

Germination of Seeds from Immature Pod and Subsequent Growth of Seedlings in *Dendrobium nobile* LINDL.

By

ITSUHIKO ITO

A culture method for germinating orchid seeds was established after many studies had been made by KNUDSON⁴⁻⁸⁾ on the sterile culture technique. Since this time many valuable studies concerning synthetic culture media have been reported by numerous investigators. Due to the fact that orchids grow so slowly and much difficulty is met in germinating some genera, it is certain that studies involving aspects others than that of the media, if orchid culturing is to improve, is to be forthcoming.

Orchid seeds have no endosperm and the embryo consists of a mass of undifferentiated tissue, with no plumule, radicle etc.. The structure corresponds to that of the early developmental stage of the embryo in other plants. It may be considered that culturing of orchid seeds is an embryo-culture in a particular sense. WITHNER¹⁶⁾ reported that, as a new method for starting orchid seedlings, an ovule-culture is possible and he achieved great success in obtaining healthy seedlings by culturing ovules three months after pollination, in *Epidendrum cochleatum* on Knudson's medium with Vitamin B₁, Vitamin B₆, niacin and adenine. At the same time he also found a slight germination with the three months old pod of *Cypripedium (Paphiopedilum) insigne*. He opened a new and exciting field in the study of orchid culture. In recent years, WITHNER²⁰⁾ reported that the *Cypripedium* could be made to germinate consistently with this new method. Previously, employing the ordinary methods, much difficulty was encountered in getting a satisfactory germination. There is however another method, recommended by BURGEFF¹⁾ and LIDDELL⁹⁻¹²⁾, in

which, the seeds are soaked prior to planting on solid media. In 1954, TSUCHIYA¹⁴⁾¹⁵⁾ published two papers along this line. In the first paper he noted the possibility of germinating orchid seeds from immature pods and reported that the germination is possible, at least with seeds older than four months. He also noted the abnormal germination of *Dendrobium (nobile strain)*, on White's medium, of the peripheral portions of a slice of its premature pod of three months age. In the second paper he reported that seeds of two months and twenty days age of a *Dendrobium phalaenopsis* hybrid (*D. phal.* x *superbiens*), *Vanda* hybrid (*V. Cooperi* x *Joaquim*) and *D. phalaenopsis* (self) showed 100 per cent germination on a nutrient medium containing tomato juice. In *Dendrobium* hybrids (*nobile strains*), four months and possibly younger are sufficient, but he suggested that in *Cymbidium* hybrids, three months, with *Cattleya* four months seemed to be a little too early. Most recently WITHNER²¹⁾ has observed the *Vanilla* species, on Burgeff's N3f medium with some organic substances, that even ovules thirty days old grew well, and that the stage of about sixty days after pollination seemed to be an optimal one for obtaining the best culture growth. The present author performed some experiments with the purpose of investigating the youngest possible stage at which the pod is capable of giving seedlings of *Dendrobium nobile*.

Materials and Methods

The material used in this work was the *Dendrobium nobile* LINDL. It is the source of the *Dendrobium* hybrids which are now widely cultured in Japan. The seeds from this plant

*Laboratory of the olericulture, Saikyo University, Kyoto, Japan.

have a high fertility and also yield an uniform growth. These plants require between nine and thirteen months for the pods to mature. The fruits which pollinated on May first of 1953 were examined periodically to check the fruits and seed development. Two pods were left to develop on each plant of medium size.

The fruits used for culture were pollinated on the 15th of May in 1953, and immature seeds from four, five and six month pods after pollination were planted in tubes containing several kinds of nutrient solutions. Because of the successful growth of the four months seeds in 1953, materials taken from younger stages were used in 1954; all were pollinated on the 16th of April, slices of the immature pods were cultured at half month intervals from one month to three and one half months. At four months after pollination, immature seeds were scraped out from the pod and inoculated. In some cultures, onion or yeast extract was added. The onion extract was prepared as follows from 'Sensyu-yellow' onions. 300gr. of the sliced onion was boiled in 600cc. of distilled water for fifteen minutes and then filtered and filled up to 600cc.. The solution thus obtained was diluted to one-half or one-fourth before use. Yeast extract was prepared by boiling 4.8gr. of dried Brewer's yeast in 600cc. of distilled water. To the extracts, two per cent sucrose, was added. The hydrogen ion concentration was adjusted to pH 5.0 and then autoclaved for 15 minutes at 15 pounds pressure.

Test tubes (diameter 18 mm., length 180 mm.) contained 15 ml. nutrient solution with 1.8 per cent agar. The tubes were placed into a glass-house, where the temperature varied markedly (maximum 40°C., minimum 6°C.). This temperature variation may be responsible for the fact that the growth of the seedlings in the present research was somewhat inferior to that of ordinary cultures carried out under a constant temperature. As a measure of growth of the seedling, the maximum length of the embryo, protocorm and seedlings of the largest 50 plants from one tube in every series was

determined by microscopy at intervals of approximately 30 days.

