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STUDIES ON THE MEDIA FOR ORCHID SEED GERMINATION

By

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With 19 Tables, 23 Textfigures and 6 Plates

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I. Introduction

Since Knudson succeeded in the asymbiotic germination of orchid seeds with a culture medium which contained inorganic salts, sugar, agar and water in 1922, many new facts have been discovered in the field of the artificial germination of orchid seeds and a number of recipes have been proposed by many workers. Among the fact reported to date, the following two are very important from the practical point of view, especially for the breeders. First, orchids hold different nutrient requirements for their germination depending upon the genera or species, and second, some orchids, like some of the terrestrials, are still difficult to germinate on the culture media artificially prepared. The reasons have not been clarified, but as to the commercially cultivated orchids, the seeds can germinate satisfactorily on culture media which are specifically recommended for that particular genus or species, or with a special cultural method. However, many of the recipes recommended to date are, except a few, somewhat troublesome to follow.

The prsent work was undertaken in order to establish the formulae which are easy to prepare and effective in germination and growth of the orchid seeds, and to find how to germinate the seeds whose germination seemed to be difficult on artificial media.

The recipes devised in this work were reported in Amer. Orch. Soc. Bull. 32: 354-355, 1963.

II. Materials and Methods

Ripe orchid seeds of the genera, *Laeliocattleya*, *Brassolaeliocattleya*, *Brassocattleya*,

Brassavola, *Dendrobium*, *Cymbidium*, *Paphiopedilum*, *Cypripedium* and *Bletilla* were used as materials. In some cases, however, immature seeds were also used in the test.

In general, four to five Ehrlenmeyer-flasks (50 ml.–200 ml. capacity) or test tubes (190 mm. × 21 mm.) were used for each trial as the culture vessels. These vessels were tightly stoppered with cotton plugs and sterilized in dry heat. After the dry heat sterilization, culture solutions which contained minerals, sugars, agar and various additions were poured into the vessels. All the media were autoclaved at 15 lbs. pressure for 20 min. The media were generally solidified with 1.5 per cent agar.

The pH value of the culture solution was adjusted, in most cases, with Na_2CO_3 and HCl and was checked with a glass electrode pH meter. Initial pH values presented in this paper show the pH values measured after autoclaving the media.

Throughout the work, filtered Wilson's calcium hypochlorite solution (Wilson, 1915) was used as the disinfectant. For the sterilization of the seeds, a desired quantity of the seeds was placed in a small glass tube or a small Ehrlenmeyer-flask and the disinfectant added. The tube or flask was then shaken vigorously for three to five min. and stored still for another five to seven min. It generally took 10 min. from the beginning of the seed sterilization to the start of planting.

The sowing method was altered depending upon the state of seeds at the end of the sterilizing procedure. When the seeds were suspended in the disinfectant solution, it was diluted with three-fold quantity of sterile water upon completion of the sterilization. A small aliquot of the diluted solution with the seeds, varied depending on the size of culture vessels used, was dropped onto the sterile agar surface in the culture vessels with an ordinary medicine pipette previously sterilized.

On the other hand, when the seeds floated on the surface of the disinfectant solution, a small aliquot of sterile water was poured onto the agar bed with the above-mentioned pipette and then the seeds were placed on it with a sterile loop needle.

The procedures described above made the seeds spread on agar surface easily when the flask was gently rotated at the completion of the sowing process. In some cases, however, the seeds were transferred directly from the disinfectant onto the agar bed with a sterile loop needle.

The immature pods were also sterilized, in the same method. Their sowing method will be stated later.

Soon after the seeds were inoculated in the flasks, in most cases, cotton plugs were replaced with rubber stoppers through each of which a fishhook-shaped glass tubing was inserted. The tubing was stuffed with a small wad of cotton at the outer end, and the whole stoppers had been sterilized previously in dry heat.

Aseptic precautions were taken throughout the work.

After inoculation the cultures were generally placed in a greenhouse. No attempt was made to control the temperature which fluctuated as the season advanced and the heat within greenhouse. Readings varied anywhere from 15°C up to approximately 30°C.

The largest 10 to 20 seedlings from one or two culture vessels were used for measurement. The seeds were counted for germination under binocular microscope and small protocorm-like materials were measured using an ordinary microscope.

III. Results

1. Preliminary experiments.

At the beginning of this study, as already tried by many workers in this field, effects of some plant saps and some chemicals which are considered at present to play important roles in the metabolism of plant development, on the germination and growth of orchid seeds were examined. And then, a simplification of the formulae was tried.

i) Effect of plant saps on the germination and growth of *Bletilla*, *Dendrobium*, *Brassolaeliocattleya* and *Laeliocattleya* seeds.

Knudson's solution C (Knudson, 1946) was used as the base, to which fresh strained juice of tomato, apple or pseudo bulb of *Cymbidium* was added at the rate of 30 per cent. Saps of *Cymbidium* pseudo bulb showed a mucilage nature. Although these saps contain a certain amount of sugars, the sugar concentration in apple juice is high enough to meet the requirement of orchid seeds even after dilution in preparing culture media. Hence, only the minerals in Knudson's formula were used in the apple juice medium.

For the checks, Knudson's medium and that in which sucrose was replaced by crude sugar were prepared.

Sugar concentration in each medium containing any plant sap was calculated from the reading of a hand sugar refractometer on the fresh strained juice. Sugar concentration in these media varied depending upon the saps added.

The pH value after autoclaving and the sugar concentration in each medium were as follows:

Medium	Sugar conc.	pH value
Knudson's soln. C (Basic soln.)	2.0%	5.2
Basic + tomato juice	2.3%	4.7
Basic + apple juice	3.6%	5.0
Basic + *Cymbi.* juice	4.0%	5.0
Knudson's mineral soln. + crude sugar	2.0%	5.3

Ehrlenmeyer-flasks of "Pyrex" glass of 100 ml. in capacity with their necks plugged with cotton were used as culture vessels.

Seeds of orchids, *Bletilla striata*, *Dendrobium*, *Brassolaeliocattleya* were planted in May, 1960, using the method described in Chapter II.

Results are shown in fig. 1-1 and Plate I. fig. 1 (A-C). As seen in Plate I. fig. 1, 60 days after seed inoculation (Plate I, fig. 1-A), *Bletilla striata* showed less growth

on the media containing apple and *Cymbi.* juices than on other media. Two months later, this tendency of growth was also observed apparently, though on apple juice medium, some plantlets showed normal growth (Plate I, fig. 1-B). On the 140th day of the seed planting, growth was generally better on Knudson's soln. C, basic plus tomato juice and Knudson's minerals plus crude sugar (Plate I, fig. 1-C). On the apple juice medium some plantlets developed normally as mentioned above, while others were inhibited in their growth as compared with those on Knudson's medium. On the *Cymbidium* juice medium, growth of the seedlings was completely inhibited. It seemed that there was no difference in the percentage of seed germination among the media.

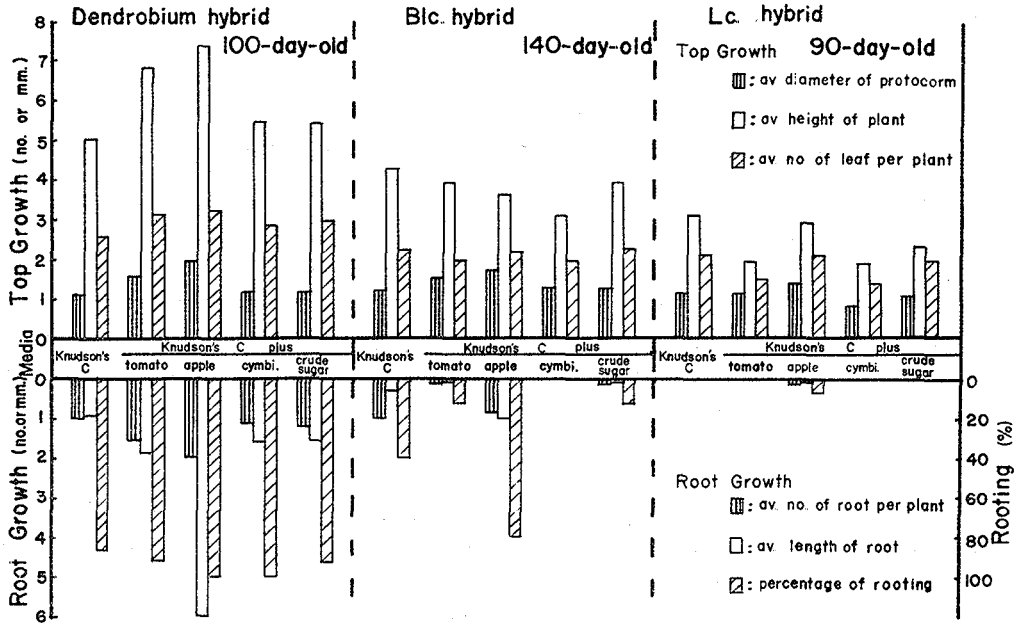

Fig. 1-I. Effect of plant saps added to the media on germinating the orchid seeds belonging *Dendrobium* and *Cattleya* group. Basic medium: Knudson's soln. C.

On the contrary, as for *Dendrobium*, growth of the seedlings was generally better on the media containing plant saps. The best growth was recorded on the medium with apple juice. Top growth of the seedlings of *Brassolaeliocattleya* and *Laeliocattleya* on the apple juice medium was worse than that on Knudson's, while in both genera the root growth was better on the former than on the latter (fig. 1-1).

ii) Effect of adenine, IAA, kinetin and fresh yeast on the germination and growth of *Dendrobium* and *Laeliocattleya* seeds.

It was reported recently that such substances as adenine, IAA, and kinetin play important roles in the growth and differentiation of plants. The effect of these substances, added to the media individually or in combinations, on the germination and growth of orchid seeds was examined in this experiment. At the

same time, the media containing fresh yeast, tomato or apple juice were prepared. Knudson's soln. C was used as a base.

The following doses were added to the Knudson's base:

10% of tomato juice
10% of apple juice
0.5% fresh yeast
40 ppm adenine sulfate
1 ppm IAA
0.5 ppm kinetin

Concentration of sugar in all the media was adjusted to two per cent adding a necessary amount of sucrose. The pH values of these media after autoclaving were between 4.5 and 4.8.

The results obtained with *Den.* and *Lc.* seeds are shown in fig. 1–2.

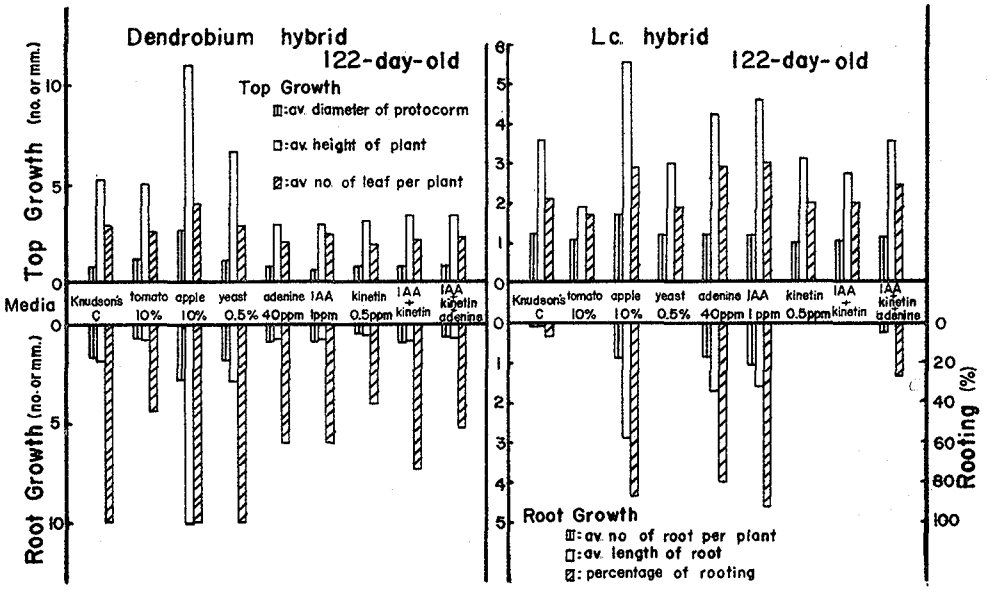

Fig. 1-2. Effect of various additions to the media on the growth of orchid seeds. Basal medium: Knudson's soln. C. Cultured in test tube with cotton plug.

The best growth was recorded on the medium containing apple juice in both genera. Those genera responded differently to the fresh yeast, adenine and IAA. Fresh yeast was effective on the growth of *Den.*, while it was ineffective on the growth of *Lc.* The response of either genera to the latter two substances was quite reverse to the former. No favourable effect of other additions was observed in the present experiment.

iii) The applicability of "Hyponex" as a base of culture solution.

In order to simplify the recipe of culture solution, Hyponex, a plant food containing nitrogen (total nitrogen not less than 7%, nitrate nitrogen not less than

5.8%, ammoniacal nitrogen not less than 1.2%), phosphoric acid (available phosphoric acid not less than 6%), potash (water soluble potash not less than 19%) and other essential elements for plant growth, and commonly used by horticulturists throughout the world, was examined for its applicability as a mineral nutrient base.

The concentration of Hyponex solution was 0.75, 1.5 or 3.0 g per liter in each medium. The applicability of Hyponex solution alone and that containing 50 per cent tomato juice solution (Meyer's tomato juice medium) or 20 per cent apple juice solution was tested. With the latter two, plots containing no Hyponex were also used. Plain Knudson's soln. C was employed as a control.

Sucrose was added at the rate of two per cent to both plain Knudson's C and Hyponex solutions, and neither sucrose nor any other type of sugar was added to those plots containing tomato or apple juice. The sugar concentrations in these two solutions were determined by hand sugar refractometer readings as already mentioned. They were approximately 2.0 per cent in the tomato juice medium and 2.4 per cent in apple juice medium. pH of these media was between 5.4 and 4.6.

As the seed material, seeds of *Dendrobium* hybrid and *Laeliocattleya* hybrid were used.

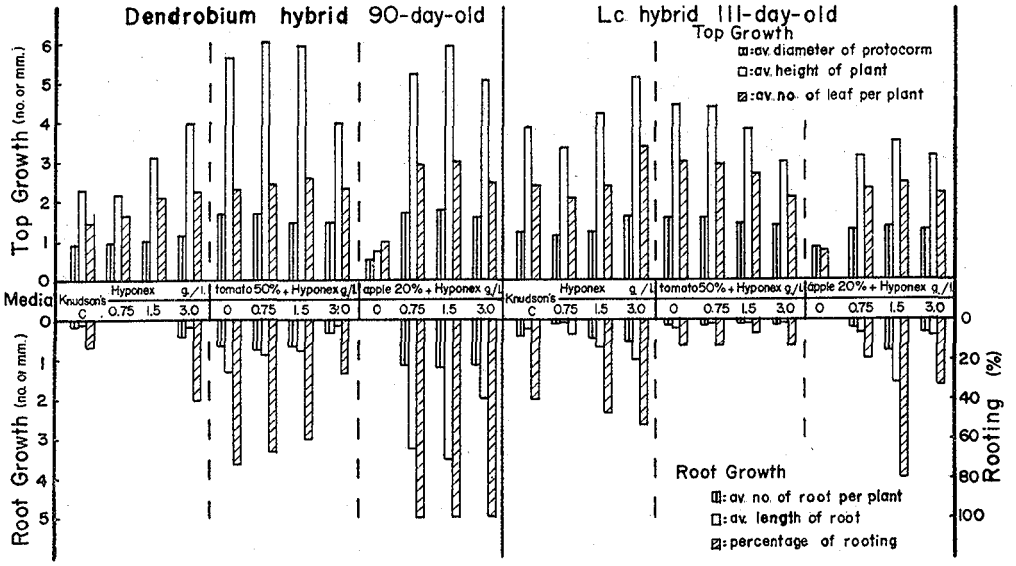

Fig. 1-3. Applicability of Hyponex as the basic nutrient in orchid seed germination.

Results are shown in fig. 1-3.

With *Dendrobium*, the best growth of whole plants was recorded on the medium with apple juice and 1.5 g Hyponex, and the seeds did not grow well on the medium with apple juice alone. In general, the root growth on the media with apple juice and Hyponex was superior to the root growth on any other media. Comparing the

growth on plain Knudson's soln. C and on plain Hyponex of 3.0g, the better growth was observed on the latter.

With *Laeliocattleya*, the best growth of root portion was recorded on the medium with apple juice and 1.5g Hyponex, as in the case of *Dendrobium*. Considering the growth of both top and root portions together, however, it may be concluded that growth is better on the medium with 3.0g Hyponex. On the media containing tomato juice, the growth was successively decreased according as the dosage of Hyponex increased.

iv) Discussion.

Through the experiments presented above, it was shown that the preferable formulae for culture solution differ depending upon the orchids used. Among the plant saps used as the supplement, apple juice was by far the most effective on the growth of both top and root in *Dendrobium*, and in other orchids on the growth of root. Hyponex is applicable as the mineral nutrient base of culture solution to asymbiotic germination of orchid seeds.

The fact that the formulae preferable for asymbiotic germination of orchid seeds are different depending upon what orchid one deals with, is clearly demonstrated in the present experiment as already shown by great many investigators (Ballion and Ballion, 1924; Breddy, 1953; Burgeff, 1936; Clement, 1924a, b; Curtis, 1947; Curtis and Spoerl, 1948; Griffith and Link, 1957; Hegarty, 1955; Spoerl, 1948; Spoerl and Curtis, 1948; Withner, 1959; etc.). This fact may be interpreted that different genera or species of orchids hold different nutrient requirements for their germination, but the exact reason for it has not yet been clarified as emphasized before. One of the ways to attack such a problem is to find first a favorable recipe for each genus from the practical point of view, setting the reason aside. Some of the experiments which will be shown in the later sections of this paper were carried out from this point of view.

The simplification of the formulae of the culture solution in asymbiotic germination of orchid seeds from the practical standpoint was reported already by several workers with success (Chang, 1953, 1955; Graeflinger, 1950; Ito, 1955; Karasawa, 1964; Meyer, 1945a and Yamada, 1952; etc.). Most of the recipes presented to date are empirically derived from Knop or Pfeffer solution, and there has been no research, except that of Wynd (1933a), with respect to a systematic determination of the optimum mineral requirements of any particular species or genus (Withner, 1959). This means there is no scientific standpoint of the recipes. Since good germination and seedling growth are aimed from the practical point of view, media should be the ones that can be prepared without difficulty. As was mentioned in the introduction, almost all the recipes now used are too troublesome to follow, for practical uses. Preparation of media, therefore, should be simplified for practical uses. These considerations must be taken into account in practically attacking the problem of asymbiotic germination of orchid seeds of unknown nature.

Hyponex, examined in this experiment, showed good results even in the plain

solution as compared with the plain Knudson's soln. C for the growth of both *Dendrobium* and *Laeliocattleya* seeds. The fact indicates obviously that this plant food is applicable as a mineral nutrient base in culture solution. Based on this fact and the considerations mentioned above, the following trials were made in order to establish recipes which are easy to follow and effective in germination and growth of the orchid seeds, using Hyponex as a mineral base.

2. Effect of types of containers on germination of orchid seeds and growth of seedlings.

Some of the results of the observation on the effect of containers on germination and growth of orchid seeds are as follows.

i) Effect of various kinds of stoppers on the germination of orchid seeds and the growth of seedlings.

Plate I. fig. 2-A shows the effect of stoppers on the growth of *Laeliocattleya* seeds 188 days after sowing. The growth was better in the flask which was completely sealed with a rubber stopper (A-b) than in the others, though the roots in that flask elongated upward with numerous hairy roots. On the 268th day after seed sowing died all of the seedlings whose roots elongated upward and top growth was depressed seriously in the sealed flask (Plate I. fig. 2-B-b). At that time,

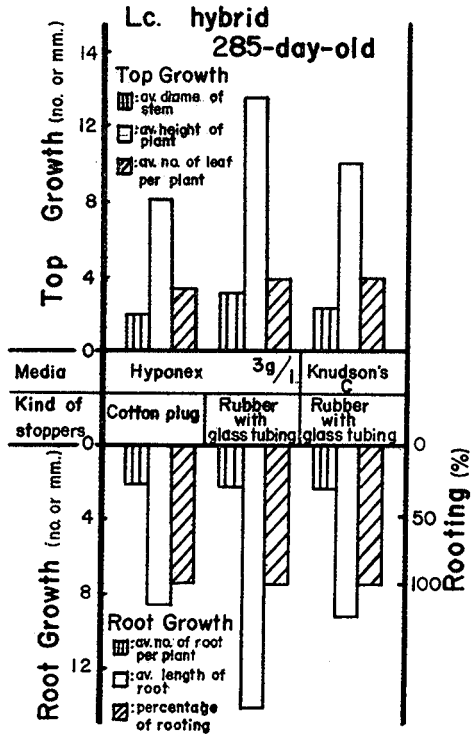

Fig. 2-1. Effect of kinds of stoppers and media on the germination of *Lc.* hybrid seeds.

temperature in the greenhouse rose drastically. This may be one of the reasons causing the death of the seedlings. The growth recorded 285 days after seed sowing is shown in fig. 2-1. The best growth of seedlings was recorded in the flask sealed with the rubber stopper with a glass tubing. In this case also, growth of the seedlings on the plain Hyponex solution exceeded the growth on the plain Knudson's solution C.

Effect of type of stoppers on the growth of orchid seeds in an early period of sowing culture was repeatedly examined. Results obtained with *Dendrobium* and *Brassolaeliocattleya* seeds are shown in tables 1 and 2. Although there are some variations depending upon the orchids used, the growth of the seedlings in completely sealed flasks is generally best as in the result shown in the Plate I. fig. 2-A.

Table 1. Effect of various kinds of stoppers on the growth of *Dendrobium* seeds.* Age: 156 days.

Stoppers	Av. height of plant	Av. no. of leaf per plant	Av. width of leaf	Av. no. of root per plant	Av. length of root	Rooting percentage
	mm.		mm.		mm.	
Cotton plugs	7.1	2.0	1.9	2.0	10.5	100
Rubber with glass tubing	7.4	2.6	1.8	1.9	11.8	100
Sealed completely	10.1	3.1	1.2	2.9	14.2	100

* Medium: Hyponex plus apple juice.

Table 2. Effect of various kinds of stoppers on the growth of *Blc.* seeds.* Age: 156 days.

Stoppers	Av. height of plant	Av. no. of leaf per plant	Av. dia. of proto-corm	Av. no. of root per plant	Av. length of root	Rooting percentage
	mm.		mm.		mm.	
Cotton plugs	3.9	2.7	1.4	1.6	3.3	100
Rubber with glass tubing	4.5	2.6	1.6	1.3	1.7	100
Sealed completely	5.3	2.9	2.0	1.2	1.7	80

* Medium : Plain Hyponex.

Plate I. fig. 3 shows the results obtained in another trial in which the effect of complete sealing on the growth of developing *Dendrobium* seedlings was examined. In this figure it is apparent that roots with numerous root hairs elongated upward and the top growth of these seedlings was depressed seriously. The results coincide with some of the results in the trials described above. Also in this figure, the excellence of the medium containing apple juice for the growth of *Dendrobium* seedlings is proved.

In Plate I. fig. 4 (A and B) is shown the result obtained in the trials in which the effect of cotton wad stuffed at the outer end of glass tubing was examined. Namely, a series of culture vessels was plugged with ordinary rubber stoppers, and another series was plugged with zigzag-shaped glass tubings instead of glass tubings with cotton wads at the outer ends. There was no appreciable difference in the

growth of the seedlings between these two series, except some contamination in the latter series.

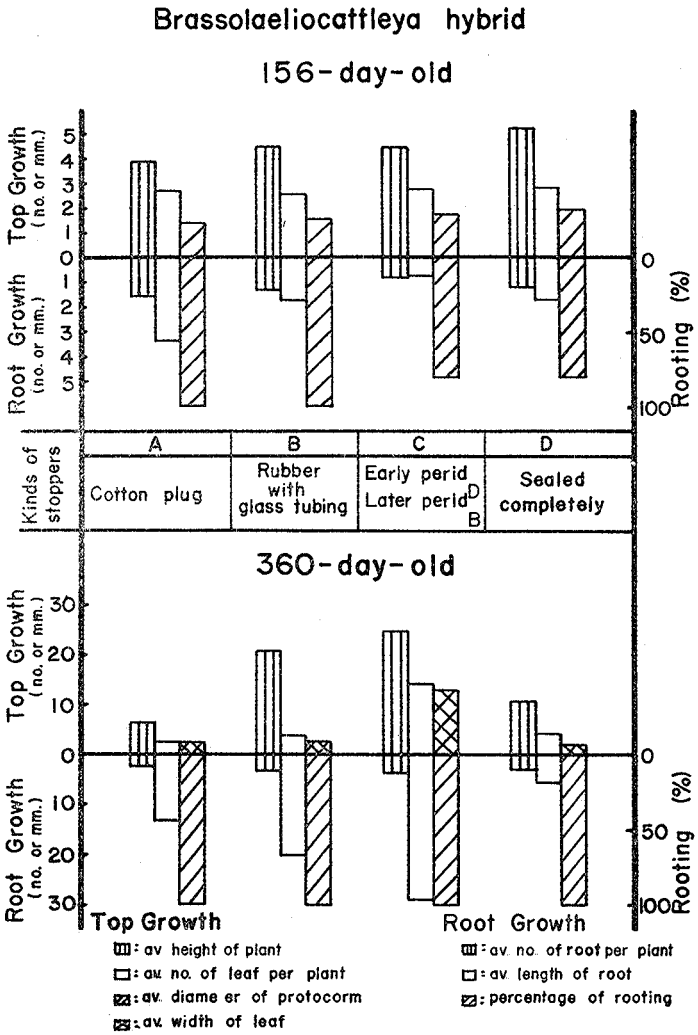

Fig. 2-2. Effect of various kinds of stoppers on the seed germination in *Blc.* In plot C the stoppers were changed on 156 days after sowing.

