

茎頂培養によるシュンラン及びカンランのライゾーム形成

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Rhizome Formation in *Cymbidium goeringii* Reichenbach fil. and *Cymbidium kanran* Makino in Shoot-Tip Culture¹

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Summary

Cymbidium goeringii Reichenbach fil. and *Cymbidium kanran* Makino are native Cymbidia of Japan and are important Oriental terrestrial orchids. When their shoot-tips are cultured, rhizomes are formed. Factors affecting rhizome formation were discussed in this paper.

1. Culturing of shoot-tips of *Cymbidium goeringii* at intervals of 2 months showed that rhizomes could be formed at any time throughout the year. However, rhizome formation depended on the starting time of cultures and the culture media. Maximum rhizome formation occurred in April when cultured in either MS or RM media both containing 1 ppm NAA and 0.1 ppm kinetin, and in June by culturing with modified *Cymbidium*-culture medium (Hyponex medium) devoid of plant growth regulators.

2. Culturing the shoot-tips of *Cymbidium goeringii* in darkness at 25°C hastened rhizome formation, and increased rhizome formation to almost twice the level obtained under conventional culture conditions with artificial illumination for 16 hours.

3. RM medium containing 1/5 concentration of inorganic components and 10% coconut water was found to be suitable for shoot-tip culture of *Cymbidium kanran*.

4. When shoot-tips of *Cymbidium kanran* were cultured in agitated liquid medium, all explants died.

5. The use of plant growth regulators was found to have no effect in cultures of *Cymbidium goeringii* and *Cymbidium kanran*.

Introduction

Oriental terrestrial Cymbidia have been selected from spontaneous mutant lineages by cymbidium lovers in East Asian countries such as China, Japan, Korea and Formosa. Until recently they have been multiplied by division. Artificial multiplication of specific cultivars of Oriental Cymbidia by cross-breeding has not been established as it has with European Cymbidia (tropical origin, epiphytic). Despite recent attempts to germinate fertilized seeds(4, 13, 14, 18), the amount of bred strains cannot meet the large demand for this type of cymbidium. Unless the problems of multiplication are overcome, production and consumption of the Oriental or-

chid may remain undeveloped.

Multiplication by aseptic germination or tissue culture is difficult for many strains of Oriental Cymbidia (7, 20) Ueda (21) noted that multiplication of Oriental Cymbidia is limited, due to complicated interactions of intractable factors such as (1) low germinability, (2) the specificity of the developmental processes (differentiation from a rhizome into buds and roots) and (3) the complexity of nutrition (closely associated with mycorrhiza in the natural environment).

Previous studies of shoot-tip culture of Japanese Oriental Cymbidia had indicated that rhizome formation in *Cymbidium goeringii* was poor and that in *Cymbidium kanran* it was totally unsuccessful(20, 21).

In this study an attempt was made to in-

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duce rhizome formation in *Cymbidium goeringii* and *Cymbidium kanran* by shoot-tip culture. This hopefully would be a basis for their subsequent multiplication. This study also evaluated the relationship between rhizome formation and various culture conditions including composition of the culture media, time of explanting, the light conditions during culture and the use of liquid medium.

Materials and Methods

Experiment 1: Seasonal effect of culture time on rhizome formation in Cymbidium goeringii

Lead bulbs of *Cymbidium goeringii* were collected from a copse in Miki-cho, Kagawa Prefecture at intervals of 2 months from June, 1971 to October, 1972. The outer leaves were removed leaving only a pseudobulb wrapped in its inner leaf sheaths. The pseudobulb was washed in tap water, immersed in 70% ethanol for 30 to 60 seconds and then surface-sterilized for 10 minutes in 7% calcium hypochlorite solution (supernatant). After washing 3 times in sterile distilled water, the most developed axillary buds (usually the axillary buds on the first three nodes from the base) were excised to a length of 1-2 mm under a stereomicroscope. These were then planted on the culture medium, about 30-40 explants for each kind of medium.

