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Organogenesis in Sterile Culture of Oriental *Cymbidium, Cymbidium kanran* Makino

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Introduction

Generally, it has been recognized by many workers[2-5] that on the characteristics of germination and developmental growth there are big differences between the tropical *Cymbidiums* which are native to India and Burma, and are epiphytic or semi-epiphytic and the Oriental *Cymbidiums* which are native to China and Japan, and are terrestrial. Namely, the seeds of the former can easily germinate to form protocorms on the culture media artificially prepared, and the protocorms continuously develop into whole plants consisting of the shoot and root, whereas the latter ones can not easily germinate, unless some particular treatments for the germination are done to them, and even when the seeds can germinate to form protocorms owing to the particular treatments, the protocorms only develop into rhizomes, and the whole plants consisting of the shoots and roots can not easily develop from the rhizomes for a few years.

Ueda and Torikata[14-19] reported that rhizome-tip of *Cymbidium goeringii* (*C. virescens*), a species of Oriental *Cymbidium*, cultured in the basal medium (KNUDSON C+NITSCH micro-element) always developed into rhizomes without shoot formation, and the addition of 10 mg/l kinetin led most of samples to shoot formation, whereas low concentration of NAA tended to promote the growth of shoot in combination with 10 mg/l kinetin, and high concentration of NAA tended to counteract the stimulation effect of kinetin on the shoot formation.

Cymbidium kanran Makino, a related species of *C. goeringii*, which is as terrestrial as the latter, is native to the mountains in Southern districts of Japan, Kishū, Shikoku, Kyushū and Ryūkyū, and to those in Formosa in China. It shows usually a low rate of vegetable propagation both under the pot culture generally carried out in this country and under natural condition of native lands. Of course, no seed propagation method of this plants has been established to date.

The present works were designed with the intention of finding how to induce the germination of the seeds of *C. kanran* which seemed to be rather difficult on the artificial media, and how to produce whole plants consisting of shoot and root through the protocorm stage after germination and the subsequent rhizome stage.

Materials and Methods

The ripe seeds of *Cymbidium kanran* used in this experiment were obtained from self-pollination conducted in this laboratory from November 27 to December 4 in 1972. The capsules were collected about ten months after pollination, from October 10 to 16 in 1973. The plants were spared from an earnest grower who has lived in this city, in 1971.

Test tubes (150 mm×15 mm) were used as the culture vessels for germination trials and Erlenmeyer flasks (100 ml capacity) as the ones for developmental growth trials of protocorms or rhizomes. Culture solutions mixed with minerals, sugar, agar and vairous additionals, were dissolved by being heated until they are turned to transparent, then 8 ml of those per test tube for the germination of the seeds and 40 ml of those per Erlenmeyer flask for developmental growth of the protocormes and the rhizomes were poured into, respectively. The pH value of the culture solution was adjusted with 0.1 N NaOH and 0.1 N HCl and was checked with a glass electrode pH meter at the completion of the mixing of media before heating. After being poured into the hot dissolved media the test tubes or the Erlenmeyer flasks were stoppered with cotton plugs, then they were autoclaved at 1.1 kg/cm^2 pressure for 20 min. Several days after autoclaving, the sterilized media were used for seeding or transplanting.

For the sterilizing of the seeds, 3% H_2O_2 water solution was used. Namely, a desired quantity of the seeds was placed into a small glass tube, then the disinfectant was added. After being stoppered, the tube was sometimes shaken in 30 min, then the seeds and the disinfectant were poured into the sterilized dishes in which aseptic water was poured, Then, with a loop of platinum needle sterilized by heating, the seeds were transferred onto the agar media previously prepared. After seeding, the test tubes were sealed up hermetically with rubber plugs, in stead of cotton ones.

The Erlenmeyer flask used as the vessel for the trials of the developmental growth of protocorms or rhizomes, was stoppered with the rubber plugs into which a fishing-hook-shaped glass tubing was inserted for aeration. The tubing was stuffed with a small wad of cotton at the outer end. The whole stoppers and all other implements used in this experiment were sterilized before experiment by autoclaving. Before inoculation, hands and arms were sterilized with 70% ethanol.