These facts give rise to an idea that better growth of seedlings may be expected when the culture is completely sealed in the early period of the cultures and plugged with ordinary rubber stoppers with glass tubings in the later period. This possibility was examined setting the plots as follows:

a: cotton plugs
b: rubber stoppers with glass tubings
c: completely sealed in the earlier period of culture and plugged with ordinary rubber stoppers with glass tubings in the later period

d: completely sealed throughout the culture

Actually, for the completely sealed plots, ordinary rubber stoppers with glass tubings whose outer ends were tightly plugged with small rubber cylinders were used. In plot C, the small rubber cylinder was removed 206 days after seed sowing. *Dendrobium grantii* was used as the seed material.

As shown in tables 3 and 4 and Plate I. fig. 5 (A–C), no favorable result was obtained in this attempt. It may be considered, however, that the timing of the removal of the rubber cylinder played an important role in the failure of this trial.

Table 3. Effect of stoppers on the growth of *Den. grantii*.* Age: 206 days, henceforth the rubber cylinder in plot C was removed.

Plot	Av. height of plant	Av. no. of leaf per plant	Av. dia. of protocorm	Av. no. of root per plant	Av. length of root	Rooting percentage
	mm.		mm.		mm.	
a	9.7	3.0	2.2	2.0	6.0	100
b	12.4	3.2	2.3	2.0	8.7	100
c	8.3	3.4	3.0	0.8	2.0	80
d						

* Medium: Hyponex plus apple juice.

Table 4. Effect of stoppers on the growth of *Den. grantii*, at the end of experiment.* Age: 365 days.

Plot	Av. height of plant	Av. no. of leaf per plant	Av. width of leaf	Av. no. of root per plant	Av. length of root	Rooting percentage
	mm.		mm.		mm.	
a	14.2	2.7	2.2	4.5	11.5	100
b	15.1	2.0	2.3	3.7	16.5	100
c	13.2	2.7	2.6	2.3	14.3	100
d	8.7	3.9	1.1	2.1	3.7	94

* Medium: same as above.

On this problem the author obtained successful result in another experiment with *Blc.* seeds as shown in fig. 2–2.

ii) Effect of size of culture vessels on the growth of *Brassavola nodosa* seedlings.

Hundred and forty-eight-day-old seedlings of *Brassavola nodosa* were transplanted into the culture flasks of various sizes, and the effect of the size of culture vessels on the growth of orchid seedlings was observed. In this experiment, the number of plants in each flask was limited to a plant per 5 ml. of nutrient solution.

Results are shown in table 5 and Plate I. fig. 6. These results show that growth of the seedlings is generally better in large culture vessels than in smaller ones.

iii) Discussion.

The results of the above experiments clearly show that the growth of the seedlings is better when culture vessels are sealed completely in the early period of culture, and is better in large culture vessels than in smaller ones in later period.

From the early period of asymbiotic culture of orchid seeds much attention has been paid by many investigators to the stopper of containers in order to minimize the desiccation of agar plate and exclude the fungal contamination which

Table 5. Effect of the size of culture vessels on the growth of *Brassavola nodosa* seedlings. Age: 177 days after transplanting.

Size of flasks	Amount of culture solution	No. of plant per flask	Av. height of plant	Av. no. of leaf per plant	Av. width of leaf	Av. no. of root per plant	Av. length of root
ml.	ml.		mm.		mm.		mm.
50	20	4	14.8	5.1	3.8	3.9	22.8
100	30	6	14.7	5.5	3.4	4.1	21.7
200	80	16	17.3	6.3	4.2	5.5	31.2
300	100	20	16.9	5.6	3.9	5.3	33.6

occurs often when a cotton plug is used as a stopper. The rubber stoppers with glass tubing eliminate these cares and assure a good growth of the seedlings as shown in the present experiment. Meyer (1948) has shown that growth of seedlings sealed in culture flasks was better than that in cotton-stoppered containers. His result coinsides with the result shown in the present experiment so far as the early period of seedling growth is concerned. Practically speaking, rubber stoppers with glass tubing may be recommended. This recommendation is supported also by Breddy's result (Breddy, 1953). When experiments are limited to germinating seeds, e.g. Oriental *Cymbidium* seed germination, however, sealed test tubes were used as containers in most cases.

As to the size of containers, Knudson (1922) observed that a better growth can be obtained in larger containers than in smaller ones. A similar result was obtained in the present experiment with *Brassavola nodosa* seedlings. From the practical standpoint containers of large size are recommended at least as transplanting vessels.

3. Effects of pH and solidity of agar bed on the germination of orchid seeds.

Effect of pH in the sowing bed was examined on *Brassolaeliocattleya* and *Dendrobium* hybrid seeds. The effect of the solidity of agar plate on the germinating orchid seeds was also examined, since it changes depending upon the pH value of nutrient solution.

i) Effect of pH on the germination of *Brassolaeliocattleya* seeds.

Plain Knudson's solution C was used as a basic culture solution, and pH was

regulated with KOH and HCl. In order to obtain a proper solidity of agar plate and hence, make planting operations easy, the following agar concentrations were employed in correspondence to different pH values.

Initial pH	Agar concentration (%)
4.55	2.0
5.25	1.5
5.65	1.0
6.72	1.0
8.38	1.0

In this experiment, the flasks were stoppered with cotton plugs.

The result is shown in fig. 3-1. The growth of the seedlings became better with the increased acidity. The best growth was recorded on the medium with pH at 4.55.

The acidity in each medium increased at the end of the experiment.

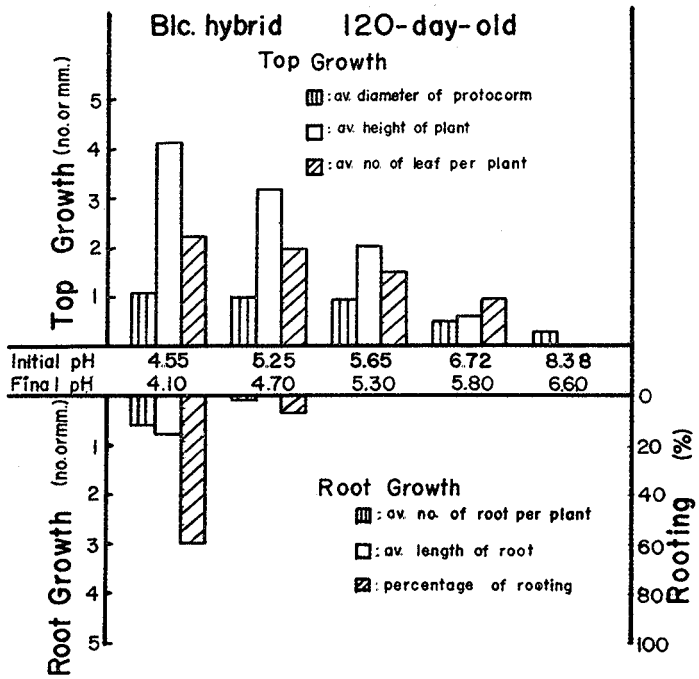

Fig. 3-1. Effect of pH concentrations in the sowing bed on the development of *Blc.* hybrid seeds. Basal medium: Knudson's soln. C.

ii) Effect of pH on the germination of *Dendrobium* seeds.

Knudson's solution C containing apple juice at 15 per cent was used as a basic culture solution. This solution is especially favorable for germinating *Dendrobium* seeds and has a strong buffer action.

Total sugar concentration in the solution was adjusted to 3.5 per cent by the

addition of sucrose. pH was regulated with Na_2CO_3 and HCl in this experiment. Agar concentration in each medium was adjusted also.

Initial pH (1 week after seed sowing)	Agar concentration (%)
4.25	2.5
4.60	1.7
4.95	1.3
5.30	1.0
5.75	0.7
6.02	0.7

As described above, initial pH values were determined one week after seed inoculation.

Fig. 3-2 shows the result. Generally speaking, there is no apparent difference among the plots. Observation record, however, shows that at first seed germination and growth of the seedlings were appreciably retarded on the media of high pH values. As shown in the figure, only small differences are seen among the final pH values which were determined after culture of six months.

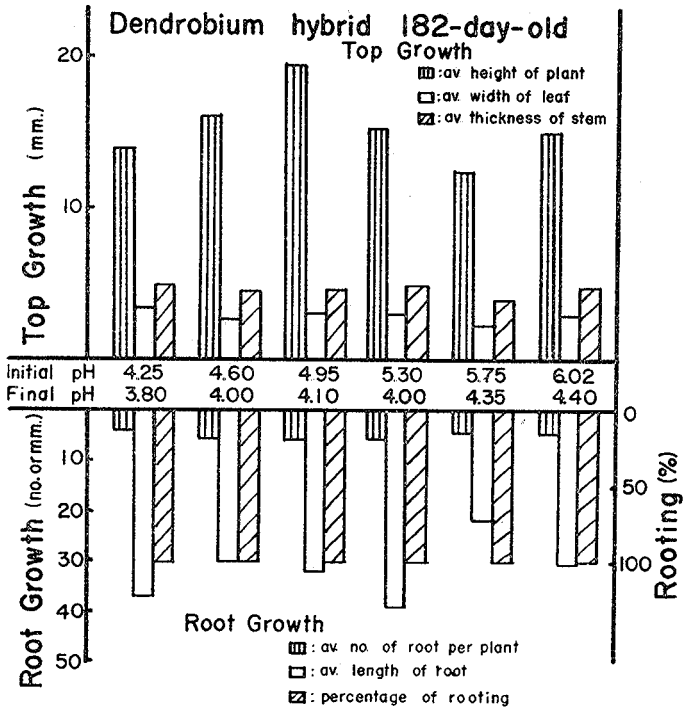

Fig. 3-2. Effect of pH concentrations in the sowing bed on the development of *Dendrobium* hybrid seeds. Basal medium : Knudson's soln. C. plus 15% apple juice.

iii) Effect of solidity of agar bed on the germination of *Laeliocattleya* seeds.

If the agar concentration is always kept constant, the solidity of agar plate

varies depending on changes of pH value of the solutions, especially after autoclaving. Therefore, the effect of solidity of agar bed on the growth of orchid seeds was investigated.

Knudson's mineral solution containing apple juice at 10 per cent was used as a basic solution. Total sugar concentration was adjusted to 2.4 per cent adding sucrose. pH of this solution was adjusted to 4.5. Agar concentrations at 0.8, 1.6 and 2.4 per cent were prepared. *Lc.* hybrid was used as seed material and the culture flasks were stoppered with cotton plugs. It was found after autoclaving that 0.8 per cent of agar is too soft.

The result is shown in fig. 3-3. As shown in the figure, the growth is better in the softish side.

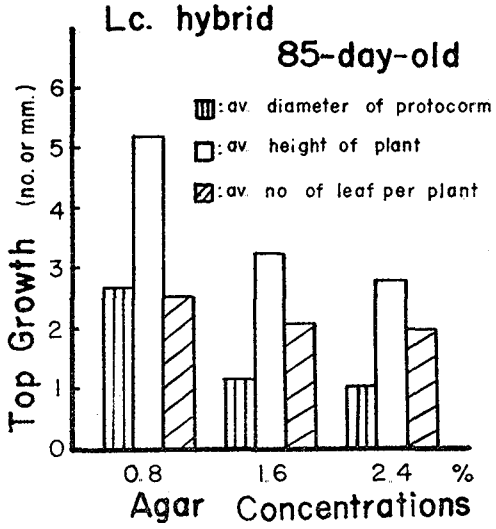

Fig. 3-3. Effect of solidity of agar plate on the development of *Lc.* hybrid seeds. Basal medium: Knudson's soln. C. plus 10% apple juice. Initial pH 4.5.

iv) Discussion.

Some workers have attempted to study pH stabilization in culture media (Burgeff, 1936; Vacin and Went, 1949a, b; etc.) and others referred to the pH concentration of germinating media (Burgeff, 1936; Breddy, 1953; Curtis and Spoerl, 1948; Downie, 1940, 1941; Hegarty, 1955; Knudson, 1925; La Garde, 1929; Lugo, 1955; Smith, 1932; etc.). However, these are of little importance as stated by Knudson (1951 and 1952). Namely, at the lower side of pH values, death of the embryo is a problem. The critical point of death in the range of pH values differs among the orchid genera or the species. The death is probably due to the increased availability of minor elements such as iron or manganese and in part due to the failure of absorption of calcium. On the other hand, when the solution is less acid, viz., pH 5.5 or above, these substances precipitate and are not utilized by the embryo. This brings no chlorophyll development in the embryo. That is, pH of the culture solution affects indirectly to the germinating orchid seeds through

the availability of iron, manganese and other substances in the solution, and if these substances could be kept available, growth of orchid seedlings would be as good at pH 6 as at pH 5.0 (Knudson, 1952).

Withner (1959) stated on the pH problem of culture solution of orchid that "except for effect of pH on availability of iron, calcium, phosphate, or other inorganic nutrients which tend to precipitate as insoluble complexes above approximately pH 5.3, pH does not seem to have a well-defined effect on growth". In studying the pH problems of nutrient solution, he suggested the applicability of complexed or chelated forms of the metal ions as an available nutrient source not so readily influenced by the pH of the solution.

In the present experiment, another indirect effect of pH, through the solidity of agar bed on the germination of orchid seeds was demonstrated. Good growth on softish agar bed is probably due to the good moisture maintenance. Thus the effect of pH of culture solution on the germination of orchid seeds is indirect and complicated matter.

In order to ascertain the direct effect of pH on the germination of orchid seeds, another type of solidifying material and of minor elements not so readily influenced by the pH variation of the solution must be employed.

With *Dendrobium*, appreciable amount of seedling growth was obtained on the medium at pH 6.02 at the end of experiment. This is probably due to the fact that in such complex nutrient solution the minor elements which readily precipitate in less acid solution would be contained in available complexed forms.

Many investigators observed that the culture medium becomes more acid during the growth period (Bahme, 1949; Burgeff, 1936; Curtis and Spoerl, 1948; Knudson, 1951; Spoerl, 1948; Vacin and Went, 1949b; Wynd, 1933b, c; etc.). The similar results were also obtained in the experiment. This may be due to the utilization of chemicals in the medium by the germinating embryos and the products of growth as suggested by Vacin and Went (1949b). The pH stabilization of culture medium is of little importance as pointed out by Knudson (1951). It seems, however, that the acidity of culture solution is only increased in every experiment in which the well developed orchid seedlings were obtained including the present author's. While in the experiment of Muir, Hildebrandt and Riker (1958) during incubation of marigold crown gall tissue the original pH levels of the liquid medium drifted toward 6.1.

As a conclusion pH about 5.0 and agar concentration about 1.5 per cent may be recommended for germinating orchid seeds from the practical point of view. Under these conditions the agar bed is hard enough for the planting procedure, and a good germination and growth of the seedlings of many orchid genera are secured.

4. Effect of sugar concentration in sowing bed on germination of *Dendrobium* and *Brassolaeliocattleya* seeds.

i) On *Dendrobium* seeds.

Sucrose concentrations of 0.5, 1.0, 2.0, 4.0 and 8.0 per cent in sowing bed

were prepared. For a basic medium Knudson's solution C was employed. pH value of these media was about 5.1.

The results are shown in fig. 4.

The best growth of the seedlings was recorded on the medium containing 4.0 per cent sucrose. The rooting became better with the increase in sucrose concentration. At 8.0 per cent growth in both top and root was suppressed as compared with the growth at 4.0 per cent.

ii) On *Brassolaeliocattleya* seeds.

The sucrose concentrations prepared and the basal medium employed were same as in the experiment on *Dendrobium*.

The results are also presented in fig. 4.

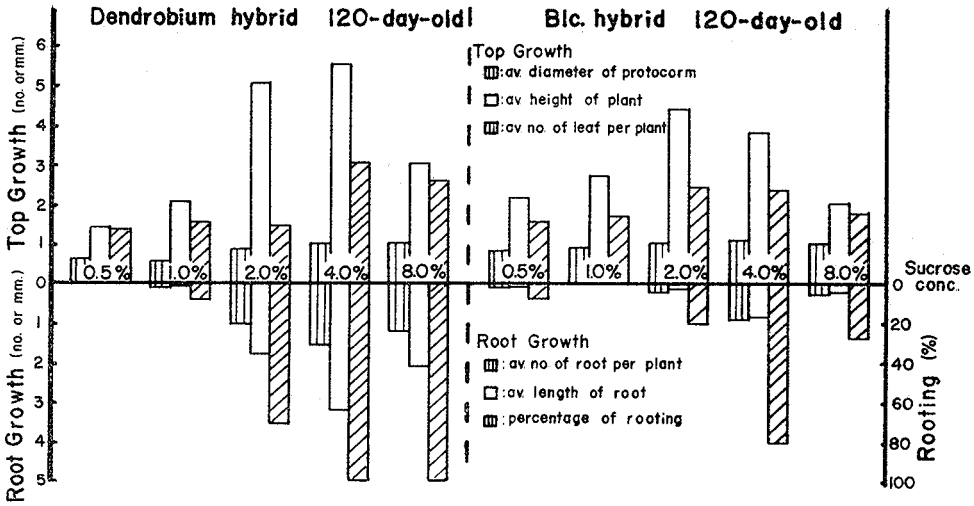

Fig. 4. Effect of sucrose concentrations in sowing bed on the development of *Den.* and *Blc.* hybrid seeds. Basal medium: Knudson's soln. C.

In general, results are the same as those obtained with *Dendrobium* seeds, though in this genus somewhat better growth in top was seen on the medium with 2.0 per cent sucrose than with 4.0 per cent medium.

iii) Discussion.

Researches in connection with the types of sugars in germinating bed have been presented by many workers on various kinds of orchids (Bouriquet and Boiteau, 1937; Bultel, 1925; Cappelletti, 1933; Knudson, 1924, 1952; as to others see Withner's chronology concerning the research on carbohydrate sources for orchids, Withner, 1959), but very few experiments can be found concerning the sugar concentrations in sowing bed. Except those of Burgeff (1936), Ito (1961) and Yates and Curtis (1949), most of the experiments were carried out making the concentrations of sugars to the extent of 2.0 per cent (Knudson, 1922; Withner, 1959). Yates and Curtis presented the results that the optimum concentrations of

sucrose for the development of shoots and roots were found to be distinct, higher concentrations favoring root growth. And concentrations which favor root growth and do not suppress top growth too much are between 0.10 and 0.15 molar, each species showing individual characteristics. This almost accords with the present results.

From the practical standpoint, sucrose concentration of about four per cent may be recommended for the germinating bed.

5. Effect of gibberellin added to the sowing and transplanting beds on the germination of orchid seeds and growth of seedlings.

i) On the seed germination and seedling growth of *Bletilla striata*.

Knudson's solution C containing 0.1, 1.0 and 10 ppm of GA were prepared. Plain Knudson's solution C was used as a control. Seeds of *Bletilla striata* were inoculated onto these media. The growth of the seedlings recorded 100 days after seed sowing is presented in fig. 5-1.

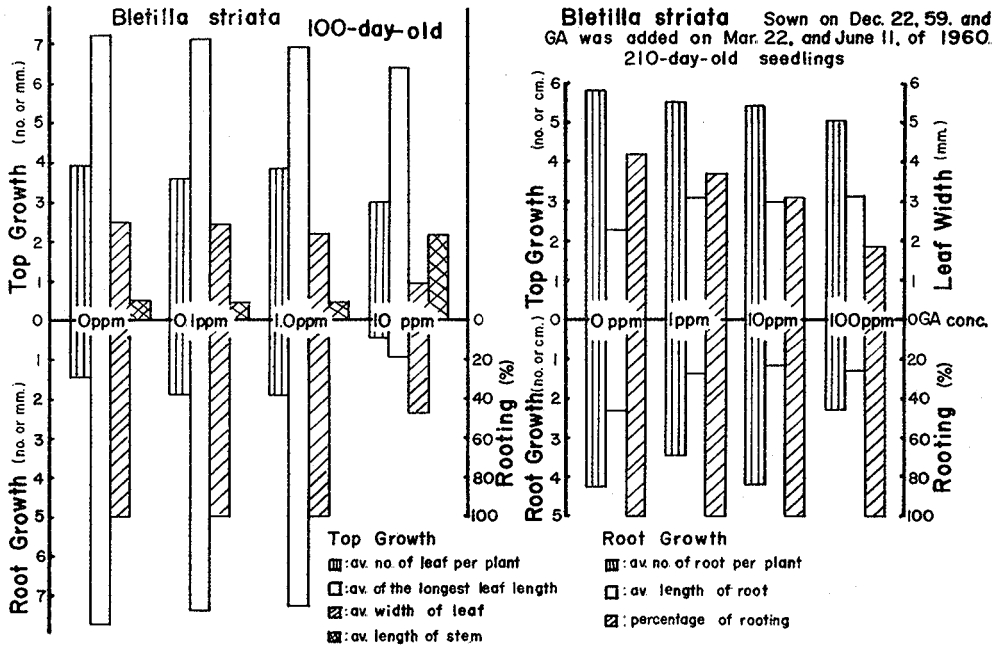

Fig. 5-1. Effect of gibberellin on the development of the seeds and seedlings of *Bletilla striata*. Basal medium: Knudson's soln. C. Flasks were stopped with cotton plugs.

The stem elongated exceedingly on the medium with 10 ppm of GA, while the number of leaves, leaf length and leaf width were reduced. Root growth was seriously suppressed on this medium.

As presented in fig. 5-1, the effect of GA on the developing seedlings of *Bletilla striata* was also examined. In this experiment, about 1 ml. of GA was poured

aseptically into the culture flask (300 ml. capacity) which contained about 100 ml. of culture solution. The concentrations of GA were 1, 10 and 100 ppm. The pouring treatment was made 90 and 172 days after seed planting. The recording was made 210 days after seed sowing.

Generally speaking, in top growth, the number of leaves per plant and the width of leaf were reduced by the GA treatment, while the length of the longest leaf was somewhat increased. GA generally suppressed the root growth except the percentage of rooting which seemed established before GA treatment.

ii) On the germination of *Dendrobium* seeds and growth of *Brassolaeliocattleya* seedlings.

Knudson's solution C plus 10 per cent apple juice was used as a basic culture solution. The concentrations of GA added to the media were 0, 0.5, 5.0 and 50 ppm.

Eighty-day-old seedlings of *Blc.* and seeds of *Dendrobium* were planted on these media.

The results are shown in fig. 5-2.

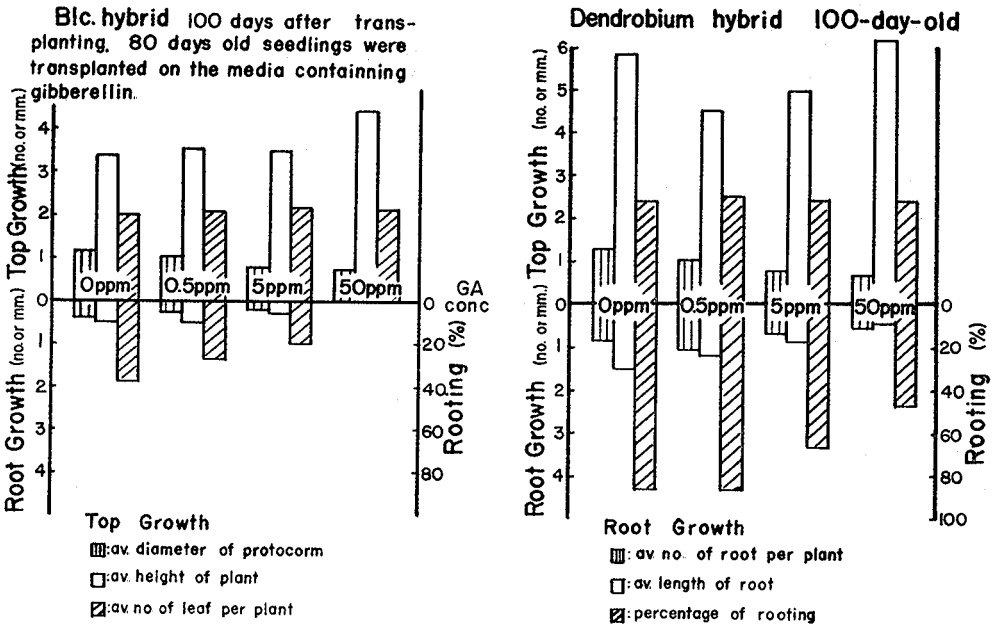

Fig. 5-2. Effects of gibberellin on the development of the *Blc.* seedlings and the *Dendrobium* seeds. Basal medium: Knudson's soln. C plus 10 percent apple juice.

