture of citrus anthers, we sectioned and stained the developed anthers for microscopic examinations every 10 days and, later, every 20 days to study the microspore development.

(1) Developmental status of explant anther before culture. The incubated pollens of the anthers were in the middle-late uninuclear developmental stage. The anther appeared light yellow and the tapetum surrounding the pollen grains could be seen clearly in the cross section of the anther (Fig. I-A). Different anthers in the same flower bud or different pollen grains in the same anther cell showed different developmental stages.

(2) Changes of the explant anther after incubation. The pollens at the middle-late stage were sampled from the anthers. In proper cultural medium and cultural conditions, we found that after 20 to 30 days, few pollen grains had undergone the first mitotic division, producing diploid or tetraploid stages.

Microscopic investigation during 40 to 60 days of anther culture showed few pollen embryoids. At the same time, pollen grains of diploids, tetraploids and polyploids were seen. This showed that the development of citrus microspores was variable, even in the same anther cell.

Those that were cultured up to 80 or 100 days appeared as big or small cellular patches or embryoids. At the same time, microspores at different stages were found.

Examination of the development of citrus explant anther culture from single cell pollen, multicell pollen and embryoid assured that the citrus seedling originated from the pollen (Fig. I-B-E). The development of microspores will be treated in another paper.

Discussion

The cultivar material used for anther development in vitro can determine the success or failure of the trial. This investigation involved 9 cultivars of citrus, and among them, only the traditionally seed-propagated 'Calamondin' and natural hybrid 'Sunkan' have been induced successfully to seedlings. Trials made by Hidaka et al. (2) on 'Ponkan' have also produced seedlings. It appears that sexually reproduced citrus can develop pollen embryoids easily from anther culture, while asexually reproduced citrus are unfavorable for the induction of pollen plants.

Cold treatment of the flower buds is important. Investigators generally employ cold treatment to promote embryoid formation and increase the induction rate. The fundamental reason is still unknown. Cytological investigations on the development of citrus microspores showed that equipartition of pollen grains was very common.

Whether cold treatment causes equipartition of citrus pollens during pollen plant induction is a problem for further study.

Temperature is an important factor in the production of citrus pollen embryoids. Results showed that 20-35°C was optimum for the production of

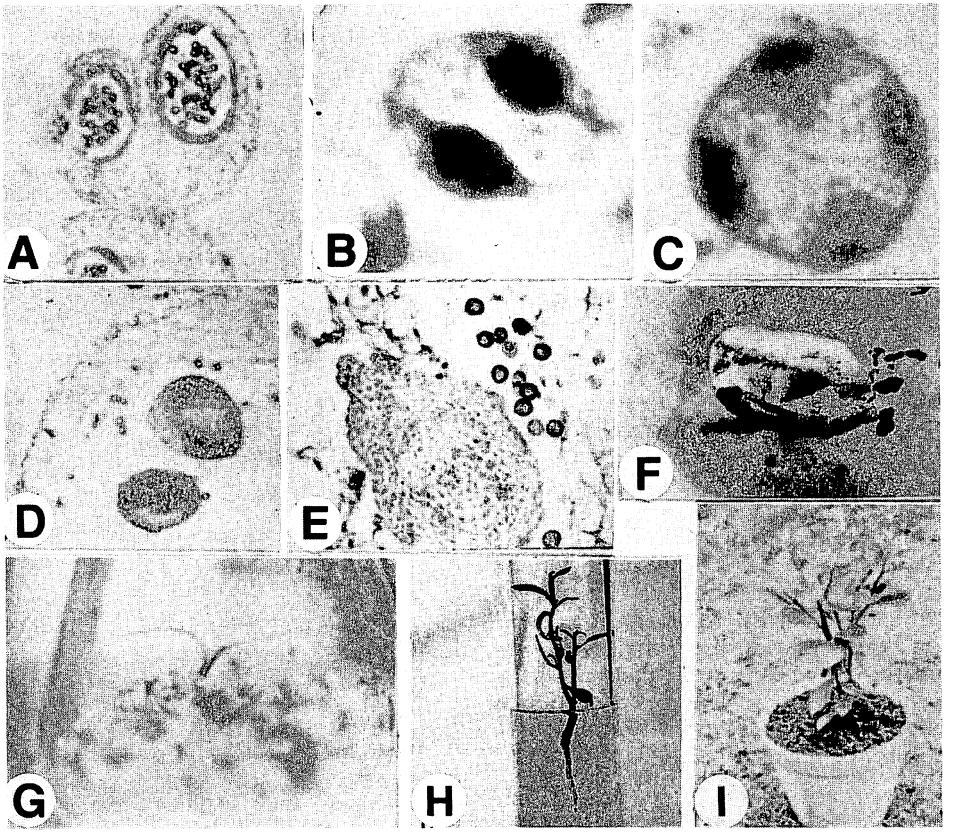

Fig. 1. A. The developmental status of citrus anthers before incubation.

B., C. Pollen grains with monoploid and polyploid nuclei.

D. From the pollen to the globular embryoids.

E. Pollen embryoid at the time of extension growth protruding from the anther wall outward.

F. The appearance of green embryoid from the fissure of another wall.

G. Further differentiation of pollen embryoid to seedlings.

H. Development of the pollen grain to an entire plant.

I. Transplanting of plantlets to the field and growing normally.

pollen embryoids. No embryoid was formed at higher temperatures. This optimum temperature coincides with the growth temperature of citrus. Thus, it is interesting to see that in the pollen culture of fruit trees, the embryoid induction temperature is closely related to the ecological requirement of tree growth.

We found that in explant citrus culture, the time for the development of microspores was very long and variable. Whether this is a phenomenon occurring in perennial woody plants or if it is generally found in fruit trees is not known. According to a study in wheat explant culture (9) 5 to 7 days after incubation, only a few pollens produced 2 to 3 cells, the peak mitosis occurring at 7 to 9 days. But, in citrus, we found diploid and tetraploid cells occurred after 20 to 30 days, and cellular patches formed at 40 to 60 days and up to 100 days. Some pollen grains formed embryoids, but still some remained in diploid and tetraploid stages. Thus, it is important to continue observations on the process of citrus microspore formation, especially the relationship between the development and cultural factors, to improve cultural techniques in order to accelerate development and improve the induction rate.

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