Murashige and Skoog (MS) medium(11), and Linsmaier and Skoog (RM) medium(10) were used, both containing 1 ppm NAA and 0.1 ppm kinetin. A cymbidium-culture medium commonly known as Hyponex medium (H medium) was also used(19), modified to contain 3 g of Hyponex (N:P:K=6.5:6:19), 1 g of Bacto-tryptone, 100 mg of inositol and 0.4 mg of thiamine·HCl. Sucrose and agar were used in all media at 30 g/l and 10 g/l, respectively. The pH prior to autoclaving was adjusted to 5.7-5.8 for both MS and RM media, and to 5.0-5.1 for H medium. After pH adjustment, 8 ml of the media was poured into each 12.5×120 mm test tube and autoclaved at 1.1 kg/cm² for 15 minutes. Cultures were maintained at 25°C under 16

hours light of approximately 500 lx from plant growth fluorescent lamps (Biolux, NEC).

Experiment 2: Effect of darkness on rhizome formation

Lead bulbs of *Cymbidium goeringii* which were grown in a 50%-shaded and unheated house, were harvested on June 1, 1983. Well developed axillary buds were excised to a size of 1-2 mm from the pseudobulb in the same way as in Experiment 1 and were planted on RM medium. Eight ml of the medium was again used in each 18×150 mm test tube. Cultures were maintained at 25°C either under 16 hours illumination (light culture) or in the dark (dark culture). Twenty-five explants were used for each of the light and dark cultures.

Experiment 3: Multiplication of rhizomes from Cymbidium kanran seedlings grown in vitro

Shoots grown from rhizomes of seed-origin, which had been successively cultured since 1968, were collected on July 5, 1976. One or two axillary buds on fairly large sized shoots were cut into 1-2 mm lengths and planted on medium as shown in Table 3. Cultures were maintained at 25°C under a 16 h photoperiod.

Experiment 4: Rhizome formation from mature plants of Cymbidium kanran

Lead bulbs were collected from mature plants of *Cymbidium kanran* on June 10-13, 1983. After the same treatment as in Experiment 1, all buds found on the pseudobulb were excised to a length of 1-1.8 mm. They were planted on RM medium with 1/2, 1/5 or 1/10 concentration of inorganic components, with or without 10% (v/v) coconut water (CW). Each 18×150 mm test tube contained 8 ml of the medium. Inositol and thiamine·HCl were excluded from the media containing CW. About 20-35 explants were used for each medium, and cultures were maintained at 25°C in the dark.

Experiment 5: Effect of agitated liquid culture and plant growth regulators on rhizome formation

Lead bulbs were collected from mature plants of *Cymbidium kanran* on August 16-

17, 1984. The shoot-tips were explanted by the same procedure as in Experiment 4. Based on the results obtained in Experiment 4, RM medium containing 1/5 concentration inorganic components plus 10% CW was used. In addition, various combinations of NAA and kinetin were tested. In the experiment to determine the effect of agitated liquid culture, medium containing 1 ppm NAA and 0.1 ppm kinetin was used. The liquid medium was shaken continuously for 2 and 4 weeks on a MONOD SHAKER MO-5 set at 30 strokes/min. The cultures were maintained at 25°C in the dark.

Results

Experiment 1: Seasonal effect of culture time on rhizome formation in Cymbidium goeringii

At any time throughout the year rhizomes developed from the explanted shoot-tips (Fig. 1). Formation of rhizomes occurred about 2-3 months after the start of culture, but occasionally it could take as long as 4 months. Rhizome formation seemed to depend on the time of explanting of the shoot-tip. This indicates the possible influence of internal factors in the lead bulb or shoot-tip. Rhizome formation was also affected by the composition of medium used. Although the level of rhizome formation appeared to be approximately equal in either MS or RM media, in which the nutrient composition was almost identical, there was an entirely different pattern in H medium, the composition of which was different.

The highest level of rhizome formation in MS and RM media occurred in April. This is when the axillary bud of the lead bulb is just about to start elongating to form a new shoot. In June, when the axillary bud is not yet fully developed as a new shoot, the level of rhizome formation was low. Thereafter it began to increase as the new shoot developed.

In H medium, rhizome formation was highest when tissue was excised in June, but in other months the level was below 50%.