In both genera, average diameter of protocorms decreased by degrees as the concentration of GA increased. The similar tendency was seen in root growth. The growth of plant in height was somewhat better on the media with 50 ppm of GA in both genera.

iii) Discussion.

From the practical standpoint no favorable effect of GA on the germination of orchid seeds or on the growth of their seedlings was observed in these experiments. This accords with the result of other investigators (Sisa and Sawa, 1963). Applicability of GA to orchid plants may be limited to grown up plants for such purposes as acceleration of flowering, shortening of juvenile phase, and controlling flowering time, as proved with other plants.

6. Effects of concentrations of Hyponex and IBA in sowing and transplanting beds on the germination and growth of *Dendrobium* and *Brassolaeliocattleya* seeds and their seedling growth.

The applicability of Hyponex as a mineral nutrient base in asymbiotic germination of orchid seeds was already proved in section 1. In this experiment, suitable amount of Hyponex in sowing and transplanting beds was determined using the seeds and seedlings of *Den.* and *Blc.* The effects of IBA on the germination of these orchid seeds and their seedling growth were also examined for its practical applicability.

Based on the results obtained in the experiments of section 1, 10 per cent apple juice solution was used as basic solution for *Dendrobium*, and plain Hyponex solution was employed for *Brassolaeliocattleya*.

i) Effect of Hyponex concentration on the seed germination and seedling growth in *Dendrobium* and *Brassolaeliocattleya*.

As described above, with *Dendrobium* 10 per cent solution of fresh strained apple juice was prepared. Then 2, 3, 4 or 5 grams of Hyponex was added per liter of the solution. Total sugar concentration of each solution was adjusted to 3.5 per cent adding sucrose. pH of these media ranged between 4.80 and 4.95 after autoclaving. With *Blc.* 2, 3, 4 or 5 grams of Hyponex was added per liter of water. Sucrose was added at the rate of 35 grams per liter of each solution. pH of these culture solutions ranged between 4.95 and 5.1 after autoclaving.

Seeds or seventy-nine-day-old seedlings of *Dendrobium* were inoculated or transplanted respectively onto apple juice media containing various amounts of Hyponex.

Similarly, seeds and ninety-one-day-old seedlings of *Blc.* were planted onto a series of plain Hyponex media.

The results are presented in fig. 6-1 and Plate I. figs. 7 and 8.

As can be seen in the figures and plate, with *Dendrobium* seeds, plant height increased by degrees according as the amount of Hyponex increased. The growth response of roots was quite reverse to that of plant height. As a whole, the same results are seen in the transplanting experiment of this genus except that the best elongation of root was recorded on the medium with three grams of Hyponex. Seedlings showed a pale green color on the sowing bed with low concentrations of

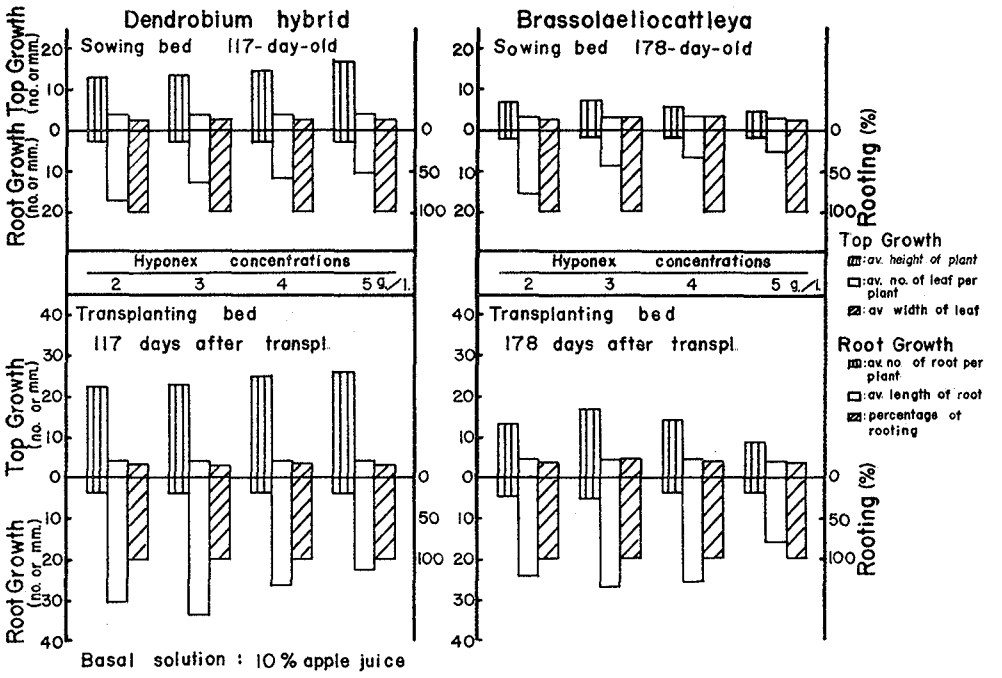

Fig. 6-1. Effect of concentration of Hyponex in the sowing and transplanting beds on the growth of seeds and seedlings of *Den.* and *Blc.* hybrids.

Hyponex as observed in the Plate. I. fig. 7.

On the other hand, with *Blc.* the best growth in plant height was recorded on the medium with three grams of Hyponex in both sowing and transplanting beds. In the sowing experiment, the root length decreased by degrees as the amount of Hyponex increased as in *Dendrobium*. In this genus, however, growth in both top and root was apparently suppressed on the medium with five grams of Hyponex. This dose of Hyponex also suppressed the growth of transplanted seedlings. The best growth in both top and root of transplanted seedlings was recorded on the medium with three grams of Hyponex.

ii) Effects of IBA concentration on the seed germination and seedling growth in *Dendrobium* and *Brassolaeliocattleya*.

As in the former experiments, solutions of various constitutions were employed as base depending upon orchids used as follows:

Orchid	Basic solution	Sugar concentration (%)
Dendrobium	Hyponex 3 g./liter of 10 per cent apple juice soln.	3.5
Brassolaeliocattleya	Hyponex 3 g./liter of water	3.5

Concentrations of IBA employed were 0, 0.1, 1.0 and 10 ppm in each group of

culture solution. pH values in apple juice media ranged between 4.8 and 4.95, and those of plain Hyponex were between 4.95 and 5.1. The same seeds and seedlings of the orchids used in the former experiment were inoculated or transplanted onto the media of each group.

The results are presented in fig. 6-2 and Plate II. figs. 1 and 2.

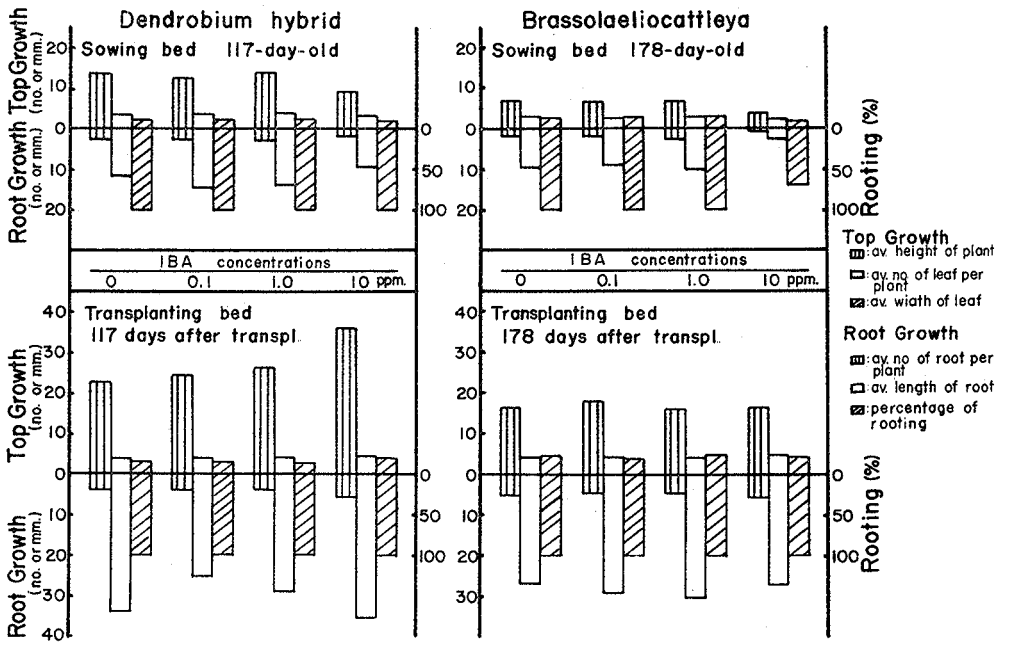

Fig. 6-2. Effect of IBA in the sowing and transplanting beds on the growth of seeds and seedlings of *Den.* and *Blc.* hybrids.

With *Dendrobium*, the seed germination and the growth of germinated seedlings were extremely inhibited by 10 ppm of IBA. On the contrary, the growth of the transplanted seedlings was apparently promoted by this concentration of IBA in both top and root, especially in plant height and number of roots per plant. Observation record shows that on this medium, however, transplanted seedlings become abnormal in forms immediately after transplanting and their roots were separated easily from the plant body at the time of sampling.

With the seeds and seedlings of *Brassolaeliocattleya*, no excellent effects were observed except that the seed germination and the growth of germinated seeds were inhibited by 10 ppm IBA as in the case of *Dendrobium*.

iii) Discussion.

Growth response of both seeds and seedlings to the concentration of Hyponex differed between *Dendrobium* and *Brassolaeliocattleya*. The former developed well on all the media examined, while in the latter the best growth of transplanted seedlings and the best top growth of the germinated seedlings were recorded on the medium with 3.0 grams of Hyponex, and the highest concentration of Hyponex apparently

suppressed the growth in both germinated seeds and transplanted seedlings. These facts suggest that the tolerance to high salt concentration, and the optimum salt concentration for best growth differ between these two orchids.

As the concentration of Hyponex in sowing beds increased, the growth in root length of germinated seedlings was inverse proportionally decreased in both *Dendrobium* and *Brassolaeliocattleya*. The similar tendency was also seen with the transplanting media of *Dendrobium*. This phenomenon is quite interesting when compared with the effect of sugar concentration on the root growth (section 4 of the present work, and Yates and Curtis, 1949), i.e. the growth in root length generally increased according as the concentration of sucrose increased. Thus, with *Dendrobium*, the effect of fertilizer and that of sucrose on the shoot-to-root ratio of the seedlings are in reverse.

The other results of Yates and Curtis (1949) concerning the effect of salt concentration on the shoot growth of other orchids, generally accord with the present results. So far as root growth is concerned, however, they conflict especially with the results obtained with *Dendrobium* in the present experiment.

Generally speaking, the present results concerning the Hyponex concentration show that optimal concentrations for root growth are lower than for top growth, although each orchid showing individual characteristics.

It is well known that many kinds of orchids grow so well on a wide variety of combinations and concentrations of inorganic substances in culture. However, very little is known about the actual mineral requirements, the optimal mineral combination or the optimal mineral concentration for the germination and growth of different orchid groups. There are many problems to be solved scientifically as to the mineral nutrition for asymbiotic germination of orchid seeds. The present work was carried out in order to determine the favorable amount of Hyponex as a mineral base of culture solution from the practical standpoint.

Concerning the concentration of Hyponex for practical use, the concentration of three grams per liter with 3.5–4.0 per cent of sugar may be recommended for the sowing and transplanting beds for *Dendrobium* and *Brassolaeliocattleya*, although for the former genus it is better to combine with apple juice solution (see also sections 1, 4, 9 and 10).

The results concerning the effect of IBA on the germination and growth of seedlings in the present work show that 10 ppm of IBA in transplanting bed is usable for *Dendrobium* in practice. This concentration of IBA inhibited the germination and growth of germinated seedlings in both *Dendrobium* and *Brassolaeliocattleya* on sowing bed and scarcely any effect was observed on the growth of transplanted seedlings of the latter orchid.

As to the work with auxins, Withner's review (1951) and his chronology (1959) are valuable. The present results generally accord with the results shown by Meyer (1945b), Meyer and Pelloux (1948), Sisa and Sawa (1963) and Withner (1951, 1955). Some of the discrepancy with their results are probably due to the differences in basal media employed.

7. Effects of vitamins, plant saps and organic acids on the germination and growth of some kinds of the orchid seeds.

i) Effect of vitamins.

Effect of thiamine and "vitamin complex" ("Panvitan solution": each cubic centimeter containing Vitamin A, 5,000 I.U.; Vitamin D_2, 500 I.U.; Vitamin B_1, 2 mg.; Riboflavin, 3 mg.; Nicotinamide, 20 mg.; Pyridoxine HCl, 2 mg.; Na-pantothenate, 5 mg.; Vitamin B_{12}, 2 mg.; Ascorbic acid, 75 mg.; and L-Lysine monohydrochloride, 25 mg.) on germinating *Dendrobium* seeds was examined using Knudson's minerals as base. A medium with 15 per cent apple juice was also prepared. Sugar concentration in all the media used was regulated to 3.5 per cent adding sucrose.

The media employed were as follows:

(a) Knudson's solution C (sucrose: 3.5 per cent)
(b) (a) plus thiamine 1 mg./l.
(c) (a) plus "Panvitan solution" 0.5 ml./l.
(d) (a) plus "Panvitan solution" 1.0 ml./l.
(e) Knudson's mineral solution 850 ml. plus fresh strained apple juice 150 ml.

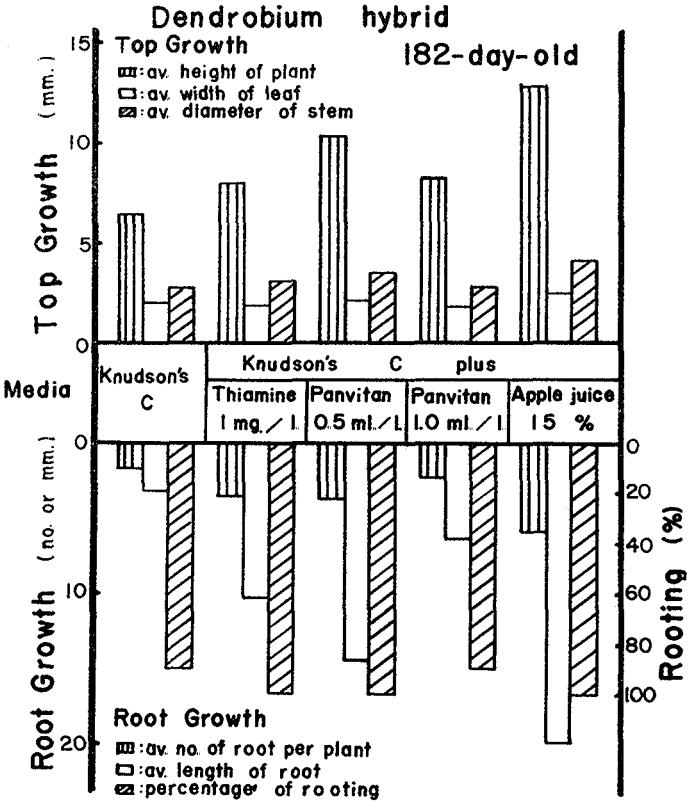

Fig. 7-1. Effects of thiamine and vitamin complex on the germination of *Dendrobium* seeds.

pH of these media after autoclaving was between 4.9 and 5.2. Measurements were recorded after six months of culture.

The result of this experiment shown in fig. 7-1 proves that all the additions examined give a good effect in germinating the seeds. The best growth, however, was obtained on the medium containing apple juice, followed by one with "Panvitan solution" 0.5 ml./l., thiamine 1 mg./l. and "Panvitan solution" 1 ml./l. The best germination was also obtained on the medium with apple juice.

A similar experiment was carried out with glutathion (10 mg./l.), DL-malic acid (8 mg./l.), glutathion (10 mg./l.) plus DL-malic acid (8 mg./l.) and apple juice (15 per cent v/v). In this experiment too, the best germination and growth were recorded on the medium with apple juice. Other additions showed little or no favorable effect on germination or seedling growth.

ii) Comparison of effectiveness of fresh strained tomato and apple juices on germination of *Brassocattleya*, *Dendrobium* and *Cypripedium reginae* seeds.

The effectiveness of apple and tomato juices for germinating orchid seeds was repeatedly examined in preliminary experiment. The results are somewhat in conflict among the experiments. Therefore, effectiveness of these juices was examined repeatedly using *Brassocattleya*, *Dendrobium* and *Cypripedium reginae* seeds.

Knudson's C minerals and Hyponex (3 g./l.) were used as bases. To each of them, 5 or 15 per cent of apple juice or 15 per cent of tomato juice was added. Meyer's tomato juice solution was also prepared. Sugar concentration in each medium was adjusted to 3.5 per cent adding sucrose, except in Meyer's solution in which the sugar concentration was 2.3 per cent.

The results are shown graphically in fig. 7-2 with the pH values determined prior and after the culture in each medium. Some of the results are also shown in Plate II. fig. 3 (A-D).

With *Brassocattleya*, the best growth was recorded on plain Hyponex, and the growth was poorer on the media containing 15 per cent tomato juice in both bases. Addition of apple juice to Knudson's minerals, suppressed the top growth while it somewhat increased the growth in root length. In the Hyponex series of experiment, however, the growth in both top and root was suppressed by addition of apple juice. The superiority of Hyponex in germinating the seeds of *Cattleya* group was confirmed again (see figs. 1-3 and 2-1). However, the effectiveness of apple and tomato juices in both basal solutions conflicted again with the results obtained in the experiments of section 1. This conflict may be probably derived from the differences in the kinds of orchids used, the differences in the concentration of the juices or the minerals used, differences in the pH values among the media, the type of sugars and its concentrations employed, etc. Another important factor was found, which will be described later and will be discussed in Chapter IV.

With *Dendrobium*, growth of the seedlings was generally promoted on the

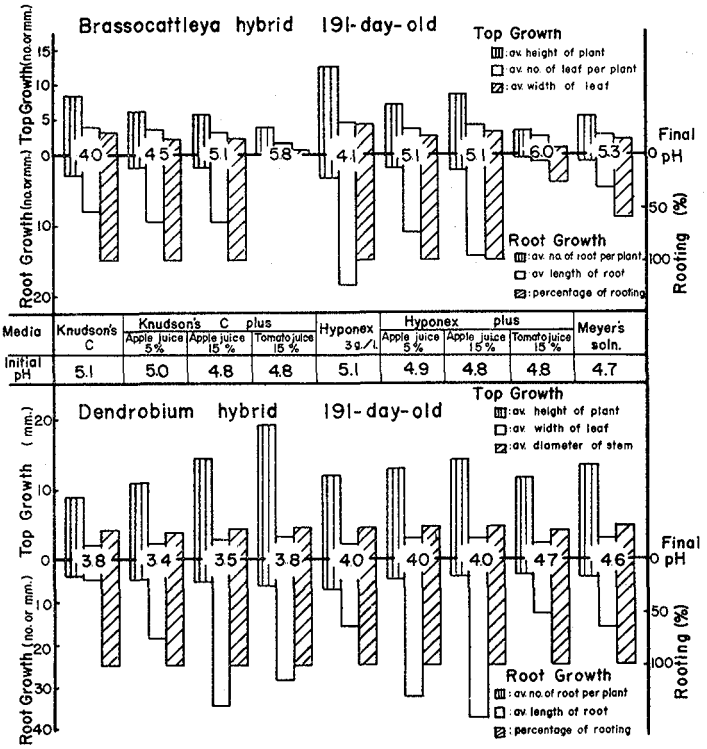

Fig. 7-2. Comparative growth of *Bc.* hybrid and *Den.* hybrid seeds on the different basal media and additions.

media to which the juices had been added. Again the growth of the seedlings on plain Hyponex exceeded that of Knudson's (see fig. 1-3). The best growth of the seedlings was recorded on the Knudson's medium with 15 per cent tomato juice. As shown in Plate II. fig. 3 (A-D), however, the germination and the growth of seedlings on the media containing tomato juice were erratic among the flasks in the same plot, while on the media containing apple juice, uniform germination and growth of seedlings were observed in all the cultures employed. This seems to be one of the reasons why the effect of tomato juice differed among the experiments.

The growth of seedlings increased by degrees especially in root length, according as the concentration of apple juice increased on both bases.

The final pH value in each medium is shown in fig. 7-2. As a whole, acidity increased during the culture period with *Dendrobium*, while with *Brassocattleya*, it generally decreased in the media with juices, especially with tomato juice, and on which the growth of seedlings was very poor. This fact probably consists in other reasons for the conflicted results on the media with apple and tomato juices among the experiments. Detailed discussions of these phenomena will be presented later.

With *Cypripedium reginae* seeds, no seedling has been obtained during about two years of culture period on any of the media examined, although on some media a few seeds have swollen and protocorm-like masses of the embryo have developed.

Rest of the embryos swelled to a certain degree on all media examined, but they became brown in color and stopped swelling.

iii) Effect of dried apple fruits on seed germination in *Dendrobium*.

The pronounced favorable effect of apple juice on germinating *Dendrobium* seeds was already shown repeatedly. The effect of dried apple fruits on germinating *Dendrobium* seeds was also examined.

Two basic solutions, Knudson's C minerals and Hyponex (3 g./l.), were used as in the previous experiment. The design of the experiment was almost the same as the experiment described above, i.e., to each group of basal solution, fresh strained apple juice (15 per cent v/v) and powder of dried apple fruit (10 or 20 g./l.) were added respectively. Sugar concentration in all the media was adjusted to 3.5 per cent adding sucrose. pH of these media after autoclaving was between 4.7 and 5.2.

The results are shown in fig. 7-3 graphically and in Plate II. fig. 4.

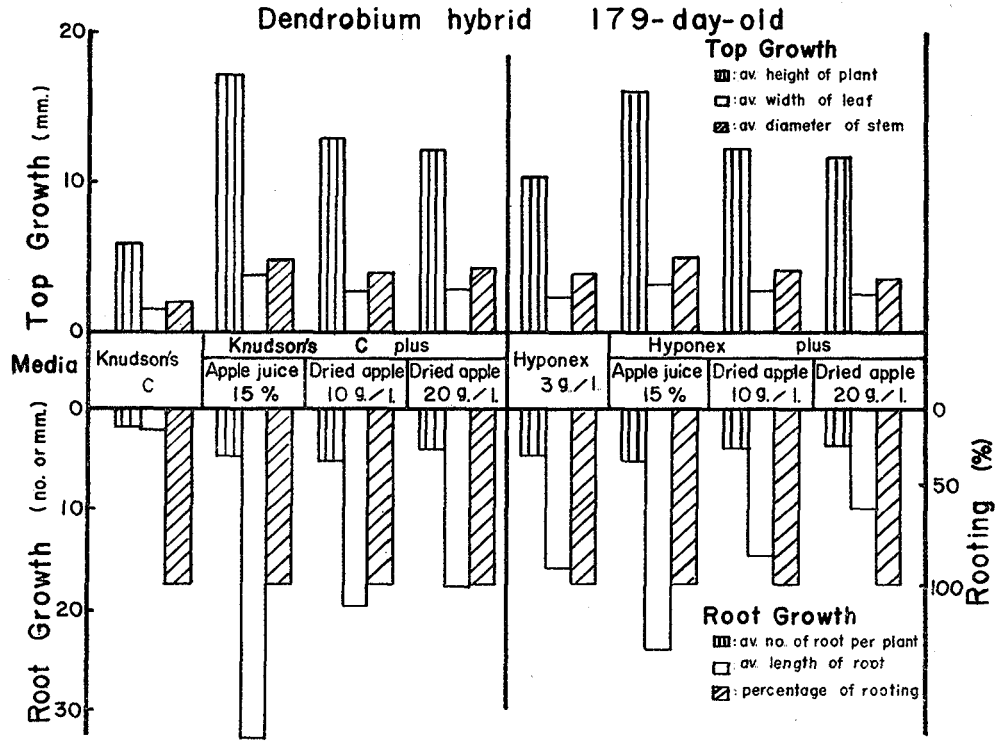

Fig. 7-3. Effect of dried apple added to the media on the germination of *Dendrobium* seeds.

