In vitro culture of ovary in orchids (II)
Influence of vitamins upon the growth of ovary of *Dendrobium nobile* and other several problems

By ITSUHIKO ITO

Summary Vitamin B_1 and B_6 seem to be effective for promoting the growth of fruit of *Dendrobium nobile*. Tocopherol acetate seems to be ineffective, though it increased seed fertility. The combination of these three kinds of vitamins obtained the best fertility. It is considered that the optimum humidity range for ovary growth is approximately 60 to 70%. At high humidity the fruit was dark green in color but the color became light green with the decrease of humidity. In the case of ovary culture of flower bud four or five days before anthesis, pollination was done 10 days after the starting of culture, and the fruit grew well and fertile seeds were formed.

Research in vitro culture of the orchid ovary was begun in 1956 by the author using *Dendrobium nobile* as the plant material and his several reports are already published.[1-5] A few subjects such as effect of different vitamins, humidity, pH value of medium and influence of age upon the growth of the ovary will be taken in this article. The material used was *Dendrobium nobile* Lindl., the same as used in the work described in the first article. "Partial sterile culture method" explained in that article was again used as a method of ovary culture, but in the last of this series of experiments an improved device as shown in Fig. 1 was used.

Results and discussion

1. *Influence of several vitamins*

The promotive effect of vitamin B_1 and B_6 upon growth of plant tissue has been reported by many workers. Reports dealing with the requirement of vitamin B_1 in fruit development are rare. Some workers[6-11] added B_1 to their media for ovary culture, but they give no information on the effect of vitamin B_1, except Kano.

In regard to the effect of vitamin B_1, B_6 and E (tocopherol acetate) on the growth of cultured fruit some experiments were carried out. 0.1, 0.5, 1 and 5 ppm tocopherol acetate was added to Nitsch's medium containing 6% sucrose. To increase solubility, it was mixed with ten times Tween 20 (Polyoxyethylene Sorbitan Monolaurate). The mixture was diluted with distilled water or Nitsch's medium to 20 times of the desired concentrations and this was added to the media to get the final concentrations. For vitamin B_1 and B_6, only 0.1 and 1 ppm solution were prepared. All vitamins were added aseptically to the medium in each vial after autoclaving. Application of 5 and 100 ppm tocopherol acetate solution was also tried by means of

京都府立大学農学部蔬菜園芸学研究室
Laboratory of Olericulture, Faculty of Agriculture, Kyoto Prefectural University, Kyoto, Japan.

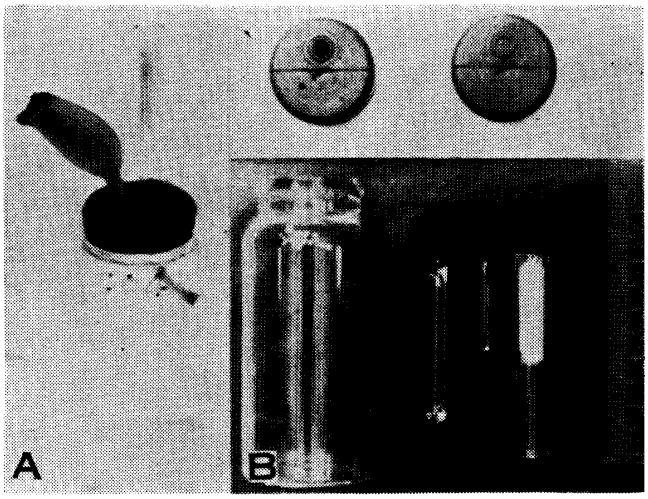

Fig. 1. Device for ovary culture in vitro. A. Fruit cultivated for 5 months on Nitsch's medium with sucrose (6%) and peptone (50 ppm). B. Device. upper left: rubber cap with two holes, upper right: rubber cap without hole, lower: left. culture vial; lower middle: two kinds of glass bar, lower right: vent.

small absorbent cotton placed on the surface of ovaries.