Observations and Results

1. Development of the embryo in immature pods.

Ovaries started to enlarge rapidly from about one half month after pollination, and reached a mature size in about four months. The embryos developed rapidly two to four months after pollination. As shown in Plate B the embryo could not be distinguished from the seed coat until two months after pollination. The ovary in this stage still has hollow spaces covering about 40 per cent of the cross section. At three months of age the seed grew to about five times the size of that of the two month specimen and the ovary was almost full of growing seeds (Fig. 1

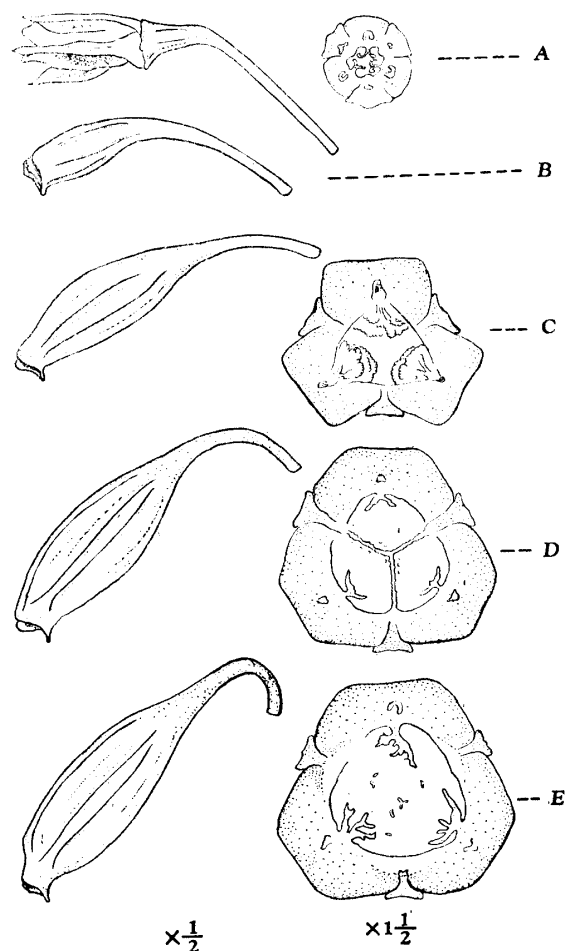


Fig. 1. Diagram showing growth and cross-section of different age pods. A, one half month after pollination. B, one month. C, two months. D, three months. E, four months.

Table 1. Growth data of immature seeds. (average of 50 seeds)

	months after pollination								
	2	3	4	5	6	8	9	12 ¹⁾ (mature)	9 ³⁾ (mature)
length of testa in μ	ca. 45	153	290 ²⁾	271 ²⁾	283 ²⁾	303	323	329	428
width of testa in μ		51	84 ²⁾	74 ²⁾	84 ²⁾			82	102
length of embryo in μ			78 100 ²⁾	93 ²⁾	114 ²⁾	112	134	143	181
width of embryo in μ			62 ²⁾	56 ²⁾	72 ²⁾			79	81

1) Seeds taken from plants of medium size.

2) Pollinated on the 15th May, the remaining ones were pollinated on the May 1st in 1953.

3) Seeds taken from a vigorous plant, pollinated on the 2nd Feb., picked up on the Nov. 4th. 1953.

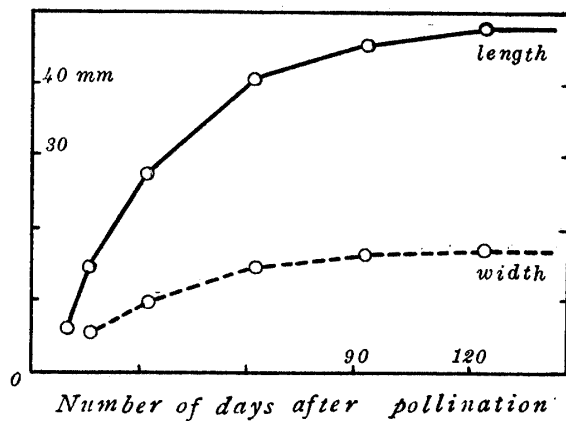


Fig. 2. Growth of pods after pollination in the *Dendrobium nobile*. (average of 10 pods)

and Plate D). The embryo was distinctly discernible from the seed coat. At four months of age, no hollow space in the ovary was observed (Fig. 1). In this stage the seeds attained the approximate shape of mature seeds, but they were somewhat more slender (plate F). At an age of six months the seeds were almost as large as the mature ones of the usual twelve months (plate I and J).