Generally speaking, the good germination and growth of the seedlings were obtained on the media in which both fresh and dried apples were supplemented. In both Knudson's and Hyponex bases, the best growth was recorded on the media containing 15 per cent fresh apple juice. Growth of the seedlings was somewhat better on the Knudson's minerals plus apple series than that on the Hyponex series

in each corresponding medium. Again, the germination and growth of the seedlings on plain Hyponex medium excelled those on plain Knudson's.

iv) Effects of organic acid and plant saps on the germination and growth of *Dendrobium grantii* seeds.

Relating to the pronounced positive effect of apple juice for germinating *Dendrobium* seeds, effects of organic acids such as DL-malic acid (160 mg./l.) citric acid (80 mg./l.) and pyruvic acid (80 mg./l.) on germinating *Dendrobium grantii* seeds were examined. These acids were added individually or in combination to plain Hyponex solution (Hyponex 3 g./l., sucrose 3.5 per cent). At the same time, a medium containing Hyponex at the rate of 1.5 g. per 500 ml. of 10 per cent apple juice was prepared. In this medium, sugar concentration was adjusted to 3.5 per cent adding sucrose. As shown in Plate II. fig. 5 (A–B), growth of germinated seeds was observed to be the best on the medium with apple juice. As to the effect of organic acids added, good germination was obtained on the media containing malic acid, while citric acid somewhat inhibited growth of the seedlings.

Effect of mashed banana (5% v/v), onion juice (10% v/v) dried apple fruit (5 g./l.) and fresh apple juice (10% v/v) was also examined using the same kind of orchid seeds as in the former experiment. The plain Hyponex solution mentioned above was also used as base in this experiment. Sugar concentration in all the media was adjusted to 3.5 per cent adding sucrose. pH of these media was between 4.6 and 5.0 after autoclaving.

The results are also shown photographically in Plate II. fig. 6 (A–B).

As a whole, the germination was promoted by the additions. The best growth was again recorded on the medium with fresh apple juice, and then followed by that with onion juice. Although banana increased the germination, it inhibited the chlorophyll formation of the young plantlets.

v) Discussion.

Throughout the experiments hitherto described, fresh apple juice was found to be most effective with uniform results for germinating *Dendrobium* seeds. It can be used satisfactorily in combination with Hyponex as a nutrient medium for germinating seeds of this orchid genus.

Though any substance or substances contained in apple juice and affecting the germination and growth of the seedlings have not been found yet, much effort to clarify this problem has not made in the present work. At least, however, malic acid seems to play some role in increasing the germination capacity.

There is a number of researches on vitamin effect (see Withner's chronology of orchid vitamin research, Withner, 1959; Crovetto, 1957a, b, and Sisa and Sawa, 1963). The results are conflicted among the investigators, and Withner (1959) stated that "there is still the shadow of a doubt about vitamin effects, but it does seem likely that for certain orchids thiamine and niacin are effective, and possibly others of the B group have an effect on occasion".

In the present work, thiamine and vitamin complex somewhat enhanced the germination and growth of the seedlings. And other additions such as citric acid, malic acid, pyruvic acid or glutathione gave little or no stimulation. On the contrary, Withner (1942) observed beneficial effect of malic and pyruvic acid using mainly *Cattleya* seeds, though the doses used were much lower than in the present work. Later he examined the effects of all types of organic compounds such as the various available intermediates in carbohydrate metabolism, many amino acids and casein hydrolysate, the purine and pyrimidine, all of the known B-vitamins, and many miscellaneous compounds and mixtures (1951). In general, the results led him to disappointment. However, as to adenine and some of the amino acids he suggested that further investigation is worth-while.

The erratic effect of tomato juice was observed also in culture of endosperm of maize by some investigators (Tamaoki and Ullstrup, 1958 and Straus, 1960). Straus postulated that the fact was probably due to differences between the different lots of canned tomatoes. In the present work, however, the erratic effect was observed among the vessels in the same plot or among the experiments. This suggests that another reason may be involved. In table 6 are shown the data concerning the pH change in media with or without seedlings. As a whole, acidity increased in the media on which growth of the seedlings was better, while it

Table 6. pH change in media, with or without the seedlings.

Media		Knudson's C	Knudson's C plus			Hyponex 3g./l.	Hyponex plus			Meyer
			Apple juice 5%	Apple juice 15%	Tomato juice 15%		Apple juice 5%	Apple juice 15%	Tomato juice 15%	
Initial	pH	5.1	4.95	4.8	4.75	5.1	4.9	4.8	4.8	4.65
Bc. hybrid	191 day old	4.0 ++	4.5 ++	5.1 ++	5.8 +	4.05 ++	5.05 ++	5.1 ++	6.0 +	5.3 +
	254 day old	3.8 ++	4.2 ++	4.5 ++	5.7 +	3.95 ++	4.95 ++	4.65 ++	5.8 +	5.7 +
Den. hybrid	191 day old	3.8 ++	3.4 ++	3.5 ++	3.8 ++	4.0 ++	3.95 ++	3.95 ++	4.70 ++	4.6 ++
	243 day old	3.7 ++	3.35 ++	3.55 ++	4.1 ++	3.9 ++	3.95 ++	4.10 ++	5.7 +	5.5 +
Cyp. reginae	254 day old	4.35 −	5.0 −	5.55 −	---	4.4 −	5.0 −	5.4 −	6.0 −	5.8 −
	543 day old	4.05 −	4.75 −	5.05 −	5.2 −	4.15 −	4.6 −	4.8 −	---	5.35 −

++, + and − show the degree of plant growth.
++: good growth
+ : poor growth
− : no growth

decreased in the media on which growth of the seedlings was poor, especially in the medium containing tomato juice. This shift of pH value was also observed in the media on which the seeds of *Cypripedium reginae* were inoculated and no germination occurred. In this case, on the 254th day from seed inoculation, the acidity increased in both plain Knudson's and Hyponex media, while it decreased in the media containing high concentrations of apple and tomato juices, especially in the media with tomato juice. On the 543rd day from seed inoculation, acidity increased in every medium in comparison with the former pH values. These facts mean that the pH in a medium is shifting from time to time regardless of whether the seedlings are alive or not. As already described and discussed in section 3, the increase of acidity during the culture period of orchid seeds seems to be a usual event. The correlation between the poor growth of the seedlings and the rise of pH on the media with tomato juice, however, calls attention to the erratic effect of tomato juice. As shown in fig. 3-2, good germination and growth of the seedlings were obtained on the media whose pH was below 6.0. Hence, with *Dendrobium*, it seems probable that the pH value itself is not the limiting factor.

Based on the considerations mentioned above, the auther believes that the products of chemical reaction which shifted the pH value in the media containing tomato juice may be concerned with the erratic effect of these media.

Nevertheless, the fact remains that tomato juice occasionally gives a pronounced effect on germinating orchid seeds. This may suggest that tomato juice is able to supply some nutritional needs to the orchid embryos.

As to the factors which are contained in tomato juice and support good growth of orchid embryos, Vacin and Went (1949b) showed that organic rather than inorganic compounds are responsible for the enhanced growth. And then, with *Cymbidium* embryo, they obtained more rapid growth than on the tomato juice media, by adding a protein hydrolysate, rich in amino acids and vitamins, to the inorganic solution. In addition, concerning the unknown factor in tomato juice which supports good growth of endosperm tissue of maize, Straus (1960) concluded that it might be free amino acids which are released into the juice by autoclaving under acid conditions.

As for the active substance or substances in tomato and apple juices, attention must be paid primarily to the amino acids as the above-mentioned considerations suggest.

Mashed banana and onion juice were already used by some investigators on germinating orchid seeds with success (banana: Withner, 1943, 1955 for germinating immature seeds of *Paphiopedilum* or *Vanilla*; Karasawa, 1964 for germinating many genera; onion: Ito, 1955, for germinating immature seeds of *Dendrobium*). No pronounced effect of them, however, was obtained in the present experiment. Differences among the genera of orchid used and differences among the developmental stages of seed may be involved in the reasons for these conflicted results.

As for the germination of the seeds of *Cattleya* group, plain Hyponex solution is worth being recommended for practice.

No well developed seedlings of *Cypripedium reginae* were obtained on the media examined. About this observation, a brief discussion will appear in section 8.

8. Effects of peptone, tryptone and other additions on the germination and growth of several kinds of orchids.

Effect of peptone and tryptone on germinating the seeds of terrestrials (*Cymbidium*, Oriental *Cymbidium*, *Paphiopedilum*, *Cypripedium* and *Calanthe discolor*) and of epiphytics (*Dendrobium* and *Brassavola*) was examined.

Throughout the experiment, mineral constitution of Knudson's C and Hyponex were used as the base. The design of experiment was the same as the one described in subsection 2 of the previous section, except that three grams of Hyponex was added to a liter of five or ten per cent apple juice solution, i.e. the amount of Hyponex was exactly three grams per liter of culture solution.

Peptone (Difco) or tryptone (Difco) was added at the rate of two grams per liter of culture solution. Sugar concentration in all the media used was adjusted to 3.5 per cent adding sucrose except Burgeff N_3f.

i) On *Cymbidium* seeds.

The media prepared were as follows:

1. Plain Knudson's solution C (sucrose: 35 g./l.)
2. Knudson's solution C plus 10 per cent apple juice
3. Knudson's solution C plus Difco Bacto-peptone 2 g./l.
4. Knudson's solution C plus Difco Bacto-tryptone 2 g./l.
5. Plain Hyponex solution (Hyponex 3 g./l. plus sucrose 35 g./l.)
6. Hyponex solution plus 10 per cent apple juice
7. Hyponex solution plus Difco Bacto-peptone 2 g./l.
8. Hyponex solution plus Difco Bacto-tryptone 2 g./l.

The pH of the media after autoclaving ranged between 4.7 and 5.05. The seeds used seemed to be considerably weak.

The results are shown in fig. 8-1 and Plate II. fig. 7 (A-B).

The enhanced growth of the seedlings was observed on the media of either bases containing peptone and tryptone. On these media good germination was also recorded, especially on the medium of Knudson's plus tryptone. Apple juice suppressed the germination and growth of the seedlings on both bases, especially on the medium of Hyponex base. Growth of the seedlings on plain Knudson's C exceeded that on plain Hyponex solution with this genus.

The effect of tryptone shown in fig. 8-1 and Plate II. fig. 7-B is incompatible with the observation record. Namely, the observation record shows that in an early period of the culture, the best germination and the enhanced developments of protocorms and seedlings were seen on the medium of Knudson's with tryptone. Therefore, competition of developing seedlings may be involved in this discrepancy. If an equal number of seeds per flask were germinated on these bases, the best

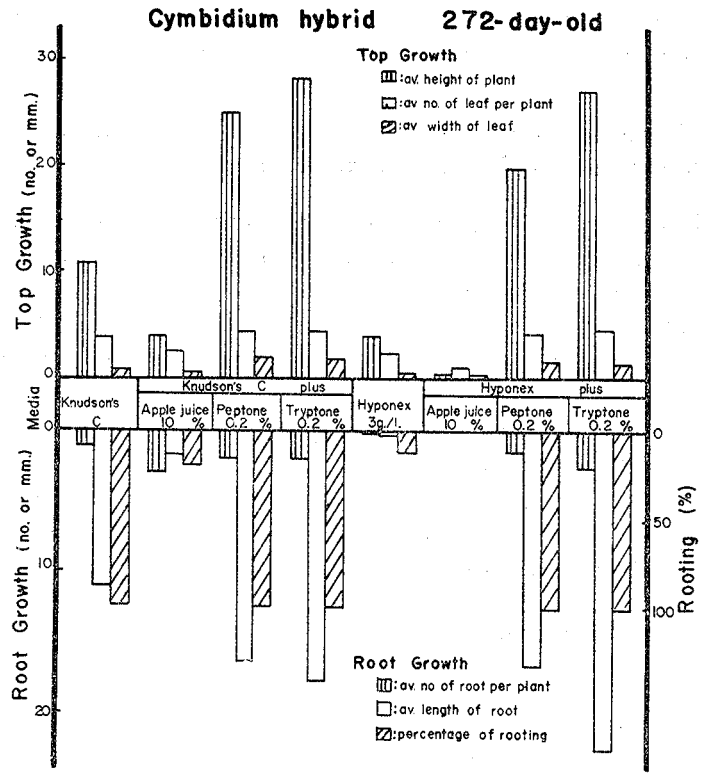

Fig. 8-I. Comparative growth of *Cymbidium* hybrid seeds on different basal media and additions.

growth of the seedlings would be expected on Knudson's base. Later in another experiment, however, superiority of the medium containing Hyponex with tryptone was confirmed. In every case the effect of tryptone was superior to that of peptone.

ii) On Oriental *Cymbidium* seeds.

Cymbidium virescence was used as the seed material.

The same constitutions of media as in the former experiment were also used in this experiment. In addition to these series of media, Burgeff N_3f solution (Burgeff, 1936) was also prepared, because seed germination of this orchid has been considered to be quite difficult. The pH of these media after autoclaving ranged between 4.7 and 5.1. Some of the vessels in each plot were placed in the cool glass-house in which the temperature often dropped below 0°C.

The results are shown in table 7 and Plate II. fig. 8, which proved the difficulty of germinating the seeds of this species. The effect of additions could not be clarified, but apple juice enhanced the development of the embryo to a certain degree. No difference in germination was found between the cultures placed in the heated greenhouse and in the cool glass-house. The cultures are now being kept for further observation.

Table 7. Degree of seed germination in *Cym. virescence* on various kinds of media. 704 days after seed sowing.

	Kind of Media	Degree of germination
Knudson	Plain	+
	+ 10% apple juice	±±
	+ peptone	++
	+ tryptone	±
Hyponex	Plain	++
	+ 10% apple juice	±±
	+ peptone	++
	+ tryptone	++
Burgeff N_3f		±

iii) On *Paphiopedilum* seeds.

Paphiopedilum callosum was used as the seed material. Exactly the same media as in the former experiment were employed.

The results are shown in table 8 and Plate II fig. 9 (A–B).

As a whole, in Hyponex series, the good germination and growth of the seedlings were obtained on the media containing peptone or tryptone, while apple juice inhibited the germination as in the case of *Cymbidium* seeds.

Table 8. Growth record of *Paphiopedilum callosum*. 267 days after seed inoculation.

	Kind of media	Av. height of plant	Av. no. of leaf per plant	Av. width of leaf	Av. no. of root per plant	Av. length of root	Rooting	No. of plant per flask
		mm.		mm.		mm.		
Knudson	Plain	2.5	2.0	0.5	2.0	3.0	2/2	2
	+ 10% apple juice	1.8	1.0	0.2	0	0	0/2	2
	+ peptone	5.3	2.7	1.4	1.6	9.4	10/10	47
	+ tryptone	2.0	1.4	0.3	0.5	2.4	5/10	10
Hyponex	Plain	2.2	2.7	1.2	1.5	6.1	10/10	13
	+ 10% apple juice	---	---	---	---	---	---	0
	+ peptone	5.6	3.1	1.8	2.1	15.4	10/10	46
	+ tryptone	6.5	3.2	2.2	1.7	15.1	10/10	34
Burgeff N_3f		2.3	2.5	0.7	0.8	2.4	5/6	6

In Knudson's C series the best germination and seedling growth were recorded on the media with peptone. Tryptone was less effective than peptone in this base. Apple juice inhibited the germination of the seeds also in this base.

The better germination and growth of the seedlings were observed on plain Hyponex medium than on Knudson's. The result on Burgeff N_3f was disappointing.

In every case, germination and growth of the seedlings were enhanced on the Hyponex media with peptone or tryptone.

Plate III. fig. 1 shows that germination was better in the flask plugged with an ordinary rubber stopper with glass tubing than in that stoppered with cotton plug.

iv) On *Cypripedium reginae* seeds.

In this experiment, three grams of Hyponex were added to a liter of five per cent apple juice, and in Knudson's base, apple juice was also added at the rate of five per cent.

The media employed were as follows:

1. Knudson's C minerals plus 5 per cent apple juice
2. Knudson's C plus peptone 2 g./l.
3. Knudson's C plus tryptone 2 g./l.
4. Hyponex 3 g. per one liter of 5 per cent apple juice solution
5. Hyponex (3 g./l.) plus peptone 2 g./l.
6. Hyponex (3 g./l.) plus tryptone 2 g./l.

pH of these media after autoclaving was between 5.0 and 5.3. In this experiment too, some of the cultures in each plot were placed in the glass-house.

The results are shown in table 9 and Plate III. fig. 2.

Table 9. Degree of embryo swelling in *Cyp. reginae* in various media. 501 days after seed inoculation.

	Media	Degree of embryo swelling
Knudson	+ apple juice	±
	+ peptone	+
	+ tryptone	+
Hyponex	+ apple juice	±
	+ peptone	+
	+ tryptone	+

As shown in Plate III. fig. 2, the embryos swelled to a certain degree, however, they became brown in color and stopped swelling about a year after seed sowing. The record presented in table 9 shows the total degree of swelling and the number of swollen embryos per flask.

No well developed protocorms or seedlings were obtained in this species as well as the experiment of subsection 2 in section 7. In this genus too, no difference in germination was found between the cultures placed in the greenhouse and in the cool glass-house. The cultures are now being kept for further observation.

v) On *Calanthe discolor* seeds.

The media used were the same as used in subsection 2 in this section. In this experiment some of the cultures in each plot were also placed in the cool glass-house.

The result are shown in table 10 and Plate III. fig. 3.

Table 10. Degree of embryo swelling in *Calanthe discolor* on various kinds of media. 704 days after seed inoculation.

	Media	Degree of embryo swelling
Knudson	Plain	−
	+ apple juice	−
	+ peptone	−
	+ tryptone	−
Hyponex	Plain	±
	+ apple juice	−
	+ peptone	±
	+ tryptone	−
Burgeff N_3f		±

With this species, germination seemed to be quite difficult on any of the media examined. However, it seems worth-while to mention that the seeds used in this experiment were stored in dry condition at the room temperature for about three months after harvesting. Some of the seeds sown on plain Hyponex solution (Hyponex 3 g. and sucrose 35 g. per liter of water) on the harvested day were germinated as shown in Plate III. fig. 4. In this genus too, no difference in germination was found between the two groups of cultures placed in the green-house and in the cool glass-house. The cultures are now being kept for continued observation.

vi) On *Dendrobium* seeds.

The culture solutions employed in this experiment are as follows:

1. Plain Knudson's solution C
2. Knudson's minerals plus 5 per cent apple juice
3. Knudson's C plus peptone 2 g./l.
4. Knudson's C plus tryptone 2 g./l.
5. Plain Hyponex solution (Hyponex 3 g./l.)
6. Hyponex solution plus 5 per cent apple juice
7. Hyponex solution plus peptone 2 g./l.
8. Hyponex solution plus tryptone 2 g./l.
9. Hyponex solution plus L-arginine, 20 mg./l., and L-lysine, 40 mg./l.
10. Hyponex solution plus L-aspartic acid 200 mg./l.

Sugar concentration in all the solutions was adjusted to 3.5 per cent adding sucrose.

pH of these media after autoclaving was around 5.1 except the solution 10, whose pH was 4.6.

The seeds used were about six months old and were weaker than those used in the other experiments.

The result is shown photographically in Plate III fig. 5.

As shown in the figure, enhanced germination and growth of the seedlings

were observed only on the media containing apple juice, but the other additions in general were disappointing.

vii) On *Brassavola nodosa* seeds.

The same series of media as used in the former experiment was employed. Unfortunately, however, the data in both plots of Hyponex plus peptone and Hyponex plus tryptone could not be obtained, because the seeds had already been contaminated before inoculation.

The results obtained are shown in fig. 8-2 and Plate III fig. 6 (A-B).

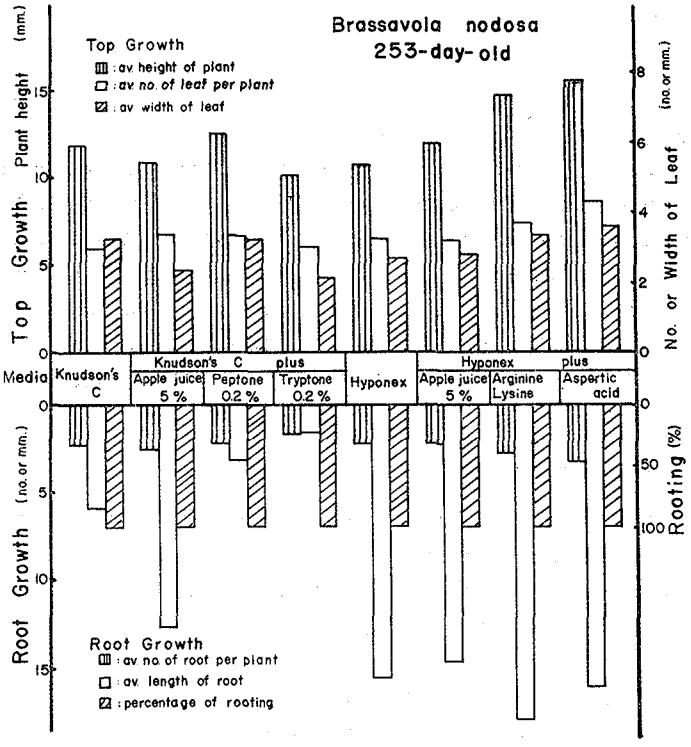

Fig. 8-2. Comparative growth of *Brassavola nodosa* seeds on different basal media and additions.

An enhanced growth of the seedlings was recorded on the Hyponex solution supplemented with arginine and lysine or aspertic acid. Peptone and tryptone in Knudson's base apparently inhibited the root growth, and apple juice increased the growth in root length on Knudson's base. Comparing plain Knudson's C medium with plain Hyponex medium, the growth in root length was enhanced on the latter medium. Chlorophyll did not develop well in the seedlings on the media with apple juice. No difference in germination capacity was observed among all the media.

viii) Discussion.

As a whole, peptone and tryptone added to both bases enhanced the germina-

tion of seeds and the growth of seedlings in both *Cymbidium* and *Paphiopedilum*, while they inhibited the root growth of *Brassavola nodosa* seedlings in Knudson's base. Apple juice, which seemed to be favored by *Dendrobium*, as repeatedly shown in the present work, inhibited the germination of seeds of both *Cymbidium* and *Paphiopedilum*. Aspertic acid or arginine and lysine in combination enhanced the germination of *Brassavola nodosa* seeds, while they exerted no favorable effect on *Dendrobium* seeds. Plain Hyponex medium was effective for the germination of *Paphiopedilum callosum* seeds, but not effective for *Cymbidium* seeds.

As already mentioned repeatedly, the present results also prove that the favorable recipe for germinating seeds differs depending upon the kinds of orchids.

A marked germination was not observed in the present work with the seeds considered to be "hard to germinate", except those of *Paphiopedilum callosum*.

It may be assumed that some of the terrestrials could not germinate well on artificial media asymbiotically, not only because their nutritional needs were not satisfied but because some other factors were involved.

Using techniques such as application of favorable nutrition, pretreatment of seeds before inoculation or solid culture, method of culture technique, various temperatures during incubation, pH of culture solution, kind of sugars, culture of immature seeds, and so on, much efforts were made by many workers to germinate the seeds of so-called "hard to germinate" species asymbiotically, mainly using seeds of *Paphiopedilum*, *Cypripedium*, *Vanilla*, etc. (Bouriquet, 1947, 1948a, b; Bouriquet and Boiteau, 1937; Burgeff, 1936, 1954; Curtis, 1936, 1943; Downie, 1940, 1941, 1943; Knudson, 1950; Liddell, 1944, 1953a, b; Lugo, 1955; Northen, 1950; Thomale, 1951, 1954; Tonnier, 1951; Withner, 1943, 1953, 1955, 1959; Yamada, 1952; etc.). Among these workers, Burgeff (1954), Knudson (1950), Withner (1953) and others referred to the mechanism of the difficulty of germination. Summarizing these works, however, it might be said that much problems remain to be solved concerning the asymbiotic germination of these "hard to germinate" seeds.

From the nutritional standpoint, various kinds of complex substance from natural source, organic substances, auxins and others were added to the culture media singly or in combination in order to obtain the well developed seedlings of "hard to germinate" orchids asymbiotically. The additions used with success were as follows: decoction of soil and mature *Vanilla* plants (for *Vanilla*, Bouriquet and Boiteau, 1937), fresh yeast (for *Vanilla*, Bouriquet, 1947), peptone (for *Cypripedium*, Curtis, 1943; Withner, 1953; for *Paphiopedilum*, Curtis, 1947; Hegarty, 1955; Liddell, 1953a, b; Northen, 1950), coconut milk (for *Paphiopedilum*, Yamada, 1952; for *Paphiopedilum* and *Vanilla*, Hegarty, 1955), mashed banana (for *Paphiopedilum* ovule culture, Withner, 1943; for *Vanilla* ovule culture, Withner, 1955), Na-nucleinate (for *Cypripedium*, Curtis, 1943), arginine and lysine (for *Vanilla* ovule culture, Withner, 1955), IBA (for *Paphiopedilum* and *Vanilla*, Hegarty, 1955; for *Vanilla* ovule culture, Withner, 1955) and so on.