Development of fruit at the end of these experiments are shown in Tables 1 and 2. Media with 0.1 ppm of B_1, 1 ppm of B_6 or 50 ppm of Tween 20 seem to be excellent for the fruit growth. It seems that the addition of tochopherol acetate inhibits fruit growth, and the growth of fruits was not improved by the presence of the three vitamins in combination (Fig. 2). Neverthless, in general, the seed fertility of fruits cultured on the media with tocopherol acetate was excellent. The seeds from fruits cultured with B_1, B_6, tocopherol

Table 1. Effect of vitamin B_1, B_6 and tocopherol acetate on the growth of cultured fruit

Kinds of vitamin	Concentration (ppm)	Number of measured fruits	Fruit cultured for four and a half months		Weight of fruit cultured five months (mg)	Method of treatment
			length (mm)	width (mm)		
B_1	0.1	3	17.5±1.1	10.1±0.7	1035±181	Dissolved in medium
B_1	1	5	17.3±0.9	8.4±1.0	685±228	〃
B_6	0.1	5	17.5±1.3	8.6±0.9	736±184	〃
B_6	1	2	18.5±0.8	9.4±0.0	920±112	〃
Ta	0.1	5	16.9±1.7	8.1±0.8	647±105	Dissolved in medium Containing 1 ppm Tw.
Ta	0.5	5	18.3±1.7	8.5±0.8	778±179	Containing 5 ppm Tw.
Ta	1	4	18.0±1.9	8.8±0.6	795±200	Containing 10 ppm Tw.
Ta	5	5	19.8±3.6	8.5±1.5	798±308	Containing 50 ppm Tw.
Ta	5	3	14.2±2.3	7.8±0.3	570±222	Spread on ovary surface with distilled water containing 50 ppm Tw. at the beginning of culture
Ta	5	2	17.3±1.1	8.1±1.2	637±101	Spread on ovary surface with 50% alcohol containing 50 ppm Tw. at the begining of culture
Ta	100	3	16.7±0.3	7.4±0.1	484±129	Spread on ovary surface with lanolin at the beginning of culture
B_1 0.5 B_6 0.1 Ta 0.5		4	17.9±1.3	8.9±0.6	768±182	Dissolved in medium containing 1 ppm Tw.
(Tw.)	50	4	19.2±1.5	9.3±0.8	966±162	Dissolved in medium
Control		4	17.3±1.7	8.6±1.2	759±240	

Start of culture: Mar. 18, 1959. Medium: Nitsch's solution with 6% of sucrose, pH 6.0. Fruits were measured after 5 months of culture.

Ta: Tochopherol acetate, Tw: Tween 20.

acetate and these in combination were superior in size to those of the control. Later experiments showed the same was true regarding seed fertility.