The seed cultures were placed in the box cotrolled at about 27°C under continuous fluorescent lighting (luminous intensity 500 Lux).

Moreover, the cultures of protocorms or rhizomes were laid in laboratory room under scattered light and variable temperature conditions.

Results

1. Germination

In order to avoid moisture evaporation, the test tubes used as culture vessels for seeding were sealed up hermetically with rubber plugs after seeding, since it was predicted by other reports to date that the seeds of this plant might need a long period for their germination[5].

The compositions of the media used for seeding are shown in Table 1[6,9,10]. Adding to the compositions shown in the Table, 30 g/l sucrose and 12 g/l agar were added to the four media, respectively, and the pH values of the medium 1 and 2 were adjusted to 5.0 and those of the medium 3 and 4 to 5.6. These four media were chosen with the purpose of clearifying whether the seeds of *C. kanran* could germinate on simple composition media without minor elements and organic nutrients.

No changes in the seeds sown could be appreciated for a long time after seeding. It was not until about 90 days after seeding that the seeds began to germinate. When the seeds began to germinate they firstly showed rather white colour, swelling up themselves, and then they developed into protocorms. About 115 days after seeding some of the protocorms developed into green rhizomes.

The germination percentages of the seeds about 150 days after seeding are shown in Table 2.

Table 1. Compositions* of media used for seeding of *Cymbidium kanran* (October, 1973)

Medium No.	Mineral salts	Organic constituents
1	Knudson's C	
2	Knudson's C + Nitsch's minor elements	Thiamine-HCl 0.10 mg/l Pyridoxine-HCl 0.10 〃 Nicotinic acid 0.10 〃 L-Arginine-HCl 0.50 〃 L-Asparatic acid 0.50 〃 α-Naphthalene-acetic acid 1.00 〃 Kinetin 0.10 〃
3	Murashige and Skoog's mineral salts (RM–1962)	
4	Murashige and Skoog's mineral salts	Murashige and Skoog's Organic constituents

*: 30 g/l sucrose and 12 g/l agar were added to above four media, respectively, and the pH values in medium 1 and 2 were adjusted to 5.0 and those in medium 3 and 4 to 5.6.

Table 2. Germination on seeding media about 150 days after sowing (March, 1974)

Medium No.*1	No. of seeds sowed	No. of germinating seeds	Do. %	Av. No. of seeds sowed per test-tube	Av. No. of germinating seeds per test-tube
1	3765	140	3.71	105	3.9
2	3897	174	4.46	97	4.4
3	4235	122	2.88	125	3.6
4	3118	105	3.37	80	3.6

*1, Same No. in Table 1.

The germination percentages of the seeds on four media were comparatively low, ranging from 2.88 to 4.46, showing no large differences among them. The average number of germinating seeds per culture test tube ranged from 3.6 to 4.4.

Although the percentages of germinating seeds were comparatively low, the number of seedling obtained in this experiment amounted to 541, in spite of the fact that all seeds used were collected from only two capsules.

For the inducement of the seed germination, some immersion treatments of the seeds into KOH water solution for several minutes before seeding as reported by Sawa[11] were conducted, but no effects to the germination were observed.

It was sometimes observed that the number of the seed germination on the test tube media increased after the time when the test tubes were unsealed in order to transplant the seedling which was developed into protocorms or rhizomes onto the transplanting media.

Accordingly, in order to increase the germination percentages, a little more aeration of the seeding vessels might be indispensable, and Erlenmeyer flasks stoppered with rubber plugs furnished with aeration apparatus, which were used as transplanting vessels, might also be better as the seeding vessels.

2. Growth of the rhizomes on transplanting media

After the seedlings developed into rhizomes, aproximate mean size 2 mm × 1 mm, they were removed from the germinating media in the test tube sealed up hermetically with rubber plugs, onto the four kinds of the transplanting media in the Erlenmeyer flask stoppered with the rubber plugs furnished with aeration apparatus mentioned above, on August 23rd in 1974.