In the present work, peptone proved to be effective to germinate *Paphiopedilum* seeds. In addition to this, the usefulness of tryptone for germinating the seeds of

this species was also proved. However, they did not exert apparent promotive effect for germinating the other kinds of "hard to germinate" orchid seeds examined in the present work. Questions may, therefore, arise whether these additions are specifically effective to germinate the seeds of that species and whether there is any other factor besides the nutritional problem on the other kinds of "hard to germinate" orchid which responded very little to these additions. As to these questions, a brief discussion will be given in Chapter IV.

In the present work, the active substance or substances which are contained in these two additions (peptone and tryptone) and have a stimulative effect in germinating the seeds of *Paphiopedilnm callosum* have not been clarified. However, so far as this species is concerned, the favorable effect of peptone or tryptone can be accepted and the recipe of Hyponex solution plus peptone or tryptone may be recommended for practice.

Kinds of sugars added to the culture media, which bring about favorable results in germinating the seeds of "hard to germinate" orchids, differ among the workers, i.e. glucose plus fractose (Burgeff, 1936; Liddell, 1953a, b; Thomal, 1954) and sucrose (Hegarty, 1955; Yamada, 1952) for *Paphiopedilum*, glucose plus fractose (Withner, 1955), sucrose (Hegarty, 1955; Lugo, 1955; Knudson, 1950) and glucose (Bouriquet and Boiteau, 1937; Knudson, 1950) for *Vanilla*, glucose plus fractose (Withner, 1953) and glucose (Curtis, 1936, 1943) for *Cypripedium*, and glucose plus fructose (Downie, 1940) for *Goodyera*. In the present work, sucrose was used in all the media examined except in Burgeff N_3f. Comparing the results obtained on plain Hyponex solution with those on Burgeff N_3f on *Paphiopedilum callosum*, better germination and growth of the seedlings were obtained on the former medium. Considering the results of the present work and of the other workers described above, and various other investigations (La Garde, 1929; Quednow, 1930; Smith, 1932; Wynd, 1933c), it may be said that kind of sugar is of little importance for germinating the seeds of "hard to germinate" orchids.

It is interesting to note here that in the present work, seeds of *Paphiopedilum callosum* were germinated easily on proper recipes, though the swelling of the protocorm was not so rapid compared with other orchids that germinate more easily.

Different kinds of culture technique or pretreatment of the seeds are recommended by several workers in order to improve the germination of "hard to germinate" orchids. Burgeff (1936, 1954) soaked the seeds in sterile water from two weeks to two months prior to planting on agar. Liddell (1944, 1953a, b) and Hegarty (1955) soaked the seeds in a liquid nutrient medium prior to planting on a solid medium. Lugo (1955) and Tonnier (1951) treated the seeds with chemicals prior to sowing. Curtis (1943) covered the planted seeds with a nutrient agar plate. Withner (1943, 1953, 1955) used the technique of ovule culture.

Curtis (1943) and Withner (1955) challenged the germination of the mature seeds of *Cypripedium reginae* asymbiotically. Although the former succeeded by covering the planted seeds with a nutrient agar plate, he failed to obtain well

developed seedlings, probably because the low temperature requirement of the plantlets was not filled. In the present work, a single seedling of this orchid has not been obtained. It is assumed that this result was partly derived from the age of the seeds after harvesting which was quite uncertain (Cf. Hegarty, 1955; Liddell, 1953a, b).

With *Cymbidium* the germination and growth of the seedlings on plain Knudson's solution C exceeded those on plain Hyponex solution. This result is quite different from the results obtained with *Dendrobium*, *Cattleya* group and *Paphiopedilum* in the present work. As to this phenomenon the type of nitrogen added to the media may be of significance. Curtis and Spoerl (1948) reported that the ammoniacal nitrogen is superior for *Cymbidium* to nitrate nitrogen. Knudson's solution C contains more ammoniacal nitrogen than plain Hyponex solution does, which seems to be one of the reasons for the superiority of knudson's solution. In the other experiment of the author, however, better germination and growth of the germinated seedlings was recorded on plain Hyponex medium than on plain Knudson's solution C, and addition of ammonium sulphate to Hyponex was inhibited the germination and growth of the germinated seedlings. With *Paphiopedilum callosum*, the present result does not accord with Burgeff's result (1936) in which he states that *Paphiopedilum* specifically requires ammonium. In the present work better germination and growth of seedlings were obtained on the plain Hyponex medium than on the plain Knudson's C medium.

It is also interesting to note that apple juice which was effective for epiphytic orchids (*Dendrobium* and *Cattleya* group) inhibited the germination and growth of the seedlings of terrestrial orchids (*Cymbidium* and *Paphiopedilum*), and that peptone and tryptone were especially favored by these terrestrials. Vacin and Went (1949) reported that *Cymbidium* seeds grew rapidly when Knudson's solution C was enriched with protein hydrolysate (Prominogen). Their results show the degree of growth on this medium was superior to that on plain Knudson's solution C or tomato juice media.

These results suggest the importance of specific substance or substances which are contained in these complex substances for germinating the seeds of terrestrial orchids.

Discussions about the effect of amino acid and the method of improving the germination of "hard to germinate" orchids will be given in the later paragraph.

In concluding this section, the following recipes may be recommended from the practical point of view.

For *Paphiopedilum*		For *Cymbidium*	
Hyponex	3.0 g.	Hyponex	3.0 g.
Peptone or tryptone	2.0 g.	Tryptone	2.0 g.
Sucrose	35.0 g.	Sucrose	35.0 g.
Agar	15.0 g.	Agar	15.0 g.
Water	1000 ml.	Water	1000 ml.
pH about	5.0	pH about	5.0

9. Effects of various amino acids and organic substances from natural sources on the germination and growth of *Dendrobium* and *Brassolaeliocattleya* seeds.

Effects of various amino acids and organic substances from natural sources such as peptone, tryptone, yeast extract and casein from milk on the germination of orchid seeds were examined on *Den.*, *Blc.* and *Cymbi.* seeds. However, no datum with *Cymbidium* was obtained, because the seeds used in this experiment were not viable.

And the effects of the various concentrations of tryptophan and glutamic acid on the germination of the seeds of *Den.* and *Blc.* were also examined.

Throughout the experiments, Hyponex (3 g./l.) was employed as the mineral base. Sugar concentration in all the media used was adjusted to 3.5 per cent adding sucrose.

i) Effects of amino acid and organic substances on the germination and growth of *Dendrobium* and *Brassolaeliocattleya* seeds.

Fourteen kinds of media were prepared with Hyponex solution as the base as follows:

Plain Hyponex
Hyponex plus apple juice (15 per cent)
Hyponex plus peptone (2 g./l.)
Hyponex plus tryptone (2 g./l.)
Hyponex plus yeast extract (2 g./l.)
Hyponex plus casein from milk (2 g./l.)
Hyponex plus L-aspartic acid (1×10^{-3} M)
Hyponex plus L-asparagine (1×10^{-3} M)
Hyponex plus L-arginine (1×10^{-3} M)
Hyponex plus Na-adenosin triphosphate (40 mg./l.)
Hyponex plus L-glutamic acid (1×10^{-3} M)
Hyponex plus L-histidine (1×10^{-3} M)
Hyponex plus L-lysine (1×10^{-3} M)
Hyponex plus L-tryptophan (1×10^{-3} M)

On these media seeds were sown on June 3, 1962.

The initial pH in each medium is presented in fig. 9–1.

The results obtained are shown in fig. 9–1 and Plate IV. figs. 1 and 2.

With *Blc.*, in general, no favorable effect of the additions was observed except peptone. Among the other additions, tryptone, yeast extract, glutamic acid and tryptophan inhibited the growth of the seedlings. Yeast extract and tryptophan especially inhibited the germination and growth of the seedlings.

With *Dendrobium*, in general, growth of the seedlings was enhanced by organic substances such as apple juice, peptone, tryptone, yeast extract and casein from milk. Among the amino acids added, only glutamic acid enhanced the growth of

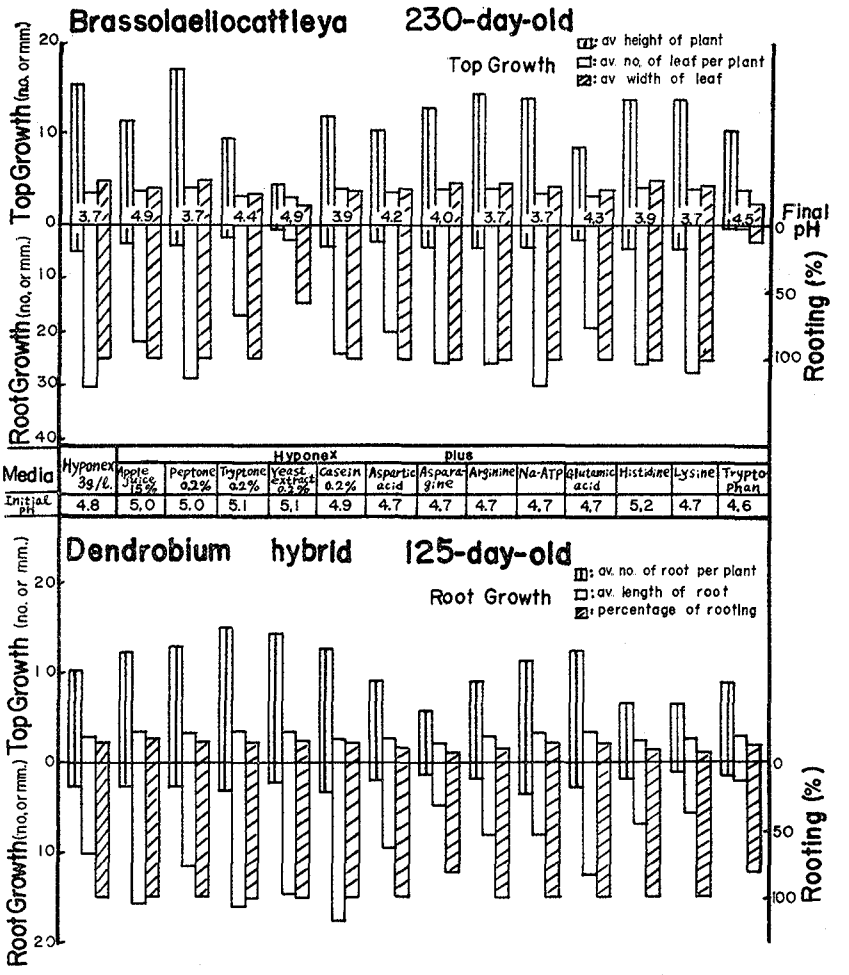

Fig. 9-1. Effect of various kinds of organic compound and amino acid on the germination of *Blc.* and *Den.* hybrid seeds. Sowing date: June 3, 1962.

the seedlings. Asparagine, histidine, lysine and tryptophan inhibited the growth of the seedlings and the former three also inhibited the germination of the seeds seriously. Tryptophan did not inhibit the germination of the seeds. Among the complex substances added, the best germination and the uniform growth of the seedlings were observed on the medium with apple juice (see Plate IV).

The contrasting responses of the two orchids to the additions were observed. Namely, tryptone, yeast extract and glutamic acid promoted the growth of the seedlings of *Dendrobium*, while they inhibited that of *Brassolaeliocattleya*. On the other hand, asparagine, histidine and lysine exerted no appreciable effect on *Brassolaeliocattleya*, while they inhibited the growth of *Dendrobium*.

On the medium containing Na-adenosin triphosphate, many undifferentiated cell masses were observed in both genera of orchids used.

In case of *Brassolaeliocattleya* the final pH value dropped in the media on which

the seedlings grew well.

ii) Effects of concentrations of tryptophan and glutamic acid on the germination and growth of *Dendrobium* and *Brassolaeliocattleya* seeds.

As shown in the results mentioned above, tryptophan inhibited the growth of seedlings in both genera used, while the responses to glutamic acid were quite contrasting between the two genera. Hence, the effects of these two chemicals were repeatedly examined at different concentrations on the same orchids.

The media containing 1×10^{-3}, 1×10^{-4} and 1×10^{-5} M of either tryptophan or glutamic acid were prepared. In addition, the media supplemented with 15 per cent apple juice or with a combination of 1×10^{-4} M of tryptophan and 1×10^{-3} M of glutamic acid were prepared.

The pH value of these media after autoclaving was around 5.0.

The results are shown in fig. 9-2 and Plate IV figs. 3 and 4.

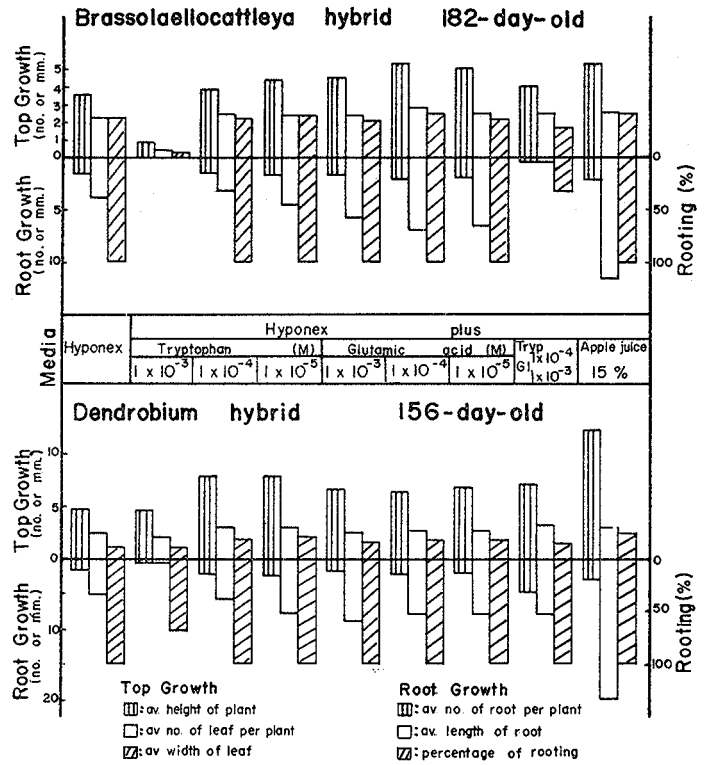

Fig. 9-2. Effects of tryptophan and glutamic acid on the germination of *Blc.* and *Den.* seeds. Sowing date: Aug. 25, 1962.

The results somewhat conflicted with the above experiment. Namely, 1×10^{-3} M of glutamic acid enhanced the growth of the seedlings in both genera, while with *Brassolaeliocattleya* the best growth of the seedlings was observed on the medium

with apple juice.

Except the effect of glutamic acid on *Dendrobium*, each chemical enhanced the growth of the seedlings apparently at the concentration of 1×10^{-4} or 1×10^{-5} molar.

The combination of tryptophan and glutamic acid enhanced the seedling growth of *Dendrobium*, while it inhibited the root growth of *Brassolaeliocattleya*.

With *Dendrobium*, the best germination and growth of the seedlings were recorded on the medium containing apple juice.

iii) Discussion.

Various kinds of complex substances such as peptone, tryptone, yeast extract and casein hydrolyzate, and various kinds of amino acids were used as additions or prime nitrogen sources for germinating the orchid seeds asymbiotically or for shoot meristem culture by many investigators (Burgeff, 1936; Curtis, 1947; Ito, 1961; Knudson, 1932; Lami, 1927; Mariat, 1952; Schaffstein, 1938; Spoerl, 1948; Spoerl and Curtis, 1948; Wimber, 1963; Withner, 1942, 1951, 1955; Yates and Curtis, 1949 and see subsection viii in section 8). Among the complex substances used as additions, the effect of peptone is comparatively consistent, while that of amino acids is not consistent among the works. This inconsistency is probably due to the different nutrient requirements by different orchid species for their germination.

In the present experiment, different responses of the genera used to the additions were observed. Moreover, the same orchid species, such as *Brassolaeliocattleya* responded differently to the same additions depending upon the sowing date i.e., upon the age of seeds after harvesting. Spoerl (1948) and Spoerl and Curtis (1948) reported that quite opposite responses to the same amino acid were observed depending upon the maturation stage of the embryo, i.e. aspartic acid allowed good growth of the mature seeds but was not good for immature ones. Besides, Schaffstein (1938) showed that the older the mature seeds become, with progressive loss of vitality, the more help they need from natural extracts. These facts mean that the nutrient requirement at the germination changes successively according to the age of embryos or mature seeds even in the same species of orchids.

Considering these facts, the conflictions among the results described in this section and the other sections of the present work may be partly understood.

Sisa and Sawa (1963) obtained the positive effect of arginine and aspartic acid in germination of *Cymbidium* seeds as supplemented to Knudson's solution C. Withner (1955) showed positive effect of arginine and lysine when he used them as additions in *Vanilla* ovule culture. The present author also obtained similar results with these chemicals and in addition to this, aspartic acid proved to be effective for germinating *Brassavola nodosa* seeds. However, they exerted no appreciable promoting effect on germinating *Dendrobium* seeds (see subsections vi and vii in section 8). In the present experiment, no definite promotive effect was observed with these acids though they were used singly and the doses employed were not the same as in the previous experiments.

The results thus again conflict. As for the effect of tryptophan, further investigation will be necessary especially on the favorable effect of peptone and tryptone for germinating terrestrial orchid, for these substances contain some amount of tryptophan.

In any case, so far as freshly harvested seeds are used, the following recipes may be recommended for *Dendrobium* and *Cattleya* group in practice:

For *Dendrobium*		For *Cattleya* group	
Fresh strained apple juice diluted to 10–20%	1000 ml.	Hyponex	3 g.
Hyponex	3 g.	Sucrose	35 g.
Sucrose to adjust the total sugar content to approx.	3.5 %	Agar	15 g.
Agar	15 g.	Water	1000 ml.
pH adjusted to approx.	5.0	pH adjusted to approx.	5.0

When characteristics of the seeds or age of the seed are uncertain, addition of peptone to the Hyponex solution is recommended.

10. Effects of sugar concentrations in transplanting bed on the growth of *Dendrobium*, *Brassocattleya*, *Brassavola*, *Paphiopedilum* and *Cymbidium* seedlings.

The effects of sugar concentrations in sowing bed were already shown in section 4.

In this experiment effects of sugar concentrations in transplanting bed on the growth of seedlings were examined for the above listed genera of orchids.

i) On *Dendrobium* seedlings.

Knudson's C mineral solution plus two per cent apple juice was used as the basic solution. To this sucrose was added at the rate of 0.5, 1.0, 2.0, 4.0 or 8.0 per cent.

The pH value of these media after autoclaving was around 5.2.

Approximately sixty-day-old seedlings were transplanted onto the agar media prepared.

The growth data recorded 175 days after transplanting are shown in fig. 10–1 graphically and the photogaph taken 235 days after transplanting is presented in Plate IV. fig. 5.

On the 175th day from transplanting, the best top growth was recorded on the medium with two per cent of sugar. The growth in root length increased according as the concentration of sugar increased.

The development of new shoot was observed in each seedling on the 235th day from transplanting, as shown in the plate, and the best growth was recorded on the medium with four per cent of sugar. Eight per cent of sucrose suppressed the top growth seriously.

ii) On *Brassocattleya* seedlings.

Hyponex (3 g./l.) was used in the basic solution. Sucrose was added to this at the rate of 0.5, 1.0, 2.0, 4.0 or 8.0 per cent.

The pH value of these media after autoclaving was around 5.2.

The seedlings of 146 days old were transplanted onto the agar media prepared.

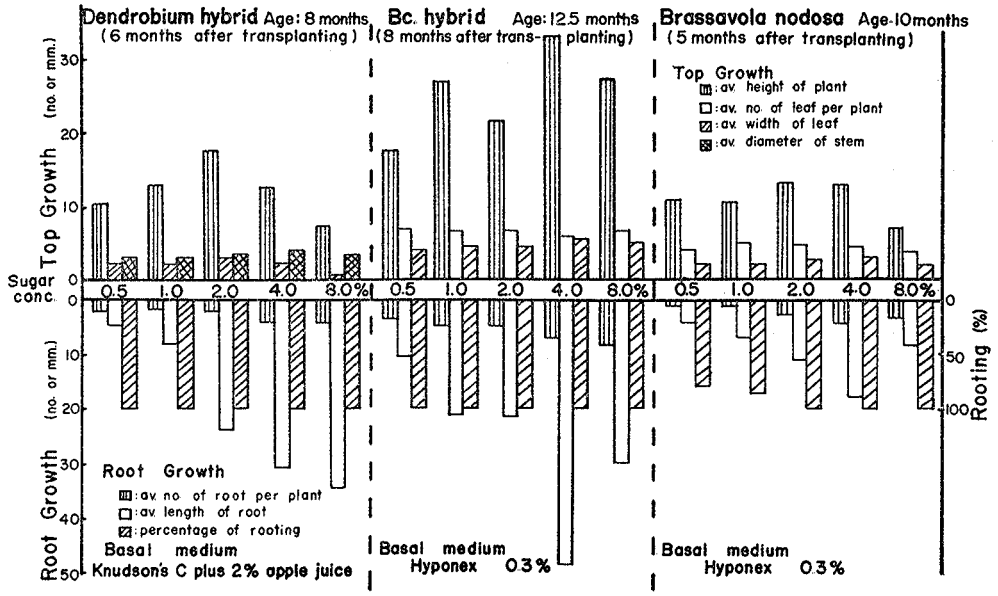

Fig. 10-1. Effect of sugar concentrations in the transplanting bed on the growth of *Dendrobium*, *Brassocattleya* and *Brassavola* seedlings.

The stages of growth 235 days after transplanting are shown in fig. 10–1 and Plate IV. fig. 6.

As clearly shown in the figure and plate, the best growth of seedlings was recorded on the medium containing four per cent of sucrose.

The number of roots per plant increased according as the concentration of sugar increased.

iii) On *Brassavola nodosa* seedlings.

The design of the experiment and the basic solution used were the same as in the former experiment.

The pH value of the media used was around 4.5.

The results are shown in fig. 10–1 and Plate V. fig. 1.

As was seen in the former experiment, good growth of seedlings was recorded on the medium with four per cent of sucrose with this orchid.

On the medium with eight per cent sucrose, seedlings became purplish red in color. This is possibly due to the development of anthocyanin pigment.

iv) On *Paphiopedilum callosum* seedlings.

Hyponex (3 g./l.) plus tryptone (2 g./l.) was employed as the basic solution.

The media containing 1, 2, 4 or 8 per cent of sucrose were prepared.

The pH values of these media after autoclaving were between 5.0 and 5.1.

Hundred and seventy-nine-day-old seedlings which germinate on Hyponex medium with 0.2 per cent peptone were transplanted onto the agar media prepared.

The growth record was made on the 151st day from transplanting.

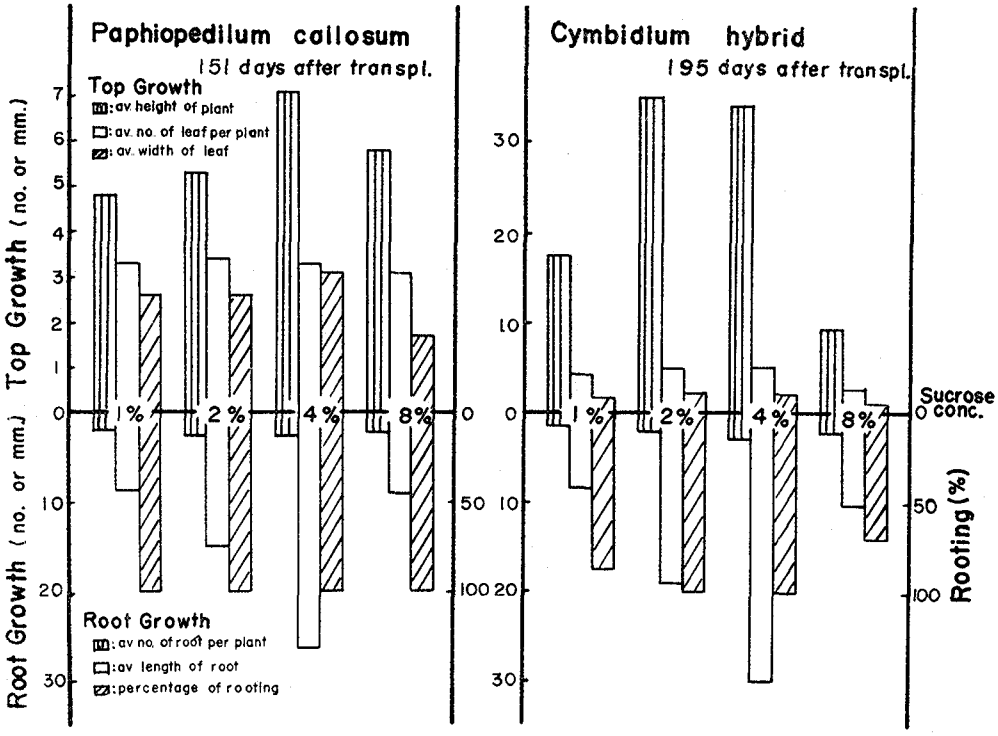

Fig. 10-2. Effects of sucrose concentrations in the transplanting bed on the growth of *Paphiopedilum* and *Cymbidium* seedlings. Basal medium: Hyponex 3g./l., tryptone 2g./l.