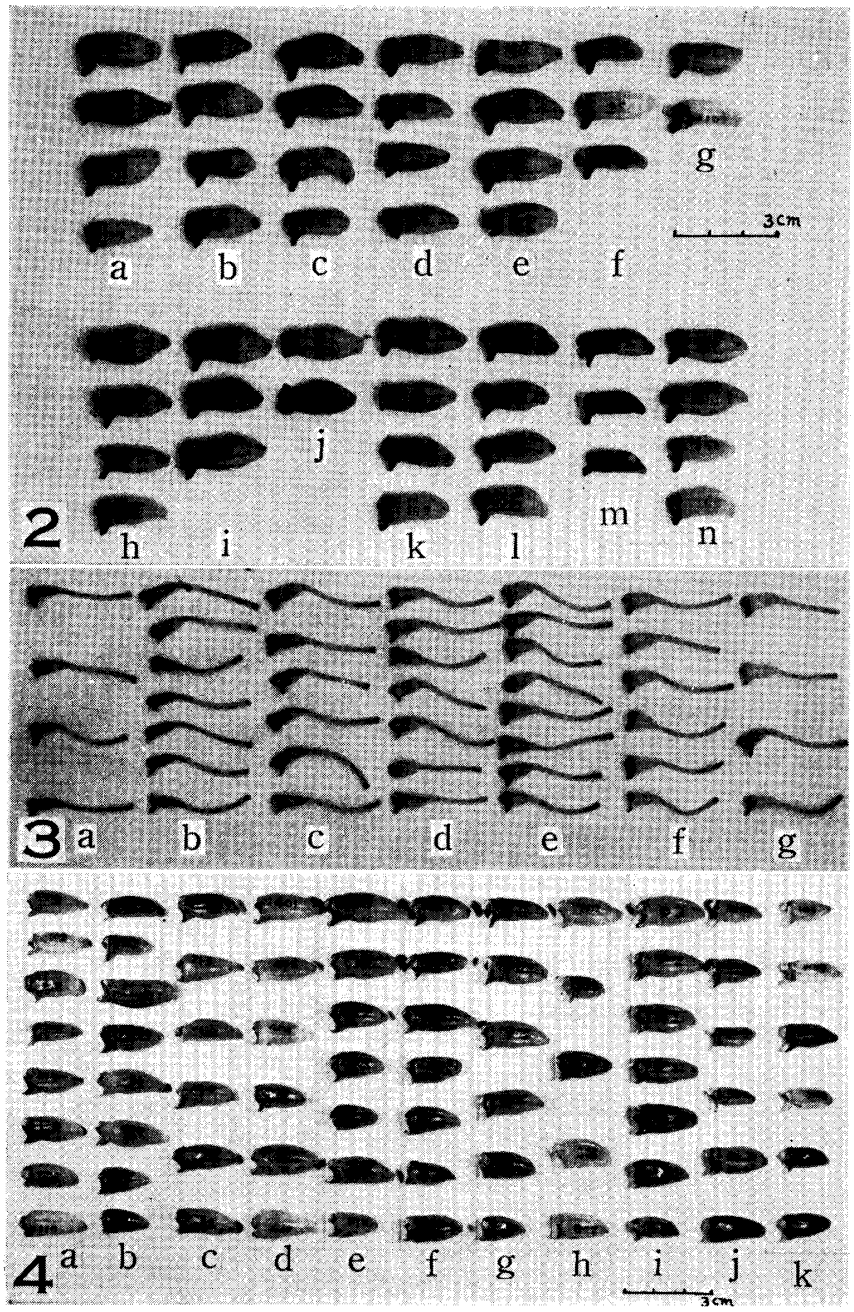

Fig. 2. Fruits cultivated for 5 months on Nitsch's media containing several kinds of vitamins with 6% of sucrose. a: tocopherol acetate 5 ppm; b: tocopherol acetate 1 ppm; c: tocopherol acetate 0.5 ppm; d: tocopherol acetate 0.1 ppm; e: $Tween_{20}$ 50 ppm; f: tocopherol acetate 5 ppm in aqueous solution (spread on ovary); g: tocopherol acetate 5 ppm in 50% alcohol solution (spread on ovary); h: B_1 1 ppm; i: B_1 0.1 ppm; j: B_6 1 ppm; k: B_6 0.1 ppm; l: vitamin mixture (Ta. 0.5, B_1 0.5 and B_6 0.1 ppm); m: tocopherol acetate 100 ppm (lanoline paste); n: control.

Fig. 3. Fruits cultivated for one month on Nitsch's media with 6% of sucrose at differing air humidity. a: 100%; b: 90%; c: 80%; d: 70%; e: 60%; f: 40%; g: 20%.

Fig. 4. Fruits cultivated for two months on Nitsch's media with 6% of sucrose; pH variously adjusted before autoclaving. a: pH 4; b: 4.5; c: 5; d: 5.5; e: 6; f: 6.5; g: 7; h: 7.5; i: 8; j: 8.5; k: 9.

Table 2. Fertility of seed and size of embryos in fruit cultured in vitro in medium containing different vitamins

Kind of vitamin	Concentration (ppm)	Method of treatment	Number of sterile fruits	No. of fruits with various grades of fertility [1]					Length of embryo (μ)	Width of embryo (μ)
				+	++	+++	++++	+++++		
B_1	0.1	Dissolved in medium	0	-	-	-	1	2	117.8±22.4	78.3±10.8
B_1	1	〃	2	-	2	-	-	1	115.9±28.7	81.6±12.7
B_6	0.1	〃	2	-	-	1	-	2	131.1±21.9	83.6± 8.8
B_6	1	〃	0	-	-	-	1	1	131.5±25.7	83.4±13.7
Ta	0.1	〃	1	-	-	1	3	-	128.8±24.5	85.0±11.7
Ta	0.5	〃	2	-	-	1	-	2	123.6±21.1	82.5± 9.5
Ta	1	〃	1	-	-	1	2	-	113.7± 6.9	82.7±14.0
Ta	5	〃	4	-	-	-	1	-	120.2±26.6	80.7±18.7
Ta	5	Spread on ovary	1	-	-	-	2	-	120.5±26.0	83.8±15.0
Ta	5	〃 (Alcohol)	1	-	-	-	1	-	121.4±23.1	85.7±12.3
Ta	100	〃 (Lanolin)	1	-	-	1	1	-	124.1±21.6	86.3±11.3
B_1 B_6 Ta	0.5 0.1 0.5	Dissolved in medium	0	-	-	-	-	4	123.9±19.9	87.0± 9.2
(Tw.)	50	〃	0	-	-	-	2	2	121.1±28.1	84.8±11.7
Control			0	-	-	2	2	-	125.4±24.8	84.3±11.0