Table 3. Developmental growth of rhizome in transplanting media*1 about 150 days after transplanting (January, 1975)

Additionals (*mg/l*)		No. of rhizome						
IAA*2	KIN*3	Observed	Green	Do. %	White	Do. %	Withered	Do. %
0	0	112	21	18.8	67	59.8	24	21.4
0.1	0	115	22	19.1	61	53.0	32	27.8
0	0.1	106	7	6.6	70	66.0	29	27.4
0.1	0.1	115	21	18.3	67	58.3	27	23.5

*1, Basal medium: Seeding medium No. 4 excluded IAA and kinetin *2, 3-Indoleacetic acid *3, kinetin

As shown in Table 3, the seeding medium 4 excluded IAA and kinetin was used as the basal one for the transplanting, and 0.1 mg/l IAA and 0.1 mg/l kinetin were added to the basal medium individually or in combinations. A platinum needle sterilized by being heated with an alcohol lamp fire were used for the removal of the rhizomes. Four rhizomes per Erlenmeyer flask were transplanted. The transplanted rhizomes were cultured under the light and temperature conditions of a laboratory room.

The developmental growths of the rhizomes on the transplanting media about 150 days after the transplanting (January 20 in 1975) are shown in Table 3. As shown in Table 3, the percentage of green rhizome was somewhat lower in the medium in which 0.1 mg/l kinetin was added to the basal medium, compared with the other media, and that of the white rhizomes in the same medium was slightly higher. Whereas, the percentages of withered rhizomes on the four media ranged from 21.4 to 27.8, and those in the two media added with IAA and kinetin individually were somewhat larger.

At this time the mean lengths of rhizomes in respective four transplanting media were about 3 mm, ranged from 1 mm to 7 mm, showing no large difference among the demia. And also, no differentiation of shoot and root from the rhizomes was observed in any four media until this time.

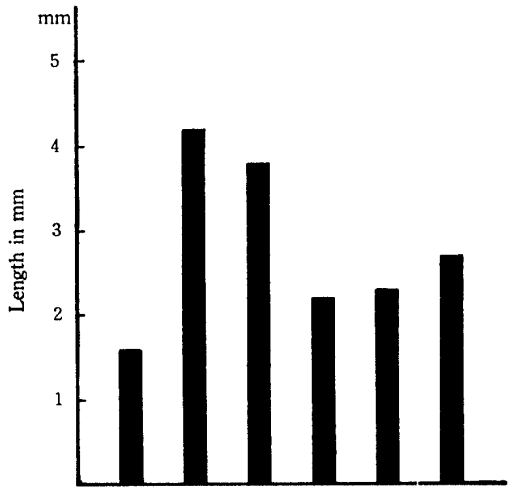
Many hair formations in the basal portion of several growing rhizomes cultured in the medium in which 0.1 mg/l kinetin was added to the basal medium were observed. Generally, the individual addition of kinetin to the basal medium seems to inhibit chlorophyll formation of the rhizomes.

3. Effects of several organic nutrients added into media on the developmental growth of the rhizomes

In order to clearify the effects of several organic nutrients added to the basal medium on the developmental growth of the rhizomes, 2 mg/l NAA, 0.2 mg/l kinetin, 2 g/l yeast extracts and 2 g/l Bact trypton were added to basal medium individually or in combinations. The basal medium used in this experiment had the same composition as the seeding medium 4, excepting that no amounts of IAA and kinetin were contained.

The materials used for this experiment were obtained from the rhizomes cultered on the four

media in the previous experiments. The elongated rhizomes were cut into about 5 mm in length by the sterilized knife. In order to avoid the effects of the previous media, these cut rhizome pieces derived from the same Erlenmeyer flask were transplanted on to different kinds of the media. Four pieces of the rhizomes per flask were transplanted. They were cultured under the light and temperature conditions of the laboratory room. All the piecies of the rhizomes transplanted were sketched under a stereoscope at the beginning of this experiment in order to make easy the discrimination of the newly elongated portion of the rhizomes from the old and original one. The data are shown in the average value of 10 rhizomes. The most vigorous rhizome in a flask was selected for the measurement. The elongated lengths in mm of the rhizomes cultured on the different nutrient media 60 days after transplanting are shown in Fig. 1.