The results are shown in fig. 10-2 and Plate V. fig. 2.

The best growth of seedlings was recorded on the medium with four per cent of sucrose.

The plant height and the root length of the seedling increased according as the sucrose concentration increased up to four per cent. The root of the seedlings grown on the medium containing eight per cent of sucrose developed purplish-red color, and many of them died at the sampling time.

v) On *Cymbidium* seedlings.

In this experiment, the same media as in the former experiment were used.

Hundred and fifty-nine-day-old seedlings which germinated on the medium of Knudson's C plus 0.2 per cent tryptone were transplanted onto each agar medium.

The results are shown in fig. 10-2 and Plate V. fig. 3. With this genus too, good growth in both top and root was observed on the medium with four per cent

of sucrose. Growth in root length increased according as the concentration of sucrose increased up to four per cent. Eight per cent of sucrose suppressed the top growth seriously.

vi) Discussion.

The optimum concentration of sugar in transplanting bed proved to be four per cent for all the genera examined. As already shown, this concentration of sugar is also favorable in the sowing bed.

The present results generally accord with the results of Yates and Curtis (1949) except in some details.

Sucrose concentration of about four per cent may be recommended for practice for the transplanting bed as well as the sowing bed.

11. Effects of the concentration of tryptone added to the transplanting bed on the growth of *Paphiopedilum callosum* and *Cymbidium* seedlings.

As shown in section 8, tryptone proved to be effective for germinating the seeds of *Paphiopedilum* and *Cymbidium* on Hyponex solution.

To determine the optimum concentration of tryptone in transplanting bed, media were prepared to contain 0, 0.1, 0.2, 0.4 or 0.8 per cent of tryptone. Hyponex solution (Hyponex 3 g./l., sucrose 35 g./l.) was used as the basal medium.

The pH values of the media after autoclaving ranged between 4.9 and 5.1.

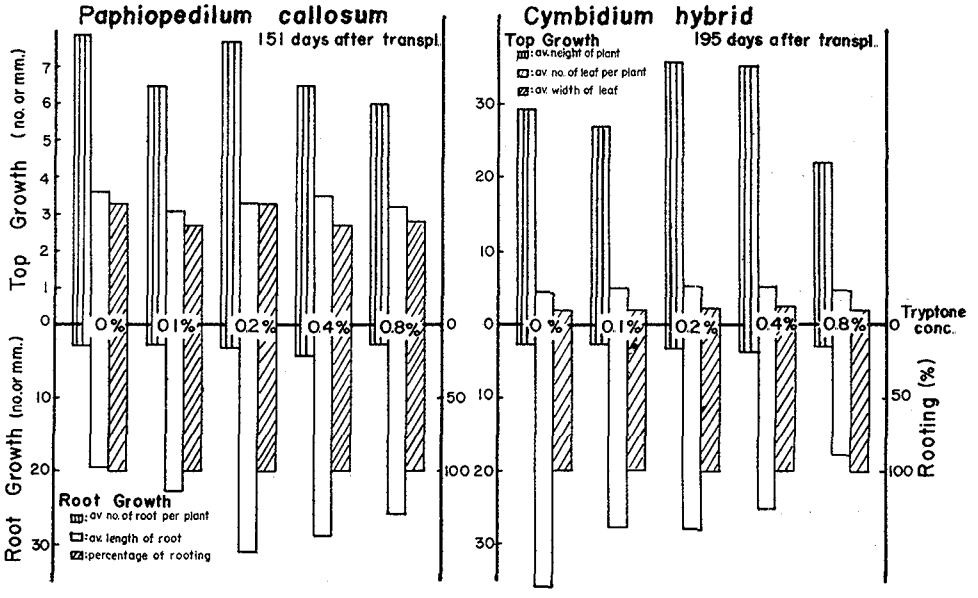

Fig. 11. Effects of tryptone concentrations in the transplanting bed on the growth of *Paphiopedilum* and *Cymbidium* seedlings. Basal medium: Hyponex 3 g/l., sucrose 35 g./l.

i) On *Paphiopedilum callosum* seedlings.

Seedlings used as the materials were the same as for the former section's experiment.

The results are shown in fig. 11 and Plate V. fig. 4.

The figures show that the optimum concentration is about 0.2 per cent.

ii) On *Cymbidium* seedlings.

Seedlings used as the materials were also the same as for the former section's experiment.

The results are also shown in fig. 11 and Plate V. fig. 5.

In this genus, too, 0.2 per cent of tryptone may be accepted as the optimum concentration.

Although the growth in root length decreased according as the concentration of tryptone increased, a good top growth was observed on the media with 0.2 or 0.4 per cent tryptone. Eight-tenths of a per cent suppressed the growth in both top and root.

iii) Discussion.

In both genera, plain Hyponex solution supported growth of the transplanted seedlings in an appreciable amount. From this fact, it may be considered that germinated plantlets do not require tryptone particularly (see fig. 8-1 and table 8). However, a good growth of the seedlings was obtained on the medium with 0.2 per cent of tryptone.

In practice, tryptone concentration of 0.2 per cent is recommended for transplanting bed.

12. Germination of the so-called "hard to germinate" species.

In order to germinate the so-called "hard to germinate" orchid seeds, some trials were carried out, using the seeds of *Cymbidium virescence* and *Paphiopedilum insigne*.

i) Ovule culture of Oriental *Cymbidium* (*Cymbidium virescence*) and *Paphiopedilum insigne*.

With *Cymbidium virescence*, cultures of ovules taken from immature green pods were tested every month after pollination. And, the cultures were terminated seven month after pollination when the seeds matured.

Test tubes used as the containers were completely sealed with rubber stoppers except in two experiments. As the culture medium, Hyponex (3 g./l.) with tryptone (2 g./l.) was employed unless otherwise noted. Thirty-five grams of sucrose was added to a liter of this solution as the carbohydrate source. In some experiments, effects of peptone and tryptone added to two different basal solutions on the germination of the ovules of this orchid species were examined.

With *Paphiopedilum insigne*, ovules from a pod of six months old were inoculated to the media of different constitutions in order to obtain a favorable recipe for the

germination of this orchid species.

The planting techniques used are as follows: after the pods were surface-sterilized with Wilson's calcium hypochlorite solution for about ten minutes, they were transferred to sterile Petri dishes each with one disc of filter paper to absorb the excess of disinfectant. The pods were opened with sterile instruments to scrape out the ovules or seeds, limiting the placental or pod tissues attached to them as little as possible. The ovules were introduced into the tubes and spread about. With this method it was difficult to avoid a certain amount of clumping when planting the immature seeds.

The cultures were generally placed in the laboratory where they were exposed to the dim room light. No control of temperature was attempted. The temperature flactuated between 0°C and 30°C depending upon the temperature flactuation of the season.

a) Determination of the germinative age in *Cymbidium virescence*.

The records obtained are shown in table 11.

Table 11. Germination record in *Cymbidium virescence*.

Days after pollination	Degree of ovule growth	Date of recording (days after inoculation)
32	−	637
61	−	607
96	±	573
123	++	546
156	###	513
184	###	485
218	###	451

As shown in table 11, germination occurred on the ovules from about four-month-old pods. After the long period of the culture, there was no difference in the degree of germination among the cultures from five-month-old to mature ones. However, the degree of the development of the protocorms was not so good as compared with the ones obtaind by water soaking after sterilization in the later experiment.

b) Effect of peptone and tryptone on the germination of the immature seeds of *Cymbidium virescence*.

Peptone and tryptone were added to the two basal media, Hyponex and Burgeff N_3f, and their effects on the germination of the ovules of *Cymbidium virescence* were examined. The media prepared were as follows:

1. Plain Hyponex solution (Hyponex 3 g./l.)
2. Hyponex solution plus peptone 2 g./l.
3. Hyponex solution plus tryptone 2 g./l.
4. Plain Burgeff N_3f.
5. Burgeff N_3f plus peptone 2 g./l.
6. Burgeff N_3f plus tryptone 2 g./l.

In the Hyponex series, 35 grams of sucrose were added to a liter of the solution.

Hundred and twenty-three-day-old and 168-day-old ovules were planted respectively onto the media listed above. In the former case, the culture tubes were sealed completely with rubber stoppers after planting the ovules, while in the latter case, the culture tubes were sealed with single hole rubber stoppers provided with a glass tube plugged with cotton.

The results are summarized in table 12.

Table 12. Germination record of *Cymbidium virescence* on different basal media and additions.

	Media	Degree of ovule growth	
		123-day-old*	168-day-old**
Hyponex	Plain	+	±
	+ peptone	±	±
	+ tryptone	+	±
Burgeff	Plain	++	±
	+ peptone	+++	±
	+ tryptone	++	±

* Recording was made 546 days after inoculation.
** Recording was made 500 days after inoculation.

With 123-day-old ovules the best germination was recorded on the medium of N_3f plus peptone, while with 168-day-old ovules there was no difference in the degree of germination among the media used. Desiccation of the agar media may be one of the reasons for the poor germination in the latter experiment.

c) Ovule culture of *Paphiopedilum insigne*.

The media prepared were the same as in the above experiment, except that sucrose was used as the carbohydrate source in Burgeff N_3f series instead of glucose and fluctose. The sucrose concentration in all the media prepared was adjusted to 3.5 per cent. To these media ovules from a 182-day-old pod of *Paphiopedilum insigne* were inoculated.

The pH values of the media after autoclaving were between 4.8 and 5.1.

The results obtained are shown in table 13.

Development of the embryo was irregular, and germination in the tubes of various media was likewise not consistent. Many of the embryos swelled somewhat, turning brown, and poorly developed seedlings were observed at the time of recording. As shown in the table, well developed protocorms were observed on the medium with Hyponex and peptone.

ii) Acceleration of germination in ripe Oriental *Cymbidium* seeds (*Cymbidium virescence*).

Attempts to improve the germination of ripe Oriental *Cymbidium* seeds were

Table 13. Comparative germination of the ovules of *Paphiopedilum insigne* on different media.*

	Media	Degree of ovule growth
Hyponex	Plain	±
	+ peptone	++
	+ tryptone	+
Burgeff	Plain	+
	+ peptone	±
	+ tryptone	+

* Recording was made 317 days after inoculation.

made using *Cymbidium virescence*. Some of the positive results obtained in these trials will be described.

Throughout the experiment, solution of Hyponex plus peptone (Hyponex 3 g./l., peptone 2 g./l., and sucrose 35 g./l.) was used as the nutrient medium. The pH value of the media after autoclaving was around 4.9.

Test tubes were used as culture vessels. They were sealed completely with rubber stoppers after seed inoculation. The cultures were placed in the laboratory as were in the former experiments.

a) Effect of duration of sterilization on accelerating the germination of ripe *Cymbidium virescence* seeds.

The seeds were soaked in Wilson's calcium hypochlorite solution for 10 min., 1.5 hrs. and 3.0 hrs. each. During the soaking treatment, the container was shaken frequently. After the soaking treatment, the seeds were planted onto the slanted agar medium.

The results are shown in table 14 and Plate V1. figs. 1 and 3 E (a–c).

Table 14. Effect of duration of sterilization on germination.*

Seeds soaked for	Degree of germination
10 min.	+
1.5 hrs.	+++
3.0 hrs.	++

* Recording was made 265 days after inoculation.
Seed age: 4-month-old after harvesting

As shown in the data, germination was accelerated by the longer durations of sterilization with calcium hypochlorite solution.

b) Effects of soaking in KCl solution prior to sterilization.

The seeds were soaked for five days in KCl solution of 0, 0.1, 1.0 or 10 per cent. Then the seeds were sterilized with Wilson's calcium hypochlorite solution for 10 min. and inoculated onto the slanted agar medium in a test tube.

The results are shown in table 15 and Plate VI. fig. 2.

Table 15. Effect of KCl soaking prior to sterilization on the germination of *Cymbidium virescence* seeds.

KCl conc. %	Degree of germination
0	++
0.1	++±
1.0	++±
10	±

* Recording was made 251 days after the beginning of the experiment. Seed age: 5-month-old after harvesting.

The results show that, except 10 per cent treatment, all the treatments accelerated the germination to a certain degree. However, the effect was not so pronounced as compared with the results of the following experiment.

c) Effects of soaking in KCl solution after sterilization.

Sterilized seeds (10 min. in Wilson's calcium hypochlorite solution) were soaked in sterile KCl solution of 0, 0.01, 0.1, 1.0 or 10 per cent for 5 hrs., 25 hrs., 5 days or 15 days.

The following method was used in the soaking treatment. An appreciable amount of seeds was transferred with sterile loops directly from the disinfectant solution, into the test tube which contained two ml. of sterilized KCl solution.

After this soaking treartment, the seeds were planted onto the slanted agar medium in the usual manner.

The result is shown in table 16 and Plate VI. fig. 3.

Table 16. Effect of KCl-soaking after sterilization on accelerating germination of *Cymbidium virescence* seeds.*

Sterilized seeds soaked for	Degree of germination				
	KCl concentration (%)				
	0	0.01	0.1	1.0	10
10 min. (control)	+				
5 hrs.	+++ +++	+++ +++	+++ ++	+++ +	+
25 hrs.	++	+±	±	−	−
5 days	−	−	−	−	−
15 days	−	−	−	−	−

* Recording was made 265 days after the start of the experiment. Seed age: 4-month-old after harvesting.

This result shows that soaking the seeds in sterile water for five hrs. is a usefull method for accelerating germination of the seeds of *Cymbidium virescence*. No positive effect was observed with KCl treatment, except that high concentrations inhibited the germination.

iii) Effects of gibberellin on the germination of ripe Oriental *Cymbidium* seeds.

Twenty-five mg. of gibberellin was added to a liter of the culture solution

which contained three grams of Hyponex and two grams of tryptone per liter of water. Gibberellin-free medium was used as the control. The pH value of the former medium after autoclaving was 4.95 and of the latter 4.90.

The seeds were inoculated onto these media after the following treatments.

1. Sterilized with Wilson's calcium hypochlorite solution for 10 min.
2. Soaked in sterile 0.5 per cent solution of KCl for five hrs. after sterilization.
3. Soaked in sterile water for five hrs. after sterilization.
4. Repeatedly sterilized after the treatment 2.
5. Repeatedly sterilized after the treatment 3.

The result obtained is presented in table 17.

Table 17. Effect of gibberellin on the germination of the seeds of *Cymbidium virescence*.*

Media	Degree of germination				
	Treatments				
	1	2	3	4	5
Plain	±	+++++	+++++	++	++
+ GA	−	−	−	−	−

* Recording was made 201 days after inoculation. Seed age: 5.5-month-old after harvesting.

As shown in the table, the best germination was recorded on the plain media of treatments 2 and 3, while GA apparently inhibited the germination of the seeds. It was also inhibited by the double sterilization. There was no difference as to the degree of germination between the soaking treatments of KCl and water.

iv) Effect of light during germination.

Seeds of *Paphiopedilum callosum* were inoculated onto the medium containing Hyponex and peptone (Hyponex 3 g./l., peptone 2 g./l. and sucrose 35 g./l.). Immediately after inoculation some of the cultures were placed in the laboratory room where they were exposed to the dim room light and others in the completely darkened chamber. The effect of light on the germination of the seeds of *Paphiopedilum callosum* was thus examined.

The result is shown in the Plate VI. fig. 4 A and B, photographically.

As can be seen in the figure the growth was better in the flasks exposed to dim room light than in those placed in the dark chamber. No difference in the degree of germination between these two treatments was observed.

v) Discussion.

As described in subsection viii of section 8, much efforts were made by various investigators to obtain seedlings of "hard to germinate" orchids.

Ovule culture techniques devised by Withner (1943, 1953, 1955) were somewhat effective on the germination of the seeds of "hard to germinate" orchids also in this experiment, when the medium was favorable for germinating the ovules of

the particular species.

The most pronounced effect on the germination of ripe seeds of *Cymbidium virescence* was observed in the plot of the seeds soaked in sterile water for five hrs. after sterilization. KCl-soaking devised by Nakamura (1962) for accelerating the germination of *Galeola septentrionalis* seeds did not exert appreciable promotive effect in the present experiment as compared with water soaking.

Gibberellin which was supplemented to the medium apparently inhibited the germination. This seems to show that the mechanism of the "hard to germinate" phenomenon of orchid seeds differs from that of dormant seeds of plants of other kinds whose germination is readily promoted by gibberellin treatment, though a question remains as to the doses used in this experiment.

On the mechanism involved in the "hard to germinate" seeds of plants, many investigations have been carried out (Crocker and Barton, 1953). In some cases, presence of the seed coat plays an important role in controlling the germination of the seeds mechanically or materially. In these cases, removal of the seed coat or cracking the seed coat assures a good germination of the seeds.

Intending to remove or soften the seed coat, trials of immersing the seeds in Wilson's calcium hypochlorite solution for such a long duration as one and a half or three hours were attempted, and these treatments appreciablly increased the seed germination. Although, when the seeds were immersed in Wilson's calcium hypochlorite solution for six hrs., the seed coats were removed completely, no seed germination was obtained. In this case, embryo might have been injured by the disinfectant. This means, appropriate softening or removal of the seed coat is necessary in order to obtain the good germination of the seeds.

The increased germination by soaking the seed in sterile water or low concentration of KCl solution for five hrs. was, therefore, probably due to appropriate softening of the seed coat.

In practice, however, as to the amount of seeds to be soaked in a volume of sterile water, the duration of soaking, the temperature during treatment, etc., further experiments are necessary for each species of orchids.

The seeds of *Calanthe discolor*, Oriental *Cymbidium* × *Cymbidium* and various kinds of *Paphiopedilum* hybrids were soaked in sterile water for five hours after sterilization. In every case, good germination was observed, however, with *Cymbidium virescence* good development of germinated seed was recorded in the older seed than in the fresh one. (unpublished data). It seems worthy to mention here that the germinated seed of *Cymbidium virescence* or its hybrid forms a rhizome at first. As for the orchids of most difficult to germinate, such as *Cypripedium*, another trials are now carried on.

Summarizing the discussion, soaking the seeds in sterile water for five hours is recommended in practice for germinating the seeds of so-called "hard to germinate" orchids. There is no need to say that it is necessary to choose a suitable medium for that particular species of orchids and to expose to proper light during the culture, or to suppress the desiccation of agar plate during the culture.

13. Effect of the storage conditions of *Dendrobium* and *Brassolaeliocattleya* seeds on their longevity.

Seeds of the above-mentioned orchids were stored in the following conditions:

1. Stored in room condition.
2. Stored in room under dry conditions.
3. Stored in 0°C with dry conditions.

Some of the seeds in each plot were planted onto the slanted agar medium in test tubes about every three months from the start of the storage.

The media used differed depending upon the kinds of orchids tested, i.e., for *Dendrobium* Hyponex plus apple juice (Hyponex 3.0 g./l. of 10 per cent apple juice) and for *Blc.* hybrid plain Hyponex (Hyponex 3.0 g./l. of water) were used.

The sugar concentration of the media was adjusted to 3.5 per cent adding sucrose.

i) On *Dendrobium* seeds.

The seeds were harvested on Feb. 24, 1962 and then were placed in a desiccator. The first sowing was made on May 22, 1962. Thereafter they were stored in the above mentioned conditions.

The result obtained is presented in table 18.

Table 18. Effect of storage conditions on the germination of *Dendrobium* seeds.

Sowing date	Storage condition	Percentage of germination	Growth of the germinated plantlets*		
			Plant height	Root length	Rooting
			mm.	mm.	%
May 22, 62		95			
July 25, 62	Room	0			
	Room+desiccation	56			
	0°C+desiccation	63			
Oct. 27, 62	Room	0			
	Room+desiccation	35	6.1	6.1	100
	0°C+desiccation	82	8.6	10.1	100
Feb. 3, 63	Room+desiccation	20			
	0°C+desiccation	72			
Sep. 18, 63**	Room+desiccation	2			
	0°C+desiccation	60			

* The recordings were made 166 days after planting.

** Planting was started three minutes after the beginning of the sterilization.

As shown in the table, the seeds stored in room conditions lost their viability within two months of storage. The best germination at the end of the experiment was recorded with the seeds stored at 0°C with dry condition. The seeds placed in room under dry condition held viability for about a year after harvesting though the viability appreciably decreased.

The seeds planted on Oct. 27, 1962 showed differences not only in the degree of germination but also in the degree of development of the germinated plantlets as also shown in table 18.

ii) On *Brassolaeliocattleya* seeds.

The seeds used were harvested on March 26, 1962. Then they were placed in room under dry condition. The first sowing was made on April 25, 1962. Thereafter they were stored in the three storage conditions as in the above experiment.

The result is shown in table 19.

Table 19. Effect of storage conditions on the germination of *Brassolaeliocattleya* seeds.

Sowing date	Storage condition	Percentage of germination	Growth of the germinated plantlets*		
			Plant height	Root length	Rooting
			mm.	mm.	%
Apr. 25, 62		88			
July 25, 62	Room	0			
	Room+desiccation	67			
	0°C+desiccation	77			
Oct. 27, 62	Room	0			
	Room+desiccation	57	3.3	0.1	6.6
	0°C+desiccation	76	4.0	3.2	100
Feb. 3, 63	Room+desiccation	33			
	0°C+desiccation	57			
Sep. 18, 63**	Room+desiccation	55			
	0°C+desiccation	69			

* The recordings were made on 166 days after planting.

** Planting was started three minutes after the beginning of the sterilization.

iii) Discussion.

In order to obtain a good germination and growth of orchid seeds, it is better to plant the seeds immediately after harvesting, as empirically recommended by the growers. If there is any necessity to store the seeds, seeds should be placed in a refrigerator under dry condition. This assures the viability of the seeds of *Dendrobium* and *Cattleya* hybrids for at least one year. This result accords with Knudson's reports (Knudson, 1924, 1934, 1940).

14. Effect of X-ray irradiations on the germination and growth of *Dendrobium* seeds.

The seeds were irradiated with 0, 3000, 6000, 12000 and 24000 r of X-ray. After the irradiation, they were planted onto the medium containing Hyponex and apple juice (Hyponex 3 g./l. of 15 per cent apple juice, sugar concentration 3.5 per cent).

The result obtained is shown in Plate VI. fig. 5 (A aud B) photographically.

As shown in the figures, high doses of X-ray irradiation inhibited both germination of seeds and the growth of the germinated plantlets, especially in the plot of 24000 r irradiation.

The dose about 10000 r of X-ray irradiaton is recommended in practice for obtaining the artificial mutation effectively.

IV. General Discussion

The present study was undertaken, as described in the introduction, in order to establish the recipes which are easy to prepare and effective in germinating the orchid seeds asymbiotically, and to find the method of germinating the seeds whose germination is considered to be difficult on artificial media. Detailed studies to clarifty the nature of orchids were not attempted.

As repeatedly shown in the present study or in the results by other investigators of asymbiotic germination of orchid seeds, different kinds of orchid genera or species respond to a medium differently, though in some cases some of the orchids are capable to germinate on media of wide range of constitutions. This means that different genera or species of orchids have different nutrient requirements for their germination, and that, for practical use, if the best result in germinating the seeds of a particular orchid species is aimed at, a suitable recipe for that species must be chosen.

Based on the above-mentioned considerations, the present study was attempted to establish a suitable instant medium for germinating the seeds of each genus of orchids which is cultivated throughout the world in commercial scale. So far as the four genera of orchids, *Dendrobium*, *Cattleya* group, *Cymbidium* and *Paphiopedilum*, are concerned, the recipes presented in the present report are effective, and they are applicable in practice.

The reason why the responses of the orchids to the media in germinating their seeds are different is probably due to the different hereditary constitution in each species of the orchids. No effort was made to clarify mechanisms of the difference in the present study. However, the differences in the nitrogen metabolism, especially in the amino acid metabolism during germination process, may be the main reason for different responses of the orchid seeds to a medium. And recently Raghavan and Torrey (1964) showed that the ability to synthesize the nitrate reductase in *Cattleya* seedlings was observed only at later stages of development. These assumptions or fact would also apply to the different responses of the ovules or seeds to a medium due to their age, as pointed out by Spoerl (1948), and Spoerl and Curtis (1948).