In cultures begun on April 24 and June 21, 1972, the rhizomes grown in H medium

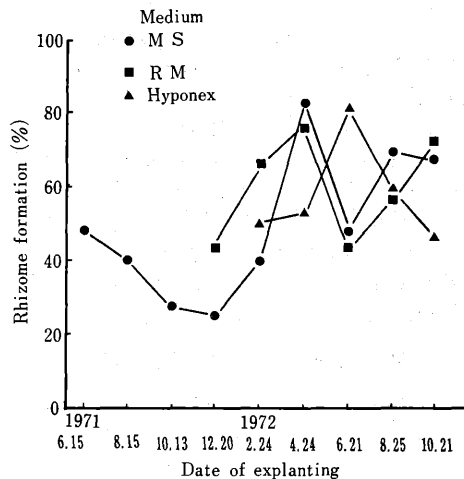


Fig. 1. Seasonal changes of rhizome formation in shoot-tip culture of *Cymbidium goeringii*. Data were recorded 180 days after the culture.

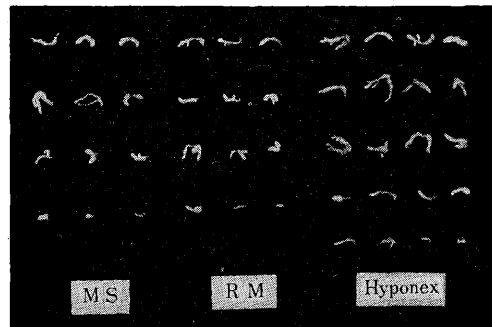


Fig. 2. Rhizome development of *Cymbidium goeringii* in shoot-tip culture. Shoot-tip culture was initiated on June 21, 1972 and the photograph was taken on March 5, 1973; after 258 days of culture *in vitro*.

were slightly larger than those grown in MS and RM media (Table 1, Fig. 2). However, with time, the rhizomes in H medium began to turn brown and die.

Experiment 2: Effect of darkness on rhizome formation

Table 2 and Figure 3 show the results in the 4th month after explanting. When cultured in the dark, rhizome formation was observed in some of the explants 30 to 45 days after explanting. Rhizome formation was much better when cultured in the dark. The rhizomes obtained in the dark were longer, thicker and 9.4 times heavier than those cultured under light. The rhizomes

Table 1. Effect of medium on the rhizome formation in shoot-tip culture of *Cymbidium goeringii*.

Date of explanting	Medium	Number of rhizomes per explant	Length of rhizome (mm)	Diameter of rhizome (mm)	Weight of rhizome (mg)
April 26, 1972 ^z	M S	1.7	12.8	2.8	48.6
	R M	1.1	12.9	2.7	45.8
	Hyponex	1.4	13.0	3.5	56.4
June 21, 1972 ^y	M S	1.4	11.1	2.3	42.2
	R M	1.6	11.5	2.6	37.9
	Hyponex	1.4	14.3	2.9	69.0

^z Data were recorded 245 days after the explanting of shoot-tip.

^y Data were recorded 258 days after the explanting of shoot-tip.

Table 2. Effect of darkness during shoot-tip culture of *Cymbidium goeringii* on the rhizome formation.

Light condition	No. of explants (a)	No. of explants contaminated	No. of explants survived	No. of rhizome-formed explants (b)	Percentage $\frac{(b)}{(a)}$	Rhizome			
						Number	Length (mm)	Diameter (mm)	Weight (mg)
Light	25	3	8	5	20	1	1.8	1.2	7.7
Dark	25	1	17	14	56	1.3	7.2	3	66.2

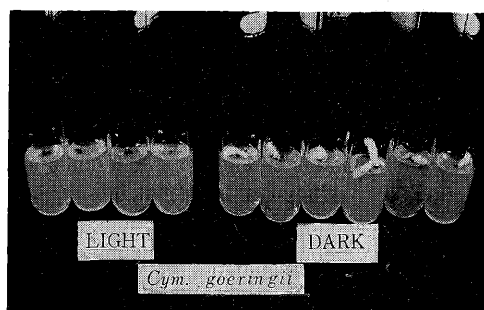


Fig. 3. Rhizomes of *Cymbidium goeringii* formed in shoot-tip culture under the light and dark conditions. Shoot-tip culture was initiated on June 1, 1983 and the photograph was taken on October 7, 1983; after 128 days of culture *in vitro*.

grown in the dark were heavier than those grown in RM medium in the light (Table 2), even though they were cultured for only about half as long.