1) +: 10^0—10^1 seeds, ++: 10^1—10^2, +++: 10^2—10^3, ++++: 10^3—10^4, +++++: 10^4—4×10^4.

It is interesting that tocopherol seems to increase seed fertility. In paticular, when used in combination with vitamins B_1 and B_6, the result is better than when used singly. This phenomenon suggests that vitamin E is effective increasing the fertility of seeds of higher plants. However, no effect or even an inhibitory effect has been found upon the growth of ovary. Effect of B_1 and B_6 on tissue culture and on cuttings of some ornametal plants has been reported by many investigators. Withner [11] recognized no necessity of addition of various vitamins such as B_1, B_2 and B_6 into the medium as the agar contains small amounts of these vitamins. Even though a medium without agar was used in the present experiments, no distinct effect could be observed by the addition of B_1 and B_6.

2. *Influence of humidity upon the growth of ovary*

In the case of normal vitro culture methods, it is difficult to study the influence of humidity on the growth of any plant tissue and organ. However, in the method employed in the present study the moisture condition around the ovary can be easily modified. In order to control humidity, different concentrations of H_2SO_4 were used at 25°C. Fifty ml desiccant in a glass beaker and two culture vials with two ovaries were enclosed in a polyethylene vessel. The experiment was started using 8 ovaries in each lot of different relative humidity. The results obtained one month after the start of culture are shown in Table 3. Under the highest moisture conditions(100%), the ovary is green and long in shape in the early stage, but later, after about one month, all the ovaries were rotted by infections originating in the stigma. In the 90% moisture lot, the ovaries was narrow in form and deep green in color, and the stigma tended to become moldy.

The ratio of length to width of ovary rose as the humidity decreased to 60%,

Table 3. Appearance and growth of fruits cultured in vitro under different humidities

Percentage of moisture	No. of fruits measured	Color of fruits	Stigma	Width of fruits (mm)	Length of fruits (mm)	Weight of fruits (mg)
20%	4	Greenish white	Dried and black	6.33±0.70	12.60±0.73	607±131
40%	6	Greenish white	Dried and black	6.54±0.80	12.62±0.73	622±115
60%	8	Light green	Healthy	6.84±0.34	12.66±0.74	657± 62
70%	7	Green	Healthy	6.64±0.37	12.26±1.50	589± 56
80%	6	Green	Healthy	6.38±0.42	13.43±1.26	645± 87
90%	6	Dark green	Frequently contaminated	5.62±0.48	12.90±1.17	500±126
100%	0	(Dark green)	(Frequently contaminated)	-	-	-

Fruits were measured or weighed after 30 days of culture. Start of culture: Apr. 7, 1958. Medium: Nitsch's solution with 6% of sucrose. pH 6.0, 25°C.

below 60% it fell. When the humidity was relatively low, the ovary became light yellowish in color, and nearly white at 20%. With decreasing humidity, the stigma dried, withered and frequently changed black in color. The optimum humidity observed for the width and weight was 60% and for length 80%. From these data, it is considered that the optimum humidity for ovary growth lies between 60 and 70% relative humidity (Fig. 3).

3. *Influence of pH of nutrient medium upon the growth of ovary*

The pH value of Nitsch's solution before adjusting is about 4.8. The buffer curve for this solution is shown in Fig. 5. Change in pH resulting from autoclaving is an interesting and important problem. In the range of pH 5-6, the change is slight. The pH of solutions adjusted to the range pH 6.2- ca. 8 are consistently reduced to ca. 6. From pH ca. 8 upward, the curve increases gradually again. The pH of all solutions which were adjusted from 6 to 8 approach pH 6 after autoclaving.