NAA (mg/l)	0	2.0	2.0	2.0	2.0	2.0
Kinetin (mg/l)	0	0	0.2	0	0.2	0.2
Yeast extract (g/l)	0	0	0	2.0	2.0	2.0
Bact trypton (g/l)	0	0	0	0	0	2.0

Fig. 1. Growth of rhizomes in length on different media 60 days after the transplanting (1976).

Note, Basal medium: Seeding medium No. 4 excluded IAA and kinetin.

The growth of the rhizomes in the basal medium was the smallest of those in the six media. About 50 percentages of the rhizomes in the basal medium showed no changes of the growth during the period of this observation.

Generally, the growing portion of the rhizomes showed yellowish green colour or light yellowish green colour, and the basal (originally transplanted) portion of the rhizomes showed light brown colour. But, in this basal medium only 20 percentages of the basal portions of the rhizomes showed light brown colour. It was a lower percentage in comparison with those in the other media. Moreover, conspicuous development of lot of harirs on the growing portions of a few rhizomes cultured on this medium was observed.

The growth of the rhizomes cultured on the medium in which 2 mg/l NAA was added to the basal medium, was the largest of those in the six kinds of media used. The growing portion of the rhizomes showed yellowish white colour or light yellowish white colour or light yellowish green colour, showing no yellowish green colour. About 80 percentages of the growing portions of the rhizomes produced many brown spots on the portion a little apart from the tips. Many basal

(originally transplanted) portions of the rhizomes cultured on this medium showed brown colour, and the percentage of them was the highest of those cultured on the six kinds of media. No remarkable development of many hairs on the growing portion of the rhizomes cultured on this medium was observed at all.

The rhizomes cultured on the medium in which 2 mg/l NAA and 0.2 mg/l kinetin were added to the basal medium, showed secondary growth among those on the six media. Many growing portion of the rhizomes on this medium showed yellowish white colour or light yellowish green colour, and a few of them showed yellowish green colour. Development of brown spots on the portion a little apart from the growing rhizome-tips cultured on this medium was in the same degree as in that on the previous media added only with 2 mg/l NAA to the basal medium. The percentage of the changing to brown colour of the basal (originally transplanted) portion of the rhizome on this medium was lower than that on the previous medium in which only 2 mg/l NAA was added to the basal medium. A few growing portions of the rhizomes in this medium showed remarkable hair development.

Both growths of the rhizomes on the medium in which 2 mg/l NAA and 2 g/l yeast extract were added to the basal medium, and on the medium in which 2 mg/l NAA, 0.2 mg/l kinetin and 2 g/l yeast extract were added to the basal medium, were almost in the same degrees and were better than that on the basal medium, but they were the lowerst of those in the additional nutrient medium.

The growth of the rhizomes cultured on the medium in which 2 mg/l NAA, 0.2 mg/l kinetin, 2 g/l yeast extract and 2 g/l Bact trypton were added to the basal medium, was better than those on the previous media. The growing portion of the rhizomes on this media showed yellowish white colour or light yellowish green colour. The development of brown spot on the basal portion a little apart from the growing rhizome-tips was somewhat in low degree.

According to these results, the effects of 2 mg/l NAA added to the basal medium on the growth of the rhizome of *C. kanran* seems to be remarkable and those of other additional nutrients dose not seem to be so clear.

The rhizomes transplanted on to these six different nutrient media (October in 1976) were continued moreover to be cultured under the light and temperature conditions in the laboratory room after this observation. They continued to grow gradually almost in the same growth-rates in the respective media as in those shown in Fig. 1.