Experiment 3: Multiplication of rhizomes from Cymbidium kanran seedlings grown in vitro

Table 3 shows the results in the 5th month after the start of cultures. Shoot-tip cultures of the axillary buds from seedlings also yielded rhizomes, although rhizome formation was poor. The medium with 1/10 concentration of inorganic components gave the highest survival rate of explants and the

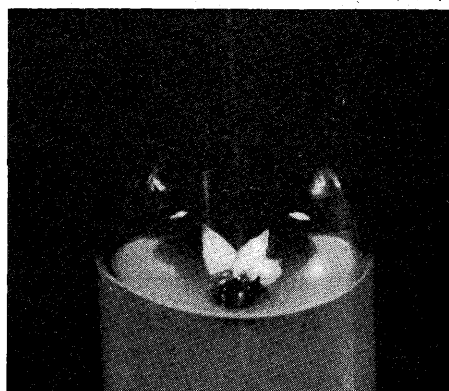


Fig. 4. A shoot-tip obtained from plantlet of *Cymbidium kanran* grown *in vitro* formed three protuberances on G medium, containing 1 ppm NAA, 5 ppm BA and 10 ppm adenine. The photograph was taken 5 months after culture.

highest level of rhizome formation, although the rhizomes were small in size.

The survival rate of explants decreased with increasing BA concentration. Adenine had no effect on the rhizome formation. All the explants turned brown and died in Lot H that contained the highest concentration of plant growth regulators (1 ppm NAA, 10 ppm BA and 10 ppm adenine). In Lots F, G and I that contained adenine, some of the explants hypertrophied and formed a few

Table 3. Effects of concentration of medium and plant growth regulators on the rhizome formation in shoot-tip culture of seedlings of *Cymbidium kanran* grown *in vitro*.

Lot	Medium	NAA (ppm)	BA (ppm)	Adenine (ppm)	Kinetin (ppm)	No. of explants	No. of explants contaminated	No. of explants survived	No. of rhizome-formed explants
A	R M	1	1	—	—	10	0	1	1
B	1/2RM	1	1	—	—	10	1	4	0
C	1/5RM	1	1	—	—	10	1	4	2
D	1/10RM	1	1	—	—	10	0	9	4
E	R M	1	—	—	1	10	0	3	3
F	R M	1	1	10	—	10	0	5	(2)*
G	R M	1	5	10	—	10	0	3	(1)*
H	R M	1	10	10	—	10	0	0	0
I	R M	1	1	5	—	10	1	2	(2)*
J	R M	1	1	1	—	10	1	1	1

(*) : Explant enlarged but did not form rhizome.

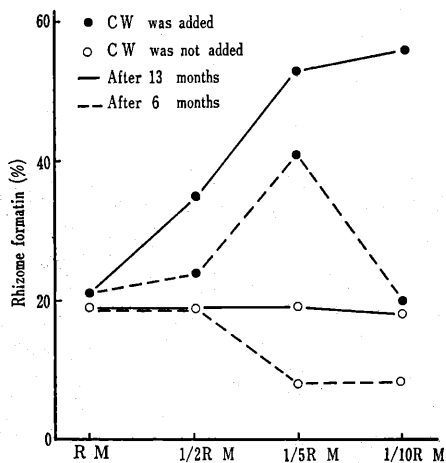


Fig. 5. Effects of concentration of medium and coconut water (CW) on the rhizome formation in shoot-tip culture of the mature plant of *Cymbidium kanran*.

protuberances (Fig. 4), but when the explants from Lot F and I were transplanted into medium containing no plant growth regulators, rhizomes were formed.