As for the active substance or substances which are contained in the additions and play an important role in accelerating the growth of cultured plants and differentiation of their tissues or organs, much attention has been paid to clarifying their nature. Many of the results, however, led the investigators to disappointment. These facts led the author to a concept that many substances take part in the formation or growth of a single plant organ, and on the contrary, a single substance takes part in the formation or growth of many organs of a plant. In other words, every substance which contributes to the growth and differentiation of a plant is a limiting factor. This is one of the reasons why only establishment of practical recipes was attempted in the present study setting the reason aside.

Various kinds of juice or decoction from plants have been used for germinating

orchid seeds. They are salep (Bernard, 1909; Burgeff, 1959), potato (Downie, 1940, 1941, 1943; Knudson, 1922; Withner, 1942), beet (Knudson, 1922). carrot (Withner, 1951), onion (Ito, 1955), banana (Graeflinger, 1950; Karasawa, 1964; Withner, 1943, 1955), tomato (Griffith and Link, 1957; Ito, 1955; Meyer, 1945; Tsuchiya, 1954; Vacin and Went, 1949b), orange (Griffith and Link, 1957), wheat (Knudson, 1922; Withner, 1951), liquid endosperms (Crovetto, 1957b; Hegarty, 1955; Ito, 1960, 1961; Withner, 1951; Yamada, 1952), seeds (Griffith and Link, 1957; Schaffstein, 1938, 1941; Withner, 1942, 1951), plant body (Schaffstein, 1938, 1941; Bouriquet and Boiteau, 1937; Burgeff, 1911), and so on. Some of them are specifically effective in germinating a particular genus or species of orchids.

In the present work, it was found that apple juice is particularly and consistently effective in germinating the seeds of *Dendrobium* as compared with tomato juice. Tomato juice was proved to be effective in fruit set of tomatoes and *in vitro* culture of plant tissues or organs as well as in germinating orchid seeds (Kent and Brink, 1947; La Rue, 1949; Leopold and Scott, 1952; Nitsch, 1951, 1952; Sternheimer, 1954; Straus, 1960; Tamaoki and Ullstrup, 1958; Tuleke, 1957). Concerning apple juice, however, there is no report except Kano's (1962) in which he found that apple juice is as effective as tomato juice in the growth of cultured tomato ovaries. As to the active substance or substances of apple juice, no attempt was made to clarify their nature in the present work, although the importance of amino acids was assumed. Schaffstein (1941) believed that the unknown material contained in natural extracts and responsible for orchid seed germination was probably a nicotinic acid derivative. The favorable effect of nicotinic acid on the germination of orchid seeds was confirmed later by several workers (Bahme, 1949; Burgeff, 1959; Mariat, 1949, 1952; Noggle and Wynd, 1943). It seems necessary to clarify the nature of these unknown active principals in the next step of research for asymbiotic germination of orchid seeds. Although there remains a question whether the promotive effect is directly due to the action of an unknown substance or substances, or indirectly to their action on other functions such as root formation.

The same question arises about peptone and tryptone.

Effects of apple and tomato juices on the germination of the orchid seeds were conflicted among the experiments. Concerning the reason for this confliction, an assumption was presented already in subsection v in section 7. Beside this, the results in section 9 and 13 seem worthy of emphasis. Namely, the response of orchid seeds to a medium differed depending upon the age of seeds after harvest and the storage condition. This may be also one of the reasons why results are conflicted.

The fact that the favorable recipe differs depending upon the genera or species of orchids and the maturity or viability of seeds, suggests the difficulty of establishing a perfect universal medium. Even if the recipe is profitable to a particular genus or species, it is preferable that the recipe has as broad adaptability as possible.

In the present work, seed germination of so-called "hard to germinate" orchids

was accelerated by soaking the seeds for five hours in sterile water after sterilization, and with *Paphiopedilum callosum*, the seeds germinated easily and grew into well developed seedlings on the favorable media. These facts mean that there are some grades in the difficulty of seed germination among the so-called "hard to germinate" orchids. As already described in section 8, much effort has been made in order to improve the germination. Withner (1952) refered to their mechanism and listed the following factors in addition to the nutrients and pH: presence of an inhibitor, dormancy, genetic factors and loss of viability. In the present work, the author assumed that the factors concerning the difficulty would be the mechanical prevention by seed coat, difference in nutrient requirement or the combination of these two, and that these factors create some grades in the difficulty of seed germination among the "hard to germinate" orchids. If germinative seeds and a profitable medium are prepared, the problem is confined to the mechanical prevention by the seed coat. Whether the disturbance is due to the inhibitor in seed coat or to the mechanical structure of seed coat, the removal or softening of the seed coat is primarily important in order to improve the germination. Hence, soaking experiments, i.e. soaking the seeds in disinfectant or in sterile water after sterilization, were made and the accelerating effect of soaking was proved. Soaking the seeds in sterile water for five hours after sterilization was applied later to germinating another kind of "hard to germinate" orchid and its effectiveness was confirmed again. Concerning the soaking treatment, however, more detailed studies will be necessary for each particular species of orchids as pointed out in subsection 5 of section 12.

As for the dormancy factor, such as one found by Curtis (1943) with native *Cypripedium*, it is better to distinguish it from germinative phenomenon and to consider it from another point of view such as developmental phenomenon.

V. Conclusion

There are many problems to be solved from the scientific point of view in the asymbiotic germination of orchid seeds. At present, however, it seems necessary to obtain useful recipes and methods for germinating orchid seeds. The author will be very happy if the recipes and the method presented in this paper are widely made use of in practice.

VI. Summary

Some trials were carried out in order to establish recipes which are easy to prepare and effective in germinating the orchid seeds asymbiotically, and to find a method of germinating the seeds whose germination is considered to be difficult on artificial media, as well as to study miscellaneous problems concerning the asymbiotic germination of orchids in practice.

The results obtained are summarized as follows:

1. Growth of the germinated seedlings was enhanced when culture vessels were sealed completely in the early period of culture. However, in the later period of culture, it was depressed. Rubber stoppers with glass tubings are recommended in practice. Such stoppers assure a good growth of the seedlings and exclude fungal contamination which occurs often when a cotton plug is used as a stopper.

2. Large sized containers are preferable as transplanting vessels to smaller ones.

3. Gibberellin added to the media exerted no favorable effect on germinating the so-called "hard to germinate" orchid, or on germination and growth of the germiated seedlings of "easy to germinate" orchids.

4. In sowing bed, the growth in root length decreased according as the concentration of Hyponex increased up to five grams per liter of solution.

5. Ten ppm of indolebutyric acid inhibited the germination and, growth of the germinated seedlings of *Dendrobium* in sowing bed, while it enhanced the growth of the transplanted seedlings. With *Brassolaeliocattleya* no appreciable effect was observed except that it inhibited the germination and growth of the seedlings in sowing bed.

6. Response to a medium differed depending upon the genera of orchids used, though some of the orchids were capable to germinate on various kinds of media. Based on these evidences, formulae which are easy to prepare and effective in germinating the seeds of a particular genus of orchids were devised for the four genera of orchids, *Dendrobium*, *Cattleya* group, *Cymbidium* and *Paphiopedilum*.

The following recipes can be recommended in practice.

a) For *Dendrobium*

Fresh strained apple juice diluted to 10–20%	1000 mls.
Hyponex	3 grms.
Sucrose to adjust the total sugar content to approx.	3.5 %
Agar	15 grms.

pH adjusted to approx. 5.0

b) For *Cattleya*

Hyponex	3 grms.
Sucrose	35 grms.
Agar	15 grms.
Water	1000 mls.

PH adjusted to approx. 5.0

c) For *Cymbidium*

Hyponex	3 grms.
Difco Bacto-tryptone	2 grms.
Sucrose	35 grms.
Agar	15 grms.
Water	1000 mls.

pH adjusted to approx. 5.0

d) For *Paphiopedilum*

Hyponex	3 grms.
Difco Bacto-peptone or Bacto-tryptone	2 grms.
Sucrose	35 grms.
Agar	15 grms.
Water	1000 mls.

pH adjusted to approx 5.0

These formulae are used for transplanting beds for each particular orchid.

7. The suitable recipes differed depending upon the age of seeds after harvesting. Storage condition of seeds after harvesting affected the germination energy of the seeds and the growth of germinated seedlings. Practically speaking, it is preferable that the recipe has as broad adaptability as possible for particular genus of orchid and that the seeds are planted as early as possible after harvest.

8. Germination of ripe Oriental *Cymbidium* seeds (*Cym. virescence*) was accelerated by soaking the seeds in sterile water for five hours after sterilization in Wilson's disinfectant. This treatment was also effective in germinating some of the so-called "hard to germinate" orchid seeds. At the same time, it seems important in germinating these seeds to choose the suitable media and to minimize the desiccation of agar plate.

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ラン種子の発芽培地に関する研究

狩野邦雄

ランの無菌発芽に際しての，簡便にして有効な処方の確立と，人工培地で発芽が困難とされている種子の発芽方法の発見を目的として，いくつかの試みをおこなった．また実際にランの無菌発芽をおこなうにあたって関係する種々の問題についても研究した．

結果はつぎのごとくである．

1. 実生の生長は，容器が完全に密閉されたとき，培養の初期では促進されたが，後期では圧えられた．実用には，ガラス管付ゴム栓がよい．この栓では，実生の生長もよく，綿栓を使った場合しばしばおこる汚染が除ける．

2. 移植床の容器としては大きな容量のものがよい．

3. ジベレリンは，いわゆる“発芽困難”な種類の発芽や，発芽容易な種類の発芽や生長に，のぞましい効果を与えない．

4. は種床での根の伸長は，Hyponex の濃度が，1ℓ あたり5.0 grms. まで増加するにしたがい減少した．

5. 10 p.p.m. の IBA は，は種床で，*Dendrobium* の発芽と生長を抑制したが，移植した実生の生長は促進した．*Brassolaeliocattleya* では，は種床で発芽と生長を抑制した以外，みるべき効果はなかった．

6. ランの幾つかの種類は，種々の異なった培地で発芽が可能であるが，ある1つの培地に対する反応は，ランの種類により異る．これ等の事実にもとづき，その種類の発芽に効果があり，しかも調合が簡単な処方を，*Dendrobium, Cattleya, Cymbidium* および *Paphiopedilum* の4属のそれぞれにつき案出した．

つぎの処方は実用に推奨される：

a) *Dendrobium* 用

10～20％リンゴしぼり汁	1000 mls.
Hyponex	3 grms.
庶糖を加えて総糖濃度	3.5%
寒天	15 grms.

pH ほぼ5.0に調整

b) *Gattleya* 用

Hyponex	3 grms.
庶糖	35 grms.
寒天	15 grms.
水	1000 mls.

pH ほぼ5.0に調整

c) *Cymbidium* 用

Hyponex	3 grms.
Difco Bacto-tryptone	2 grms.
庶　糖	35 grms.
寒　天	15 grms.
水	1000 mls.

pH ほぼ5.0に調整

d) *Paphiopedilum* 用

Hyponex	3 grms.
Difco Bacto-peptone または Bacto-tryptone	2 grms.
庶　糖	35 grms.
寒　天	15 grms.
水	1000 mls.

pH ほぼ5.0に調整

これ等の処方は，それぞれのランの移植床としても使える。

7. 好適な処方は，種子の収穫後の令によっても異なった。収穫後の種子の貯蔵条件は，種子の発芽力と発芽後の生長に影響した。実用上からは，処方は，その種のランに，可能なかぎり広い適応性をもち，また種子は採種後できるだけ早くは種するのがよい。

8. 春ランの成熟種子の発芽は，Wilson 液で殺菌後，殺菌水に5時間浸漬することによって，促進された。この処理はまた，いわゆる"発芽困難"な種類のいくつかの発芽にも効果があるが，同時に，これ等の種子の発芽に際しては，好適な培地を選ぶこと，および寒天の乾燥をさけることが重要であろう。

Explanations of Plates

Plate I.

Fig. 1 (A, B and C). Comparative growth of *Bletilla* seedlings on Knudson's media with additions, (a) plain Knudson's medium; (b) 30 per cent tomato juice; (c) 30 per cent apple juice; (d) 30 per cent *Cymbidium* pseudo bulb juice; and (e) crude sugar. (A): 60 days (B): 120 days and (C) 140 days old.

Fig. 2 (A and B). Effect of various kinds of stoppers on germinating the seeds of *Lc.* hybrid. (A): 188 days, and (B): 268 days after seed sowing. (a)-(c): Hyponex media, (d): Knudson's medium.

Fig. 3. Effect of complete sealing on the growth of developing *Dendrobium* seedlings. (c) and (d) sealed about six months after seed inoculation. Photograph taken seven months after sealing. (a) and (c): Knudson's C plus 20 per cent apple juice, (b) and (d): plain Knudson's C.

Fig. 4 (A and B). Effect of cotton wad stuffed at the outer end of glass tubing on the germination of *Dendrobium* seeds. (A) with ordinary rubber stoppers, (B) with zigzag-shaped glass tubings without cotton wads. Photographs taken 274 days after seed inoculation.

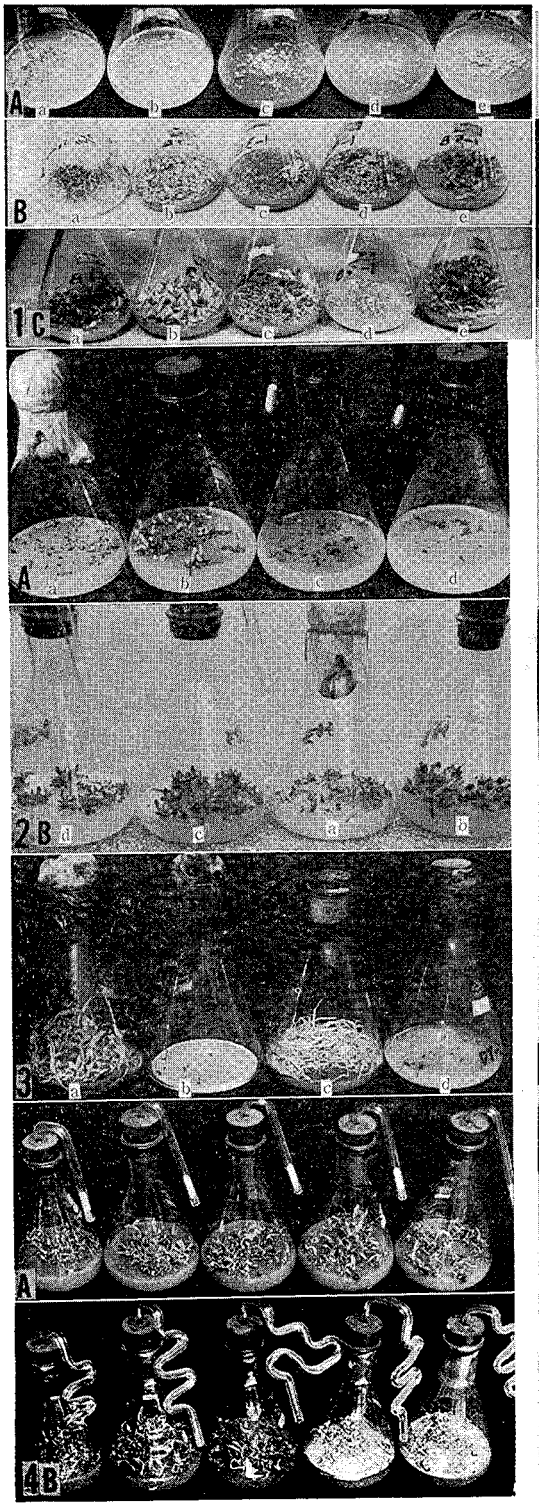
Fig. 5 (A, B and C). Effects of stoppers on the growth of *Dendrobium grantii*. (A): 206 days old, (a) cotton plugs; (b) rubber stoppers with glass tubings; (c) and (d) completely sealed, henceforth the rubber cylinders in plot c were removed. (B) and (C): 365 days old.

Fig. 6. Effect of the size of culture vessels on the growth of *Brassavola nodosa* seedlings. Photograph taken 177 days after transplanting. (a) 50 ml., (b) 100 ml., (c) 200 ml. and (d) 300 ml. capacities of flasks.

Fig. 7 (A and B). Effect of concentration of Hyponex in the sowing and transplanting beds on the growth of seeds and seedlings of *Dendrobium*. Photographs taken 117 days after seed inoculation or transplanting. (a)-(d): in sowing beds and (e)-(h): in transplanting beds. (a)-(e): Hyponex 2 g., (b) and (f): Hyponex 3 g., (c) and (g): Hyponex 4 g., and, (d) and (h): Hyponex 5 g. per liter of 10 per cent apple juice solution respectively.

Fig. 8 (A and B). Effect of concentration of Hyponex in the sowing and transplanting beds on the growth of seeds and seedlings of *Brassolaeliocattleya*. Photographs taken 178 days after seed inoculation or transplanting. (a)-(d): in sowing beds and (e)-(h): in transplanting beds. (a) and (e): Hyponex 2 g., (b) and (f): Hyponex 3 g.; (c) and (g): Hyponex 4 g.; and (d) and (h): Hyponex 5 g. per liter of water respectively.

PLATE I



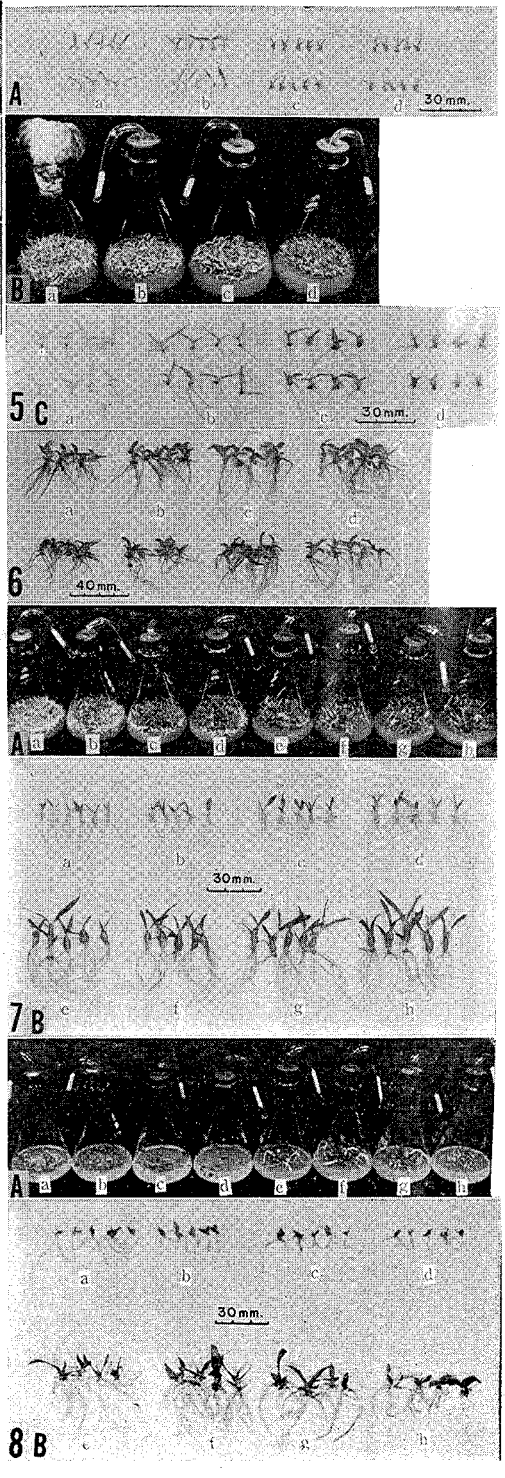


Plate II.

Fig. 1 (A and B). Effect of IBA concentration in the sowing and transplanting beds on the growth of seeds and seedlings of *Dendrobium*. Photographs taken 117 days after seed inoculation or transplanting. (a)-(d): in sowing beds and (e)-(h): in transplanting beds. (a) and (e): basal medium (Hyponex 3 g./liter of 10 per cent apple juice); (b) and (f): IBA 0.1 mg./l.; (c) and (g): IBA 1.0 mg./l.; and (d) and (h): IBA 10 mg./l.

Fig. 2 (A and B). Effect of IBA concentration in the sowing and transplanting beds on the germination of seeds and growth of seedlings of *Brassolaeliocattleya*. Photographs taken 178 days after seed inoculation or transplanting. (a)-(d): in sowing beds and (e)-(h): in transplanting beds. (a) and (e): basal medium (Hyponex 3 g./liter of water); (b) and (f): IBA 0.1 mg./l.; (c) and (g): IBA 1.0 mg./l.; and (d) and (h): IBA 10 mg./l.

Fig. 3 (A-D). Erratic effect of tomato juice on the germination of *Dendrobium* seeds. Photographs taken 189 days after seed inoculation. (A): Hyponex plus 15 per cent apple juice, (B): Meyer's solution, (C): Hyponex plus 15 per cent tomato juice, and (D): Knudson's C plus 15 per cent tomato juice.

Fig. 4. *Dendrobium* seedlings grown on Knudson's and Hyponex media with dried apple fruits or apple juice. Photograph taken 175 days after seed sowing. (a): plain Knudson's soln. C, (b): Knudson's plus 15 per cent apple juice, (c): Knudson's plus dried apple 10 g./l., (d): Knudson's plus dried apple 20 g./l., (e): plain Hyponex solution, (f): Hyponex plus apple juice 15 per cent, (g): Hyponex plus dried apple 10 g./l., and (h): Hyponex plus dried apple 20 g./l.

Fig. 5 (A and B). *Dendrobium grantii* seedlings grown on Hyponex media with additions. (a) plain Hyponex; (b) citric acid 80 mg./l.; (c) DL-malic acid 160 mg./l.; (d) pyruvic acid 80 mg./l.; (e) citric acid 80 mg./l. and DL-malic acid 160 mg./l.; (f) citric acid 80 mg./l. and pyruvic acid 80 mg./l.; (g) pyruvic acid 80 mg./l. and DL-malic acid 160 mg./l.; (h) DL-malic acid 160 mg./l., pyruvic acid 80 mg./l. and citric acid 80 mg./l.; and (i) 10 per cent apple juice. Photographs taken 264 days after seed sowing.

Fig. 6 (A and B). *Dendrobium grantii* seedlings grown on Hyponex media with additions: (a) plain Hyponex; (b) 5 per cent mashed banana; (c) 10 per cent onion juice; (d) dried apple fruits 5 g./l.; and (e) 10 per cent apple juice. Photographs taken 264 days after seed inoculation.

Fig. 7 (A and B). *Cymbidium* seedlings grown on Knudson's and Hyponex madia with additions. (a)-(d): Knudson's media and (e)-(h): Hyponex media. (a) and (e): plain media, (b) and (f): 10 per cent apple juice, (c) and (g): peptone 2 g./l., (d) and (h): tryptone 2 g./l. Photograph taken 272 days after seed inoculation.

Fig. 8. Germination of *Cymbidium virescence* seeds on Knudson's, Hyponex and Burgeff N_8f media with additions to the former two media. Explanations are same as in fig. 7 for (a)-(h), except (i) Burgeff N_3f. Photograph taken 416 days after seed inoculation.

Fig. 9 (A and B). *Paphiopedilum callosum* seedlings grown on Knudson's, Hyponex and Burgeff N_8f media with additions to the former two media. Explanations are same as in fig. 7 for (a)-(h), except (i) Burgeff N_8f. Photographs taken 264 days (A) and 267 days (B) after seed inoculation.