Table 4. Shoot and root formation of rhizome in different media (June, 1978)

Basal medium	Organic additionals (mg/l)				No. of rhizome				
	NAA*2	KIN*3	YE*4	BT*5	observed	withered	Do. %	formed shoot and root	Do. %
Seeding	0	0	0	0	103	4	3.9	0	0
medium	2	0	0	0	91	25	27.5	1	1.0
No. 4	2	0.2	0	0	138	40	29.0	4	2.9
(RM-1962)*1	2	0	2	0	91	22	24.2	3	3.3
	2	0.2	2	0	136	29	21.3	4	2.9
	2	0.2	2	2	65	16	24.6	3	4.6
Total					624	136	21.8	15	2.4

*1, Provided that 3-Indolacetic acid and kinetin were excluded *2, α-Naphtaleneacetic acid
*3, kinetin *4, Yeast extract *5, Bact trypton

About 10 months after transplanting on these media (August in 1977), the differentiations of shoots from the rhizomes cultured on these media except for the basal one, were observed. The number of the rhizomes which differentiated the shoot, increased gradually thereafter. After the shoot development, the rhizomes soon differentiated the root on the basal portion of the shoot. The percentages of the shoots and roots formation from the rhizomes cultured on different media 20 months after transplanting (June 2, 1978) are shown in Table 4. Until this time no shoot and root formation from the rhizomes cultured on basal medium was observed. The numbers of the rhizomes which formed the shoots and roots did not show so large a difference among the additional nutrients media.

According to the observations after this time, the shoots and roots formation from the rhizomes were observed even in the basal medium.

Basing on the results shown in Table 4, the addition of organic nutrients to the basal medium seems to be effective to the shoots and roots differentiation as well as to the acceleration of the growth of the rhizomes as mentioned above.

4. Shoot differentiation from the rhizomes on high kinetin content media

The effects of NAA and kinetin added to basal medium on the developmental growth of the rhizomes individually or in combinations, especially those of high kinetin concentration on the shoot differentiation were examined. The constitution of the basal medium was the same one as that in the seeding medium 4 (RM-1962) except that no amounts of IAA and kinetin were contained.

The amounts of NAA and kinetin added to the basal medium are shown in Fig. 2, respectively. Namely, the concentrations of NAA were 0, 0.2 and 2.0 mg/l, and those of kinetin 0, 0.2, 2.0 and 10 mg/l, respectively.

The growing rhizomes which had been cultured on the previous experiments (transplanted October in 1976) were cut into about 5 mm pieces sterilizedly, and transplanted on to twelve kinds