Experiment 4: Rhizome formation from mature plants of *Cymbidium kanran*

Rhizomes were formed 70 to 90 days after explanting. Figures 5 and 6 show the results in the 6th and 13th month after the start of culture. In the 6th month, rhizome formation was almost the same in RM and 1/2 RM media regardless of the presence of coconut water (CW). In 1/5 RM medium, though, rhizome formation increased remarkably when CW was present, but in 1/10

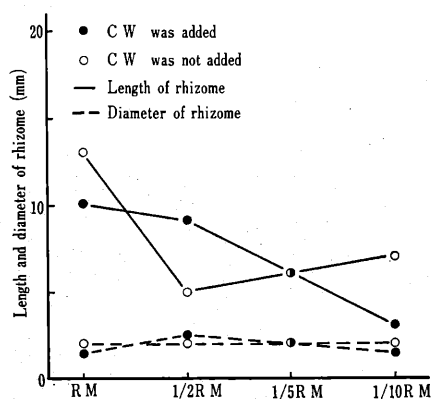


Fig. 6. Effects of concentration of medium and coconut water (CW) on the development of rhizome of *Cymbidium kanran* cultured for 13 months.

RM medium, CW had only a slight effect on rhizome formation.

In the 13th month, rhizome formation was almost the same at all concentrations of RM medium without CW. In the presence of CW, however, rhizome formation increased with a decrease in the content of inorganic components, indicating the effectiveness of CW on rhizome formation.

The concentration of inorganic components or CW in the medium had no effect on the diameter of rhizomes (Fig. 6). Rhizome length tended to be shorter in the CW-containing media with lower concentrations of inorganic components but there was no consistent trend in the media without CW.

These results indicated that, in terms of rhizome formation and size, the medium

Table 4. Effects of plant growth regulators and agitated liquid culture on the rhizome formation in shoot-tip culture of the mature plant of *Cymbidium kanran*.

Lot	NAA (ppm)	Kinetin (ppm)	Medium condition	No. of explants (a)	No. of explants contaminated	No. of rhizome-formed explants (b)	Percentage $\frac{(b)}{(a)}$
A	1	0.1	Agar	23	1	5	21.7
B	1	0.1	Liquid 2 weeks	23	1	(1)*	(4.6)*
C	1	0.1	Liquid 4 weeks	23	1	0	0
D	0	0	Agar	22	4	6	23.4
E	0.1	0.1	Agar	23	0	3	13.0
F	0.1	1	Agar	23	0	2	8.7
G	1	1	Agar	23	2	4	17.4

(*)*: This rhizome turned brown and died during the course of culture.

with 1/5 concentration of inorganic components and 10% (v/v) CW is best for shoot-tip culture of *Cymbidium kanran*.

Experiment 5: Effect of agitated liquid culture and plant growth regulators on rhizome formation

Table 4 shows the results at the 6th month of culture. In Lot A, using the medium which yielded the most rhizomes in Experiment 4, the level of rhizome formation was 21.7%. In Lot B, however, which was in agitated-culture for 2 weeks, only 1 explant formed a rhizome, and even this turned brown and died. Likewise in Lot C, which was in agitated culture for 4 weeks, no rhizomes were formed at all.

In agar media, no distinct effects of NAA or kinetin on rhizome formation were observed. In Lot D, where no plant growth regulators were added, rhizome formation was rather better.

Discussion

In shoot-tip culture of *Cymbidium goeringii*, it takes approximately 2 to 4 months for rhizomes to form(1). In Experiment 1 and 2 under artificial illumination, it took about 2 to 3 months for rhizome formation to occur, while in darkness it only took 30-45 days. Thus, culturing in darkness enhanced rhizome formation. Since under natural conditions a rhizome shows subterranean growth and is negatively phototropic, culture in the dark seems to be reasonable, and is recommended for shoot-tip culture as for seed germination(3).

Previous studies have shown that in shoot-

tip culture of *Cymbidium goeringii* the level of rhizome formation is less than 20%, but is increased to 60% by addition of coconut water to the medium(21). Aoye(1) reported that shoot-tip culture carried out in December resulted in a different level of rhizome formation (from 22 to 63%) depending on the media used. The experiments in this study also showed that the level of rhizome formation differed with the media used. Rhizomes were obtained even in H medium without plant growth regulators. In Experiment 5 of shoot-tip culture of *Cymbidium kanran*, a high level of rhizome formation was obtained in the medium without plant growth regulators. These results indicate that plant growth regulators are not necessary for rhizome formation. The rhizomes of *Cymbidium goeringii* grown in H medium finally turned brown and died. Auxin is known to accelerate the growth and multiplication of rhizomes (9,16). Therefore auxin may not be necessary for rhizome formation but it may be necessary for the survival and multiplication of rhizomes once formed.