PLATE II

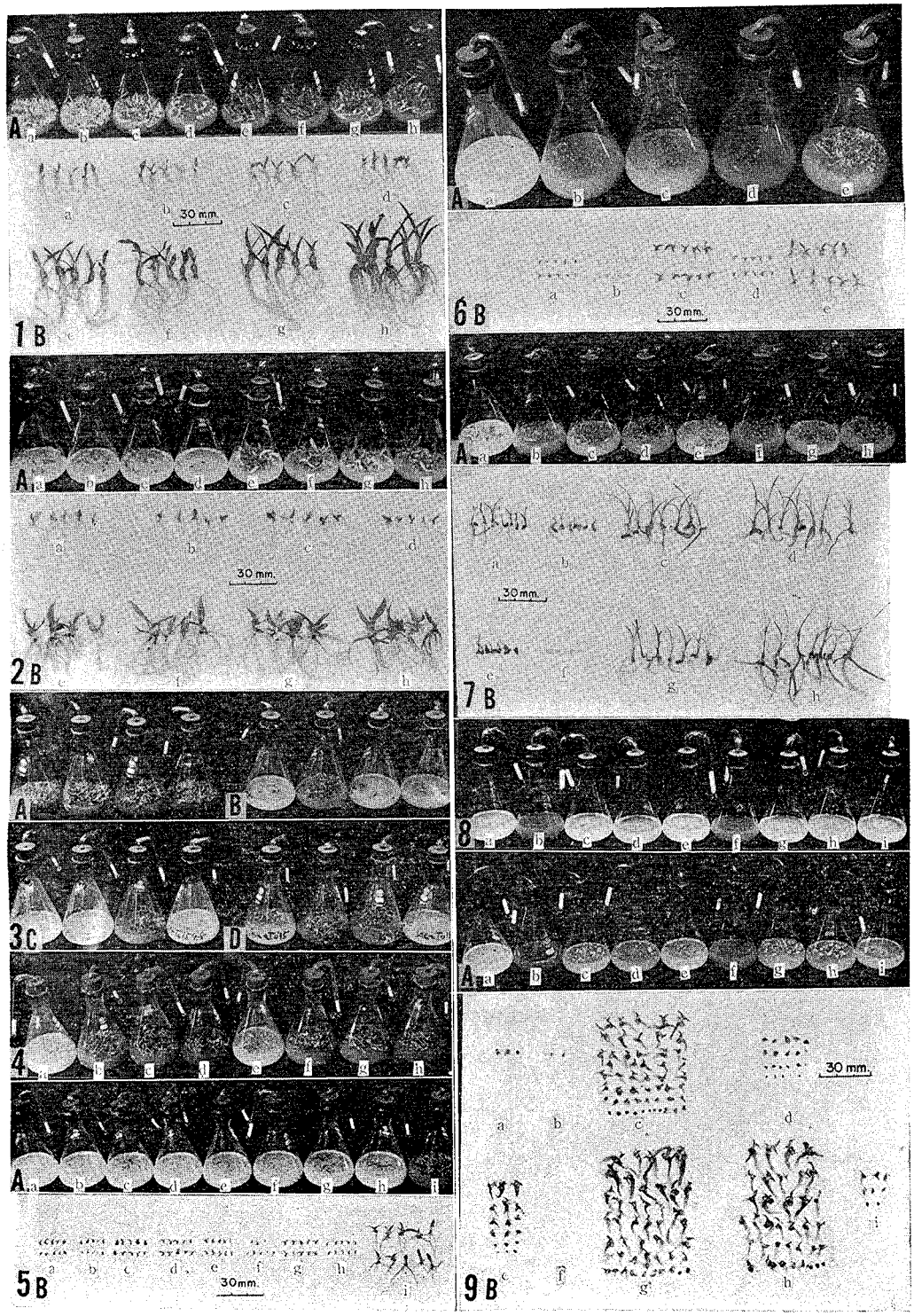


Plate III.

Fig. 1. Effect of stoppers on the germination of *Paphiopedilum callosum* seeds. Medium: Hyponex plus tryptone. Age: 182 days old.

Fig. 2. Germination of *Cypripedium reginae* seeds on Knudson's and Hyponex media with additions. (a)-(c): Knudson's media and (d)-(f): Hyponex media. (a) and (d): 5 per cent apple juice, (b) and (e): peptone 2 g./1., and (c) and (f): tryptone 2 g./1., respectively, were added. Photograph taken 508 days after seed sowing.

Fig. 3. Germination of *Calanthe discolor* seeds on Knudson's, Hyponex and Burgeff N_8f media with additions to the former two madia. Explanations are same as in fig. 8 of Plate II. Photograph taken 416 days after seed sowing.

Fig. 4. Germination of *Calanthe discolor* seeds on plain Hyponex medium. Seeds were sown on the harvest day. Left two flasks: seeds sterilized. Right two flasks: seeds not sterilized. Photograph taken 416 days after seed inoculation.

Fig. 5. Germination and growth of *Dendrobium* seeds on Knudson's and Hyponex media with additions. (a)-(d): Knudson's media and (e)-(j): Hyponex media. (a) and (e): plain media, (b) and (f): 5 per cent apple juice, (c) and (g): peptone 2 g./1., (d) and (h): tryptone 2 g./1., (i): L-arginine 20 mg./1. and L-lysine 40 mg./1., and (j): L-aspertic acid 200 mg./1., respectively, were added. Age: 210 days old.

Fig. 6 (A and B). Germination and growth of *Brassavola nodosa* seeds on Knudson's and Hyponex media with additions. (a)-(f): explanations are same as in fig. 5. (g): Hyponex plus L-arginine 20 mg./1., and L-lysine 40 mg./1., (h): Hyponex plus L-aspartic acid 200 mg./1. Photographs taken 210 days (A) and 253 days (B) after seed sowing.

PLATE III

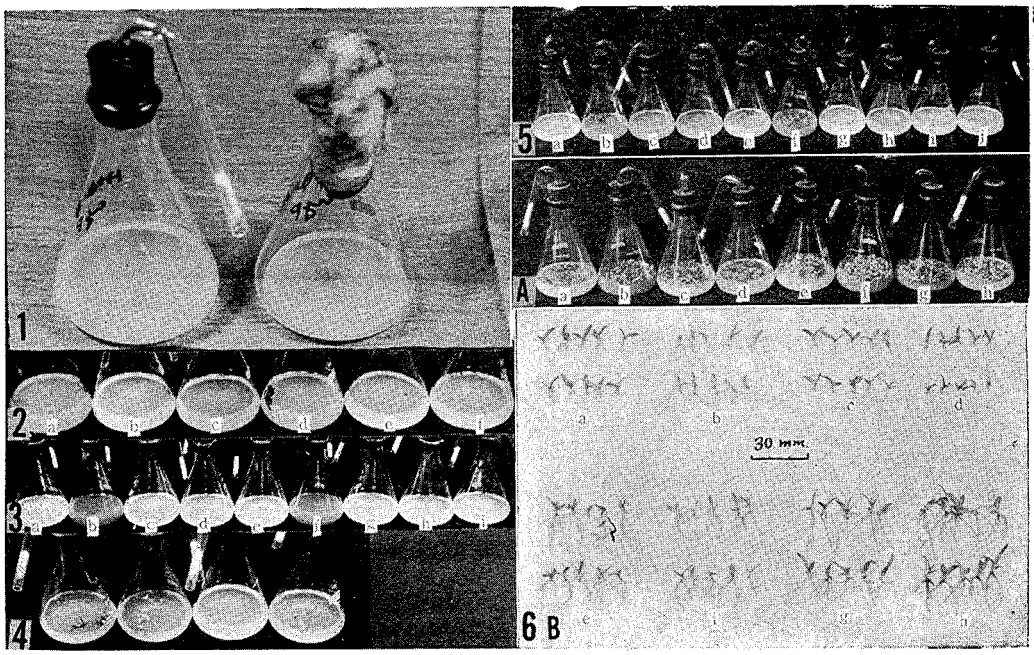


Plate IV.

Fig. 1 (A and B). Effects of complex organic substances and amino acids on the germination and growth of *Dendrobium* seeds. The seedlings were removed from a culture flask to be spread out in Petri dishes. Basal medium: Hyponex. (A-a) and (B-a): plain Hyponex, (A-b) and (B-j): 15 per cent apple juice, (A-c): peptone 2 g./l., (A-d): tryptone 2 g./l., (A-e): casein from milk 2 g./l., (A-f): yeast extract 2 g./l., (B-b): L-aspartic acid 1×10^{-3} M., (B-c): L-asparagine 1×10^{-3} M., (B-d): L-arginine 1×10^{-3} M., (B-e): Na-adenosin triphosphate (40 mg./l.), (B-f): L-glutamic acid 1×10^{-3} M., (B-g): L-histidine 1×10^{-3} M., (B-h): L-lysine 1×10^{-3} M., and (B-i): L-tryptophan 1×10^{-3} M., respectively, were added. Photographs taken 141 days (A) and 144 days (B) after seed sowing.

Fig. 2 (A and B). Effects of complex organic substances and amino acids on the germination and growth of *Brassolaeliocattleya* seeds. The seedlings were removed from two culture flasks to be spread out in Petri dishes. The explanations are same as in fig. 1. Photographs taken 230 days after seed sowing.

Fig. 3 (A and B). *Dendrobium* seedlings grown on Hyponex media with various concentrations of L-tryptophan or L-glutamic acid. (A-a) and (B-a): plain Hyponex, (A-b) and (B-b): L-tryptophan 1×10^{-3} M., (A-c) and (B-c): L-tryptophan 1×10^{-4} M., (A-d) and (B-d): L-tryptophan 1×10^{-5} M., (A-e) and (B-e): L-glutamic acid 1×10^{-3} M., (A-f) and (B-f): L-glutamic acid 1×10^{-4} M., (A-g) and (B-g): L-glutamic acid 1×10^{-5} M., (A-h) and (B-h): L-tryptophan 1×10^{-4} M. and L-glutamic acid 1×10^{-3} M., (A-i) and (B-i): 15 per cent apple juice, respectively, were added. The seedlings were removed from a culture flask to be spread out in Petri dishes. Photographs taken 156 days after seed inoculation.

Fig. 4 (A and B). *Brassolaeliocattleya* seedlings grown on Hyponex media with various concentrations of L-tryptophan or L-glutamic acid. The seedlings were removed from a culture flask to be spread out in Petri dishes. The explanations are same as in fig. 3. Photographs taken 182 days after seed inoculation.

Fig. 5. Effect of sugar concentrations in transplanting bed on the growth of *Dendrobium* seedlings. Photograph taken 235 days after transplanting. (a): 0.5, (b): 1.0, (c): 2.0, (d): 4.0 and (e): 8.0 per cent. Basal medium: Knudson's C mineral solution plus two per cent apple juice.

Fig. 6. *Brassocattleya* seedlings grown on Hyponex media with various concentrations of sucrose. (a): 0.5, (b): 1.0, (c): 2.0, (d): 4.0 and (e): 8.0 per cent. Photograph taken 235 days after transplantng.

PLATE IV

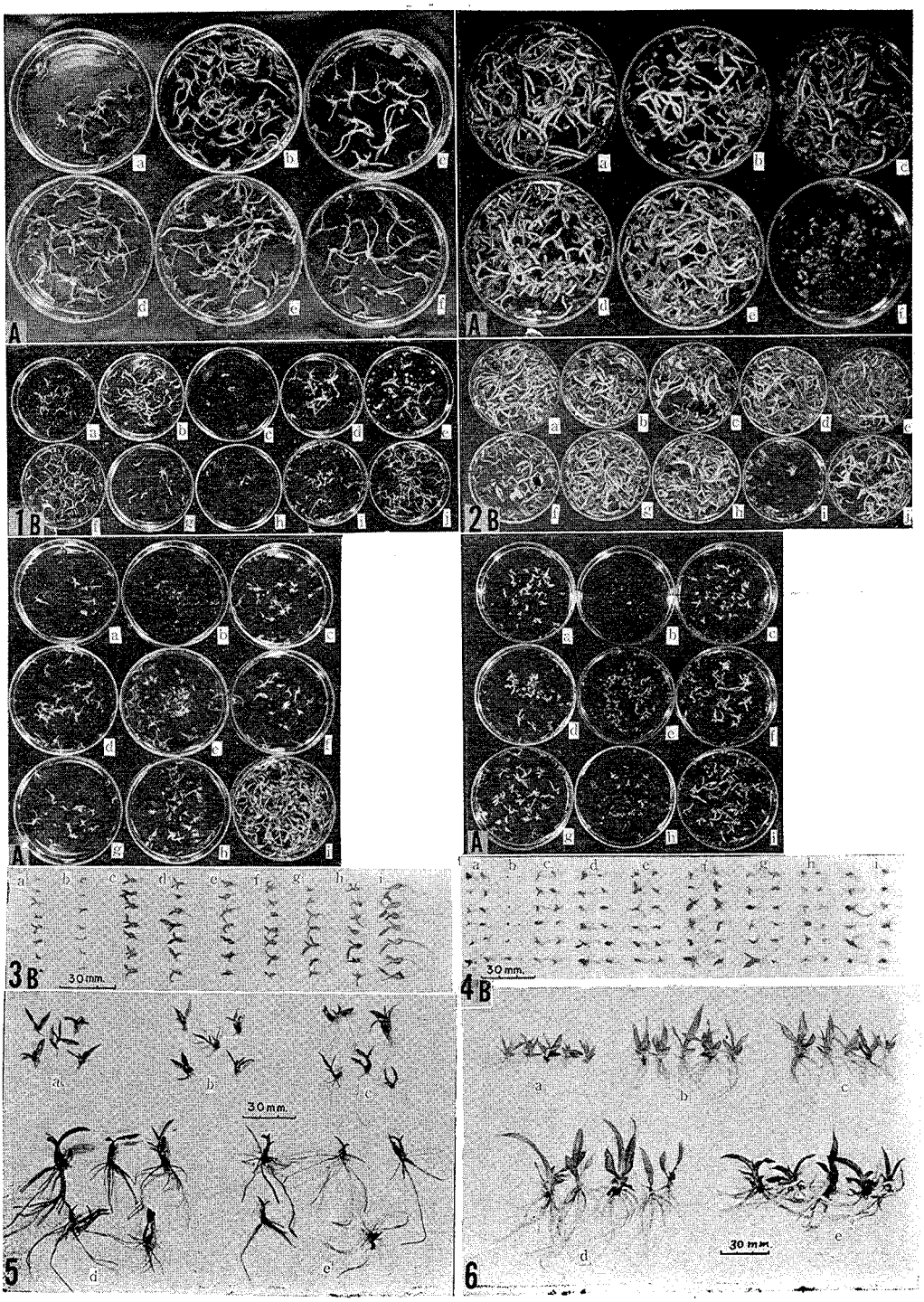


Plate V.

Fig. 1. *Brassavola nodosa* seedlings grown on Hyponex media with various concentrations of sucrose. (a): 0.5, (b): 1.0, (c): 2.0, (d): 4.0 and (e): 8.0 per cent. Photograph taken 149 days after transplanting.

Fig. 2. *Paphiopedilum callosum* seedlings grown on Hyponex and tryptone media with various concentrations of sucrose. (a): 1.0, (b): 2.0, (c): 4.0 and (d): 8.0 per cent. Photograph taken 151 days after transplanting.

Fig. 3. Effect of sucrose concentrations in the transplanting bed on the growth of *Cymbidium* seedlings. Explanations are same as in fig. 2. Photograph taken 195 days after transplanting.

Fig. 4. *Paphiopedilum callosum* seedlings grown on Hyponex media with various concentrations of tryptone. (a): 0, (b): 0.1, (c): 0.2, (d): 0.4 and (e): 0.8 per cent. Photograph taken 151 days after transplanting.

Fig. 5. Effect of tryptone concentrations in the transplanting bed on the growth of *Cymbidium* seedlings. Explanations are same as in fig. 4. Photograph taken 195 days after transplanting.

PLATE V

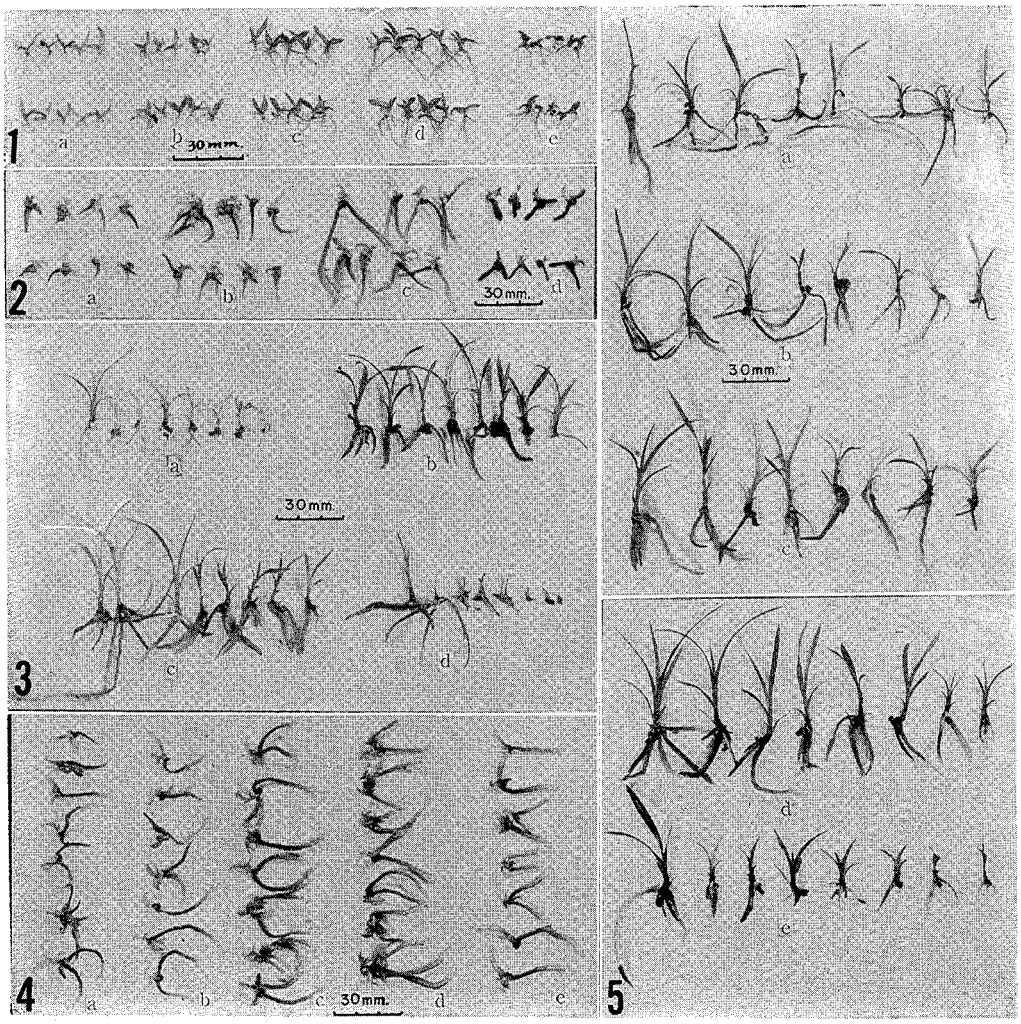


Plate VI

Fig. 1. Effect of duration of sterilization in accelerating the germination of ripe seeds of *Cymbidium virescence*. The seeds were soaked in Wilson's calcium hypochlorite solution for (a) ten min., (b) 1.5 hrs., and (c) 3.0 hrs. Basal medium: Hyponex with peptone. Age: 239 days old.

Fig. 2. Effect of KCl soaking prior to sterilization in accelerating the germination of ripe seeds of *Cymbidium virescence*. The seeds were soaked in various concentrations of KCl solution for five days before sterilization. (a): 0, (b): 0.1, (c): 1.0 and (d): 10.0 per cent. Basal medium: Hyponex with peptone. Age: 221 days old.

Fig. 3 (A-E). Effect of KCl soaking after sterilization in accelerating the germination of ripe seeds of *Cymbidium virescence*.

(A): soaked for five hrs. after sterilization except (a).
- (a): control (sterilized for ten min. with Wilson's disinfectant),
- (b): sterile water,
- (c): 0.01 per cent KCl,
- (d): 0.1 per cent KCl,
- (e): 1.0 per cent KCl,
- (f): 10.0 per cent KCl.

(B): soaked for 25 hrs. after sterilization.
- (a): sterile water,
- (b): 0.01 per cent KCl,
- (c): 0.1 per cent KCl,
- (d): 1.0 per cent KCl,
- (e): 10.0 per cent KCl.

(C): soaked for five days after sterilization.
Explanations are same as in (B).

(D): soaked for 15 days after sterilization.
Explanations are same as in (B).

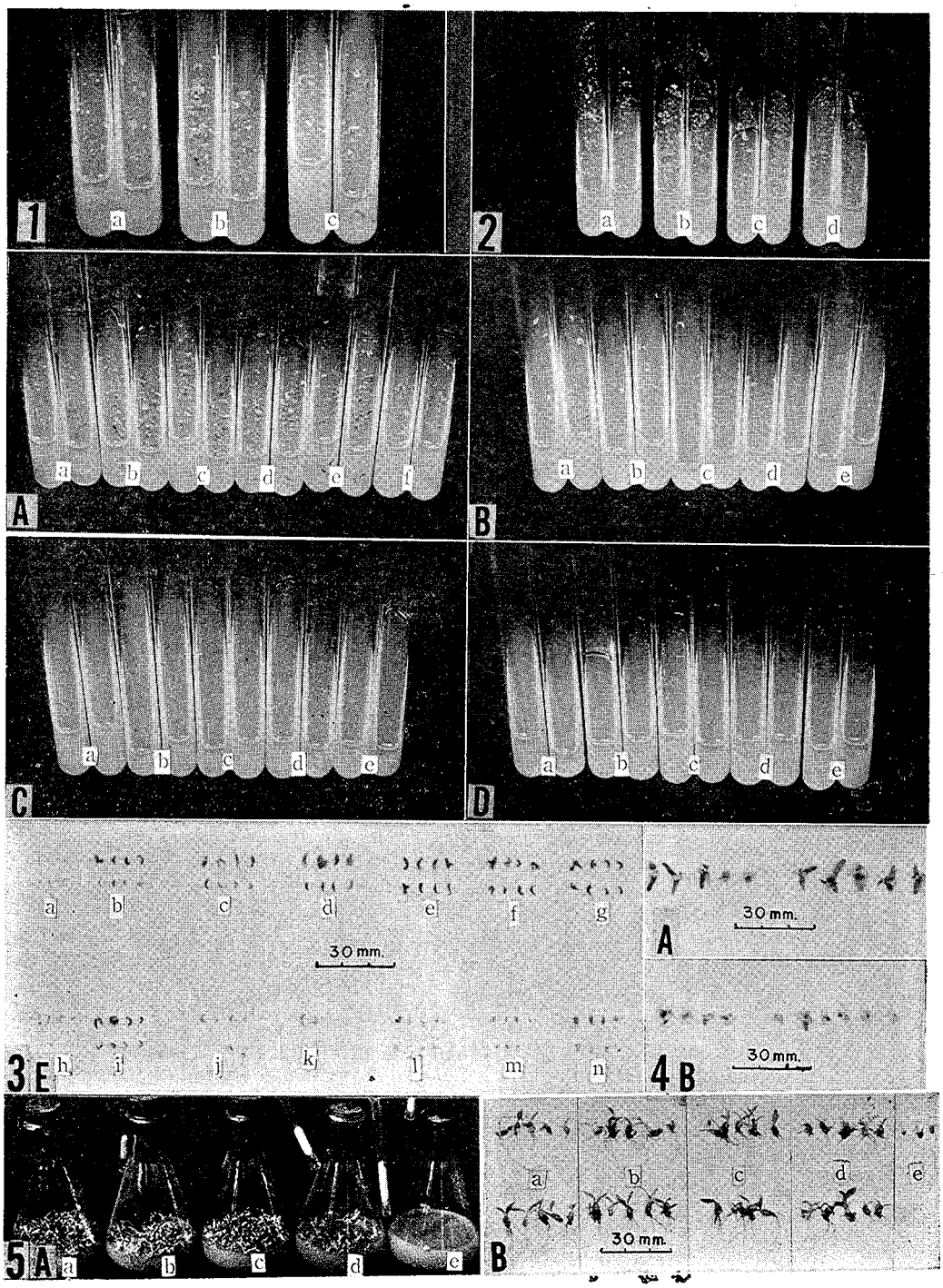
Photographs taken 239 days after the start of the treatments.

(E): state of the seedlings 265 days after the start of the treatment.
- (a): control (sterilized for 10 min. with Wilson's disinfectant (fig. 1-a and A-a),
- (b): sterilized for 1½ hrs. (fig. 1-b),
- (c): sterilized for 3 hrs. (fig. 1-c),
- (d): soaked in water for five hrs. after sterilization (A-b),
- (e): soaked in 0.01 per cent KCl solution for five hrs. after sterilization (A-c),
- (f): soaked in 0.1 per cent KCl solution for five hrs. after sterilization (A-d),
- (g): soaked in 1.0 per cent KCl solution for five hrs. after sterilization (A-e),
- (h): soaked in 10.0 per cent KCl solution for five hrs. after sterilization (A-f),
- (i): soaked in water for 25 hrs. after sterilization (B-a),
- (j): soaked in 0.01 per cent KCl solution for 25 hrs. after sterilization (B-b),
- (k): soaked in 0.1 per cent KCl solution for 25 hrs. after sterilization (B-c),
- (l): soaked in water for five days after sterilization (C-a),
- (m): soaked in 0.01 per cent KCl solution for five days after sterilization (C-b),
- (n): soaked in 0.1 per cent KCl solution for five days after sterilization (C-c).

Fig. 4 (A and B). Effect of light on the germination and growth of *Paphiopedilum callosum* seeds. (A): exposed to dim room light. (B): cultured in the completely darkened chamber. Photograph taken 311 days after seed inoculation.

Fig. 5 (A and B). Effect of X-ray irradiation on the germination and growth of *Dendrobium* seeds. (a): 0 r, (b): 3,000 r, (c): 6,000 r, (d): 12,000 r and (e): 24,000 r. Basal medium: Hyponex with apple juice. Photographs taken 210 days (A) and 252 days (B) after seed inoculation.

PLATE VI



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