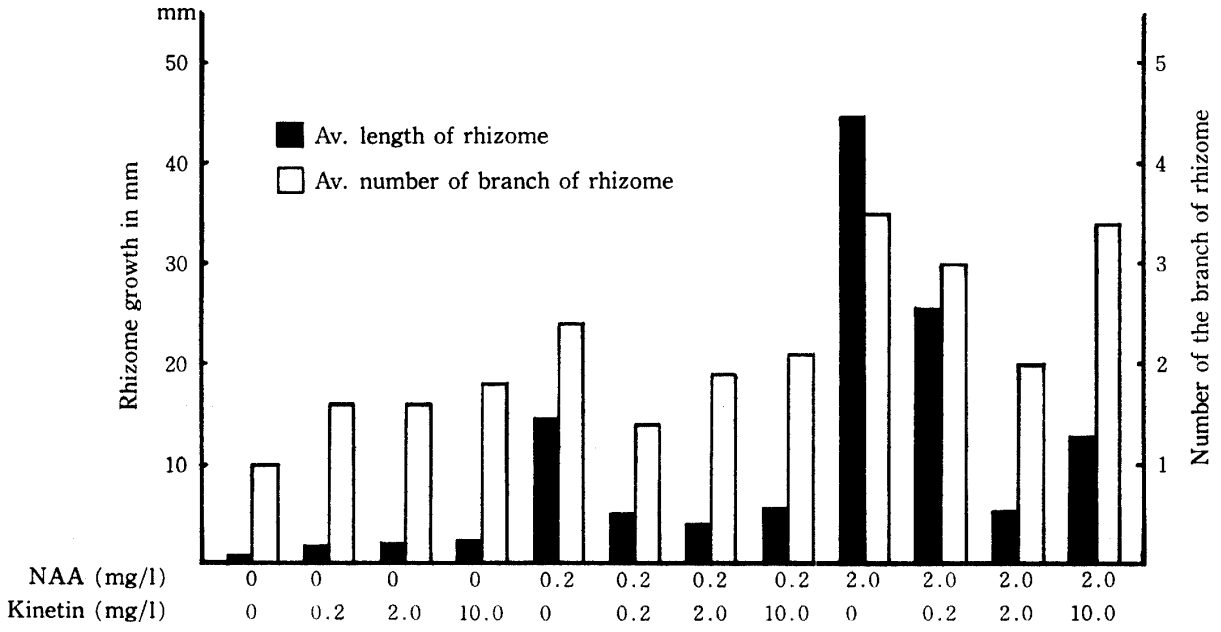


Fig. 2. Effects of NAA and kinetin added to basal media on the growth of the rhizomes of *Cymbidium kanran* 12 weeks after planting (1978).

Note, Basal medium: Seeding medium No. 4 excluded IAA and kinetin.

of the media with the different concentrations of NAA and kinetin individually or in combinations (Fig. 2). The rhizome pieces obtained from the flask were transplanted on to different kinds of media, in order to put out the effects of the previous media. Four cutting rhizomes per flask were transplanted at the same distance from each other. The transplanted rhizomes were cultured in an incubator controlled at 22°C under the light condition in a laboratory room. The data obtained are shown in average values of ten rhizomes. The most vigourous rhizomes in a flask was selected for the measurements. The effects of NAA and kinetin added to basal medium on the growth of the rhizomes 12 weeks after transplanting are shown in Fig. 2. As shown in Fig. 2, the rhizome growth increased with NAA concentration, but the effects of NAA on the rhizome growth was remarkably counteracted due to high concentation of kinetin. The number of the branch of the rhizome also increased with NAA concentration, and the effect of NAA on the number of the branch of the rhizome was not so much counteracted due to high concentration of kinetin as in the case of the rhizome growth.

It was a result of much interest that about 50 percentages of newly growing rhizome-tips cultured on the media in which 0.2 mg/l NAA, 0.2 mg/l NAA and 0.2 mg/l kinetin, and 0.2 mg/l NAA and 2.0 mg/l kinetin were added to the basal medium, grew in the media.

Moreover, all the tips of growing rhizomes cultured on the media containing 10 mg/l kinetin, and 0.2 mg/l NAA and 10 mg/l kinetin showed initial appearance of shoot formation (Fig. G) and also about 90 percentages of the growing rhizome-tips cultured on the medium containing 2.0 mg/l NAA and 10 mg/l kinetin showed the initial appearance of shoot formation. This initial appearance of shoot formation was clearly discriminated from the appearance of the rhizome-tips continuing only the growth of themselves (Fig. F). According to the later observations after this time, the rhizome-tips which showed initial appearance of shoot formation, have not developed to date into the whole plants consisting of shoots and roots under the same cultivating conditions. The growth rates of these rhizomes cultivated on high kinetin concentration media are extremely slow.

Discussion

1. Germination

Generally, it has been recognized that Oriental *Cymbidiums* are usually difficult to germinate on the culture media artificially prepared[4,5].

Kako[3] reported that the seeds of *C. virescens* contained the substances inhibitory to germination which consist of phenol compounds and the inhibitory substances were removed by the treatments in which the seeds were immersed in 1.0 M KOH water solution for 3 minutes or in water solution saturated with KOH for 10 to 15 minutes.

Though the same treatments as reported by Kako[3] were practiced for the seeds of *C. kanran* in order to induce their germination, no effects of the treatments on the germination were observed.

Sawa et al.[12] reported that the germination rate of immature seeds of *C. kanran* (about 6 months after pollination) collected from the plants cultured in a green-house, was 40 percentages when no sterilizing treatments were practiced to the seeds, and the germination of the immature seeds were obstructed remarkably with KOH treatment.

In this experiment the sterilizing treatment for the seeds of *C. kanran* was practiced with the method in which the seeds were immersed in 3% H_2O_2 water solution for 30 minutes, with the expectation of the harmless of this solution to the seeds. With this treatment the seeds of *C. kanran*

were perfectly sterilized.