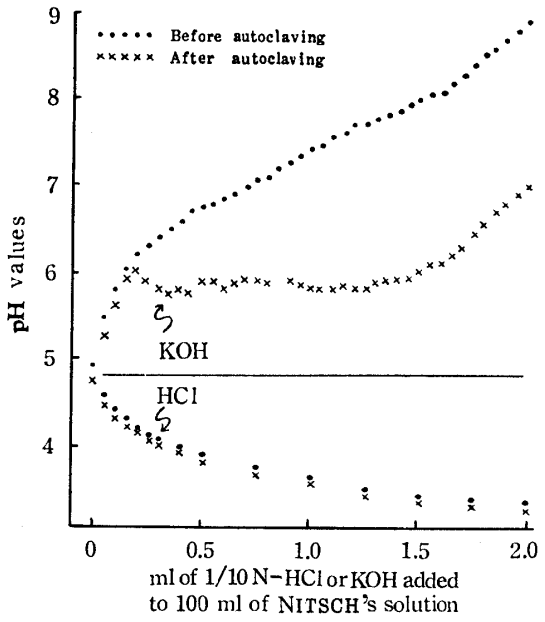

Fig. 5. Buffer curves for Nitsch's solution without sugar and agar, and befor and after autoclaving. Autoclave pressure: 15 Lbs.; time: 15 min.

At pH 4 and 9 (before autoclaving), some ovaries became yellowish and withered. Excellent ovary growth was observed between pH 6 and 8 (Fig. 6). In such wide range of pH value, no remarkable difference in the ovary growth was observed. It may be concluded, therefore, that regarding orchids, ovaries can grow in a wider pH range than in the case of seed germination and seedling growth. After two months the pH of all media suitable for culture fell to 3.8~4.5.

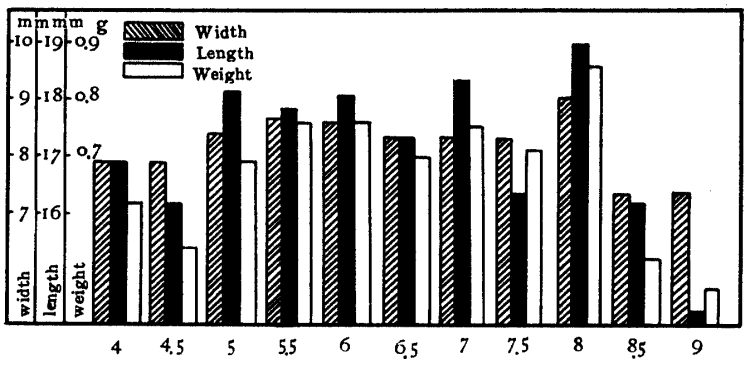

Fig. 6. Influence of pre-autoclaving pH of media upon the growth of fruit. Medium: Nitsch's solution with sucrose (6%) and peptone (100 ppm). Culture: Mar. 6–May 6, 1961. Autoclaving: 15 min. at 15 Lbs. in^2.

4. *Culture of bud ovary*

To investigate the growth of the juvenile ovary of *Dendrobium nobile*, flowers and buds were excised at different stages. The four stages are as follows: four or five days before anthesis (small green bud, ca. 36 mm long, ovary color is light green), one day before anthesis (large bud tinged with purple, ca. 41 mm long), on the day of anthesis, and at three days after anthesis (full bloom) (Table 4). As maturity

Table 4. Configuration of *D. nobile* flower used for in-vitro culture experiment made at various stages

Stages of floral development	Days before or after anthesis	No. of fruits measured	Width of ovary (mm)	Length of ovary (mm)	Length of sepal in bud (mm)
Anthesis	3 days after	15	2.9±0.1	9.5±0.5	–
	3 days after	15	2.9±0.1	9.1±0.6	–
	on the day of	15	2.7±0.1	8.9±2.4	–
	on the day of	15	2.8±0.1	8.5±0.7	–
Flower bud	1 day before	15	2.7±0.1	8.4±0.5	32± 2
	1 day before	15	2.7±0.1	8.2±0.7	33± 1
	1 day before	15	2.7±0.2	8.2±0.7	33± 2
	4 or 5 days before	30	2.5±2.6	6.9±0.6	27± 2

** Significant at the 1% level, *** at the 0.1% level.

of stigma was delayed in young buds, immediate pollination was impossible. And as the buds did not bloom, but tended to wither in a week, petals and sepals were cut off, and pollination was done ten days after starting the culture with pollinia of certain nobile strain.