2. Germination and growth of immature seeds from four, five and six months old ovaries.

From November to December in 1953, immature seeds from young pods of four, five and six months, after pollination, were planted aseptically. These pods resulted from the flower which had been pollinated on May 15th. For

planting, the immature pods were first surface sterilized with 75 per cent alcohol, then immersed in calcium hypochlorite solution for 10 minutes, and then washed with sterile water. The pods were opened and the seeds were scraped out by means of a rather thick nicrome wire and immediately inoculated into the tubes.

For the culture, three media, i. e. onion (one half sol.) and yeast-extract each contain-

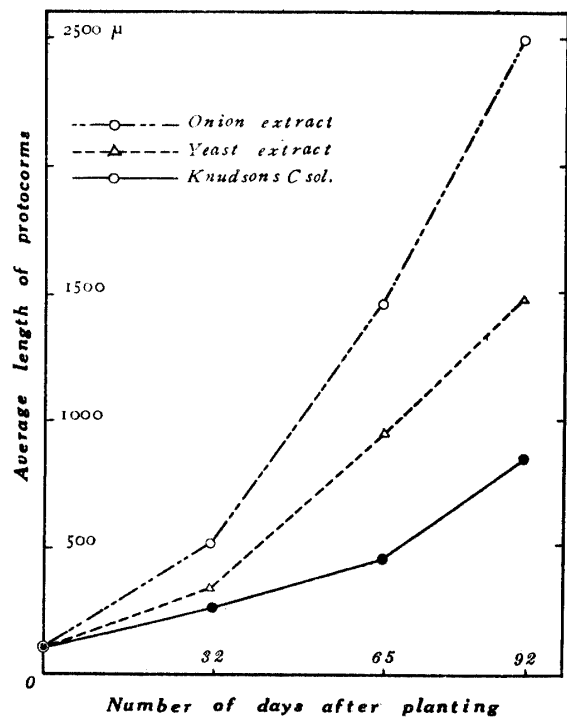


Fig. 3. Rate of growth of the protocorms grown from immature seeds of four months of age. (onion extract: 1/2 sol.)

ing two per cent sucrose and Knudson C solution were used. The pH values after autoclaving were 4.8, 5.0 and 4.25. Growth curves of seedlings from the four months old pod, during the 92 days on each medium, are shown in Figure 3. As a result of this culture, it was recognized that immature seeds should be at least four months old, if a high germination rate and good growth are being sought. Each medium shows a distinct difference as to the growth of the seedling. As to the onion extract, one half of the original solution gave the best results. Immature seeds of five and six months of age also germinated and grew well.

3. Germination of seeds and growth of seedlings, from immature pods, within three months after pollination.

With the materials pollinated on the 16th of April in 1954, slices of immature pods from one month after pollination to three and one half months were cultured at half month intervals. At four months of age the pod became too big to be handled with ease, instead of culturing the pod slices it was preferable to plant the immature seeds only. A one half concentration Knudson's B solution, 0.8 per cent yeast extract with two per cent sucrose and a half concentration onion extract with two per cent sucrose were used. But, these experiments failed because the temperature was too high (maximum 40°C.) during the experiment, the cultures suffered severely and even the seeds of four months age could not be grown as well as in 1953. In the immature seeds of less than three and one half months of age no germination occurred. There was only one successful tube in which seedlings

from the immature pod, of three months of age, had grown on yeast extract (Fig. 4). This particular case was examined by taking out the plants from the tube at three and a half months after inoculation; 107 seedlings with two leaves, 134 with one leaf and 11 white protocorms were observed. The total weight was 0.351 gr. The weight of the largest 50 seedlings with two leaves was 0.126 gr. whereas the corresponding weight of the control seedlings, obtained from the mature seeds, was 0.130 gr. (Table 2).

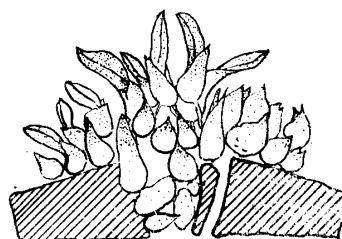


Fig. 4. Seedlings growing on a slice of a three month old of pod. $\times 3$.

4. Comparison of growth of seedlings from mature and immature seeds.

The mature seeds used as the control were available when the seeds attained six months of age. The seeds were inoculated on a media of onion and yeast extract both containing two per cent sucrose. The growth of the immature seeds was superior to that of the mature seeds (Table 3). The initial length of the embryo of the immature seeds was 114 microns in an average of 50 seeds, while that of the mature seeds was 181 microns (Fig. 5). It is interesting that the growth of plants resulting from immature seeds was better after about 100 days, although at the start of the experiment the seeds were obviously smaller than the mature ones.