Generally, it was pointed out that the seeds need high oxygen tension for their germination[13]. Mukade[7,8] reported that the germination rates of immature seeds of wheat increased up to 90% with the treatment in which the seeds were immersed in 1% H_2O_2 water solution at 25°C for 17 hours, after then in the same solution at 11°C for 30 hours, though the germination rates of the seeds were 9% when the seeds were immersed only in water. Ikeda[1] reported that dormancy of rice seed is caused by the restriction of the entry of oxygen of hull, pericarp and testa, and their restriction rate is variable according to the age of seed and variety.

In this experiment it was not until about 90 days after seeding that the seeds of *C. kantan* began to germinate, and the germination percentages were low. So, it may be difficult to decide that this sterilizing treatment due to H_2O_2 solution was also effective to induce the germination of the ripe seeds of *C. kanran*. It is necessary to examine in detail whether H_2O_2 solution rearlly increases the germination rate of *C. kanran* seed.

2. Effects of organic nutrients on the growth of the rhizomes

The growth of the rhizomes was promoted by the addition of 2 mg/l NAA to the basal medium. The addition of kinetin to the basal medium was not so effective to the geowth of the rhizomes and rather counteractive to the effects of NAA on the growth of the rhizomes. But, the media containing high concentration kinetin, were effective to shoot formation of the rhizomes. Namely, almost all tips of growing rhizomes cultured on the media in which 10 mg/l kinetin was added to the basal medium, showed the initial appearance of shoot formation, although these tips could not develop into the shoot when they were continued to be cultured on the same media.

Ueda and Torikata[15] reported that rhizome-tips of *C. goeringii* cultured on the basal medium (KNUDSON C+NITCH micro-elements)[6,10] always developed into rhizomes without shoot formation, but addition of 10 mg/l kinetin led in most of the samples, to shoot formation.

In this experiment the whole plants consisting of shoot and root were produced from the rhizomes cultured on the media in which 2 mg/l NAA was added to the basal medium. Basing on the results obtained in these experiments, in order to produce whole plant consisting of shoot and root from the rhizomes of *C. kanran* it may be effective firstly to promote the growth of the rhizomes due to the addition of 0.2–2 mg/l NAA and 0.1–0.2 mg/l kinetin to the basal medium. Moreover, it may also be a useful method for the production of the whole plant consisting of shoot and root, that the rhizomes showing shoot premordia tips under the condition of the high kinetin concentration medium are to be transplanted onto low kinetin medium.

Summary

Seeds of *Cymbidium kanran* Makino were sowed aseptically on an artifitial medium. Effects of growth substances added to the basal medium on the growth of the rhizomes which developed from the seeds were studied. The resuts obtained were summarized as in the following.

1. It was not until about 90 days after seeding that the seeds began to germinate. The germination rates of the seeds were low, average values 3–4 percentages.

2. The growth of the rhizomes was promoted due to the addition of 2 mg/l NAA to the basal medium (RM–1962 excluded IAA and kinetin). The addition of kinetin to the basal medium was not so effective to the rhizome growth, but rather counteractive to the effects of NAA on the rhizome growth. But almost all rhizome-tips cultured on the media in which 10 mg/l kinetin was added to the basal medium, showed the initial appearance of the shoot formation, although these tips could

not develop into the shoot when they were continued to be cultured on the same media.

3. Whole plants consisting of shoot and root were produced from the rhizomes derived from the seeds when they were cultured on the media in which 2 mg/l NAA and 0–0.2 mg/l kinetin were added to the basal medium.