The results after three or five months of culture are given in Table 5. There was no significant difference in size of fruit obtained from the flowers pollinated three days after and one day after anthesis and from the bud pollinated one day before anthesis. But, in the case of seed fertility five months later, the ovary from

Table 5. Seed formation in fruit of *Dendrobium nobile* cultured from flower bud in vitro

Days before or after anthesis	Self or cross polli-nation	Days from starting of culture to polli-nation	No. of fruits meas-ured	of fruit after 3 months of culture		No. of fruits with various grades of fertility[3)]					Rate of fertile seed (%)	width of embryo (μ)	
				Width (mm)	Length (mm)	+	++	+++	++++	+++++			
3 days after	self	0	4	8.2±0.4	21.4±1.7	0	0	1	1	0	–	–	
3 days after	self	7	9	8.7±0.9	22.6±1.8	0	1	0	0	2	69	76.9	12.7
on the day of	self	0	7	8.6±0.5	21.4±1.0	0	3	0	0	0	–	–	
on the day of	self	7	10	8.5±0.9	21.4±2.5	1	0	0	1	0	–	–	
1 day before	self	7	3	8.8±1.1	20.9±1.7	2	0	0	0	0	–	–	
1 day before	self[1)]	7	6	7.9±0.6	21.1±1.9	1	0	0	1	1	47	79.5	12.2
1 day before	cross[2)]	7	5	8.8±1.0	18.3±2.4	0	0	0	0	1	86	88.3	11.6
4 or 5 days before	cross[2)]	10	15	9.2±0.9	21.9±2.1	1	0	0	1	8	93	82.5	9.3

1) Pollinated with pollen of other flower of *D. nobile*. 2) Cross pollination by pollen of certain hybrid. Starting of culture: Mar. 9, 1963. On the Nitsch's solution with 4% of sucrose and 100 ppm of peptone. pH 6.5. 20cc; 25°—36°C. 3) +: 10^0—10^1 seeds, ++: 10^1—10^2, +++: 10^2—10^3, ++++: 10^3—10^4, +++++: 10^4—4×10^4.

the youngest bud showed highest value, though the size of the ovary was smallest. It is interesting to note that growth of fruit from the youngest bud at the start of culture was superior to that of the mature flower (Fig. 7). It seems to be due to two points: early supply of nutriment and continuous growth without dormancy at anthesis.

摘 要 ビタミンB_1およびB_6はデンドロビウム・ノビルの果実の発育促進に効果がみられ，トコフェロール・アセテートの添加は種子稔性を高める傾向があって，これらの併用によって，最も良好な稔性が示された．子房の生長に対する最適の湿度は60～70%であった．高い湿度では，果実は濃緑色を呈するが，湿度の低下とともに淡緑色となる．開花前・4—5日の蕾の培養では，培養開始10日後に受粉されたが，果実は良好に生育し，種子が形成された．

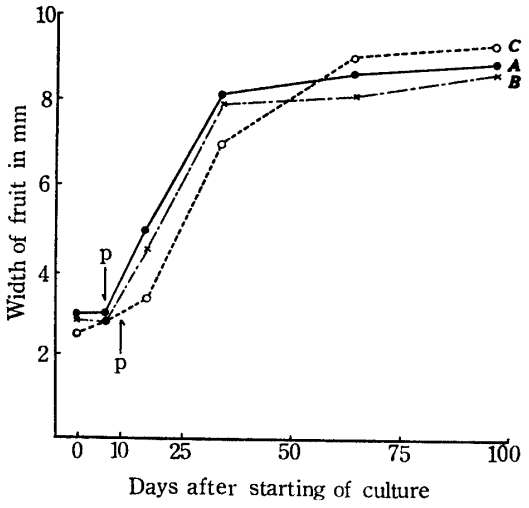

Fig. 7. Growth curves of fruits cultured in vitro, taken at different stages of floral development. A: Average growth curve of 9 fruits taken from the flowers 3 days old after anthesis and pollinated 7 days after stating of culture in vitro. B: Average growth curve of 10 fruits taken from the flowers, of which both culture in vitro and pollination were started just at anthesis. C: Average growth curve of 15 fruits taken from the flower buds 4 or 5 days before anthesis and pollinated 10 days after starting of culture in vitro.

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