Table 2. Measurement of seedlings in a one slice culture from a three month old pod in *Dendrobium nobile*. (Measurement taken three and one half months after inoculation. Largest 50 plants observed)

	total weight (gr.)	average length of seedlings (mm.)	average width of seedlings (mm.)	total number of seedlings with roots
immature seeds	0.126	4.40	1.57	6
mature seeds (control)	0.130	5.34	1.20	39

Table 3. Growth of protocorms from immature seeds of six months age and mature seeds of nine months age (initial size of seeds: mature 181 μ , immature 114 μ)

days after planting	maturity	onion extract		yeast extract 0.8% sol. (pH*5.2)
		1/2 sol. (pH*5.0)	1/4 sol. (pH*5.15)	
thirty	immature	275	254	224
	mature	318	323	319
sixty	immature	646	877	718
	mature	704	784	606
ninty	immature	2465	1742	1296
	mature	1404	1698	1076

* initial pH

In this experiment the number of inoculated seeds was unequal in both series. The immature seeds had a tendency to stick together, and were inoculated about four times thicker than the mature seeds.



Fig. 5. Mature seeds of *Dendrobium nobile* taken from large pod on very vigorous plant. Nine months old. (scale indicates 0.1 mm.)

Discussion

Practically, immature seeds scraped out from four months old pods may be considered to be most useful, because in this stage the manipulation is very easy, moreover the germination rate and healthiness of the seedlings obtained are not inferior to those from mature seeds.

It has been recognized by many investigators that some organic substances have an excellent effect upon germination and the subsequent growth. The substances used for orchid germination were arginine²¹), adenine¹⁷), lysine²¹), glycine¹⁵), peptone²⁰), Vitamin B₁¹⁴), Vitamin

B₆¹⁴) thyroxin¹⁹), estrone¹⁹), insulin¹⁹), organic acids such as indole butyric³)²⁰)²¹), fumaric¹⁷), glutamic¹⁷), succinic¹⁷), tartaric¹⁷), pyruvic¹⁷), malic¹⁷), nicotinic¹⁴), and juice and extracts from natural sources such as tomato juice¹³)¹⁵)¹⁶), carrot juice¹⁹), coconut milk³)²¹), yeast¹⁴), wheat⁴), potato⁴)¹⁷), orchid seeds¹⁷) and wheat germ¹⁹) ; also fish emulsion²) and mashed bananas²⁰)²¹) were used. After having tried more than a dozen kinds of plant extracts, the present author found that onion extract is very suitable and practical for the growth of the protocorm of *Dendrobium nobile*.

Proper usage of plant tissue extracts should be more thoroughly explored in the future, if the purpose is only to obtain a vigorous growth of the seedlings and not to study the nutritional requirements. The seedlings on onion extract in the present experiments were rather yellowish green in color. This may be due to the high sugar concentration. Onion tissue originally contains 6.5–8.5 per cent sugar and the basic solution of the onion extract used includes therefore 2.5–3.0 per cent sugar. Although it was diluted to only one half or one quarter, two per cent sucrose was added again. Thus the sugar concentration for orchid seedlings might become supra-optimal in this solution.

It is a remarkable fact that the growth of the immature seeds can later exceed that of

mature seeds. It may be ascribed to the thick inoculation in the case of the immature seeds. Orchid seeds are apt to germinate better in thick inoculations.

Summary

1. Morphological observations on ovules and immature seeds in young fruits of different stages as well as experiments concerning the possibility of germination and growth of immature seeds or pods in *Dendrobium nobile* on the media containing onion- and yeast-extract and on the Knudson's solution.

2. Ovary starts to enlarge rapidly from about one half month after pollination, and reaches a mature size in about four months; embryos develop rapidly two to four months after pollination. The outline of the seeds is completed in four months and attains almost a full size in six months.

3. Immature seeds from four, five and six months after pollination germinate and grow well. Growth of immature seeds of six month age exceeds even that of mature ones of nine months age.

4. An addition of onion extract to the sugar medium produces vigorous seedlings with leaves and roots, although somewhat yellowish, when compared with that on Knudson's C solution. On yeast extract medium, with sugar, intermediate growth was observed.

5. Slices of immature pods from one month to three months and a half after pollination were cultured. The cultures were unsuccessful, in one tube of 0.8 per cent yeast extract with 2% sucrose in which the immature pods of three months age were cultivated, healthy seedlings were obtained.

6. As the results of the present culture experiments it was revealed that the use of immature seeds from young pod of four months after pollination is practical for *Dendrobium* (*nobile* strain) cultures.

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Explanation of Plate

Development from ovule to seed. A, one and one half month after pollination. B, two months. C, two and one half months. D, three months. E, three and one half months. F, four months. G, four months and one week. H, five months. I, six months. J, twelve months (mature seed). Scale indicates 0.2 mm..

Plate