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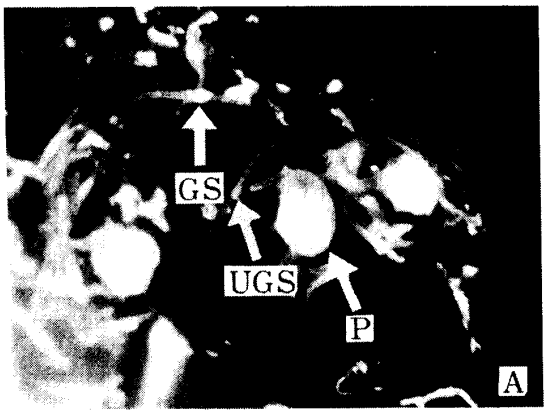
Explanation of plates

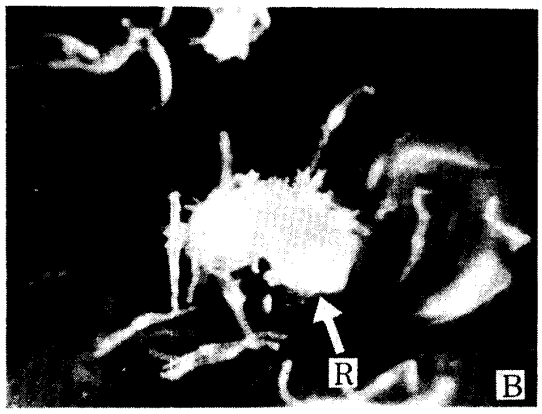
Plate 1

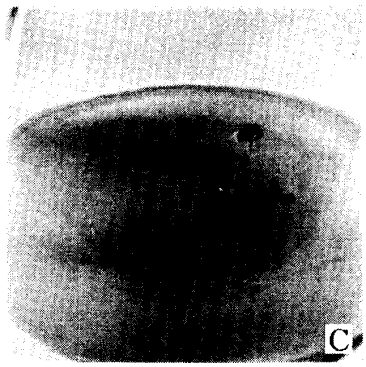
Fig. A. Ungerminated seed (UGS), germinating seed (GS) and their development to protocorm (P).
Fig. B. Rhizome (R) development from protocorm.
Fig. C, D, E. Growth process of rhizome on transplanting medium.

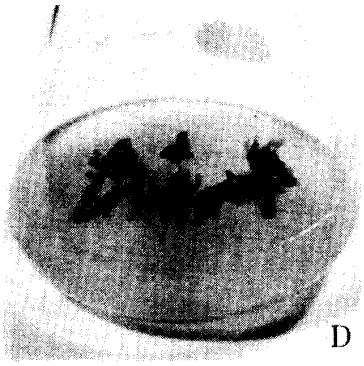
Plate 2

Fig. F. Rhizome-tip growing on low kinetin medium.
Fig. G. Shoot primordia development from rhizome on high kinetin medium.
Fig. H. Shoot (St) and root (Rt) development from rhizome.
Fig. I. Whole plant consisting of shoot and root.









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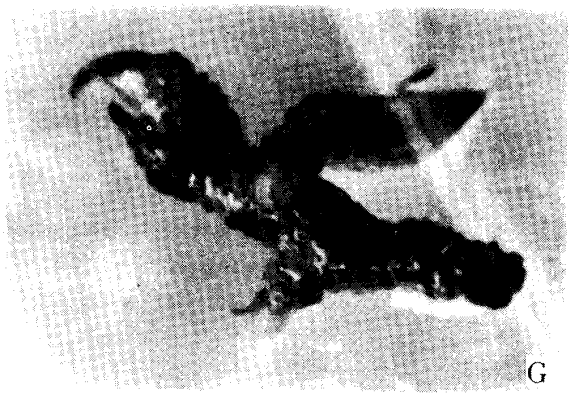

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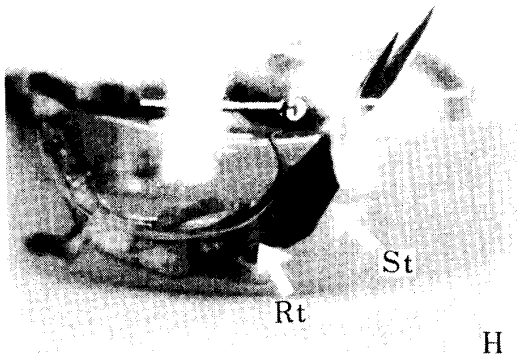

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