The explanting time is another important factor in shoot-tip culture. A similar result was reported in the culture of *Hamamelis intermedia*(2). July is the best season for culture of this plant, when the content of browning substances that diffused from the shoot-tip into the medium is lowest. The relationship between the browning substances in the medium and the level of rhizome formation could not be defined in our study, but browning was less when cultured

in the dark than in the light. The rhizomes also grew and developed better in the dark culture. As pointed out in many orchid culture studies, a low content of browning substances is considered to be an important factor for multiplication of tissue.

Nitsch *et al.* (12) founded that in the culture of internodal tissue of *Plumbago indica* and pith tissue of *Nicotiana tabacum*, adenine as well as cytokinin was indispensable for the formation of adventitious buds. In the shoot-tip culture of *Cymbidium kanran* from the seedlings grown *in vitro*, adventitious bud-like protuberances were produced when adenine was added to the medium containing auxin and cytokinin. These protuberances failed to develop further in the same medium, but some of them developed into rhizomes after transplanting into a growth regulator-free medium.

Coconut water has been reported to be effective for protocorm multiplication of European Cymbidia (8). In shoot-tip culture of *Cymbidium kanran*, a higher level of rhizome formation was obtained in the CW (10%)-containing RM medium with 1/5 concentration of inorganic components. This result indicates that the successful shoot-tip culture of *Cymbidium kanran*, which so far has been a very difficult species to propagate, may be possible. Rhizome formation of *Cymbidium kanran* in shoot-tip culture differed between cultivars and strains. From past experience, the level of rhizome formation is relatively high in *Cymbidium kanran* of medium quality and calico strains but low in white-flowered strains.

Since Wimber (22) demonstrated the effect of agitated liquid culture on protocorm multiplication in shoot-tip culture, the effect of this method in many protocorm-forming orchids has been reported (5, 6, 15, 17). However, its effect has not yet been confirmed in rhizome-forming orchids. The present results showed no beneficial effect could be derived from shoot-tip culture of *Cymbidium kanran* in agitated liquid culture; in fact this method was found to be harmful, because all explants died after 2 or 4 weeks of shaking. The reason for this is unknown.

When rhizome tips (1~5 mm) of *Cymbidium faberi* were cultured in agitated liquid culture, Hasegawa *et al.* (4) found that most of the explants survived and continued to elongate in medium containing no BA, while in media containing 1 or 10 ppm BA, the terminal bud and axillary buds of explant formed a number of bracts to develop into hypertrophic organs with short plastochron. The shoot-tip from the pseudobulb and the growing point of the rhizome were found to respond differently in agitated liquid culture.

Acknowledgement

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茎頂培養によるシュンラン及びカンランのライゾーム形成

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摘 要

重要な東洋系（地生）シンビジウムとして知られ、日本に自生するシュンラン *Cymbidium goeringii* Reichenbach fil. 及びカンラン *Cymbidium kanran* Makino について、茎頂培養によるライゾーム形成に影響を及ぼす幾つかの条件について検討した。

1. 2か月間隔でシュンランの茎頂培養をした結果、ライゾームは周年形成されたが、ライゾーム形成率は培養開始時期と培地に左右され、NAA 1 ppm, Kinetin 0.1 ppm を含む MS 及び RM 培地では4月に、そして植物生長調節物質を含まないシンビジウム用修正 Hyponex 培地では6月にそれぞれ最高となった。

2. シュンランの茎頂を 25°C, 暗黒で培養すると、

従来の 16 時間人工照明下での培養に比べ、ライゾームが形成されるまでの所要日数が短縮され、ライゾーム形成率が高まり、ライゾームの生長量がほぼ2倍となった。

3. カンランの茎頂培養には、RM 培地の無機成分を5分の1に希釈し、ココナツウォーターを10%添加した培地が適した。

4. カンランの茎頂を液体振とう培養すると、すべての外植体が枯死した。

5. シュンラン及びカンランの茎頂培養において、ライゾームの形成に対して植物生長調節物質は不可欠でないことが明らかとなった。