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Micropropagation of a terrestrial *Cymbidium* species using rhizomes developed from seeds and pseudobulbs

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Abstract

Growth of *Cymbidium kanran* rhizome was enhanced by higher NAA:BAP ratios in modified Murashige & Skoog (MS) media. Only vegetative shoots resulted from rhizomes cultured in vitro when lower NAA:BAP ratios were used. The rhizomes were induced from the axils of leaves when shoots were explanted to medium containing higher concentrations of NAA. Root formation of *C. kanran* was inhibited by the addition of either auxin or cytokinin to the culture media. Differentiation of the rhizomes into plantlets occurred when the concentrations of ammonium nitrate and potassium nitrate in MS medium were reduced. The modified MS medium containing lesser amounts of potassium nitrate and ammonium nitrate than those of the original MS media, and was optimal for the production of plantlets from rhizomes of *C. kanran* without addition of auxin and cytokinin.

Abbreviations: NAA – α-naphthaleneacetic acid, BAP – N^6-benzylaminopurine, MS medium – Murashige & Skoog medium

Introduction

Cymbidium kanran Makino is a typical terrestrial *Cymbidium* species distributed in temperate Eastern Asia (South Western Japan, Tsushima, Ryukyus, Chejudo, Formosa and China) and has been known as a temperate species among *Cymbidium* which is difficult to germinate. Efficient micropropagation of this species by in vitro culture of seeds, and organs has been difficult to achieve. Usually, underground rhizomes of *C. kanran* result from in vitro cultures of seeds, tissues, and organs. Growth of rhizomes developing from seeds, rhizome tips and shoots has been observed under various cultural conditions. However, differentiation of the rhizomes into plantlets in vitro does not occur easily and cultural conditions for differentiation of the rhizomes into plantlets have not been optimised.

Several investigators have reported cytokinin induced shoot formation in rhizome cultures of *C. kanran* and closely related species [2, 3, 11–14, 24–26]. Usually, elongation and proliferation of rhizomes occurs under high auxin concentrations in culture medium. On the contrary, only protocorm-like shoots without root were induced from *C. kanran* rhizomes treated with high concentrations of cytokinin [3, 11, 12]. These plant growth regulators added in the media have little or no effect on efficient induction of roots from shoots emerging from rhizome cultures of *C. kanran*. Concentrations and ammonium:nitrate ratios in the culture media have been known to affect the germination of orchid seeds and subsequent organogenesis considerably [4–7, 15, 17–19, 27, 28]. However, few studies have examined the effects of concentrations and ammonium-nitrate ratios on the growth and development of the underground

rhizomes of temperate terrestrial *Cymbidium* in vitro.

In the present paper, we describe a highly efficient method for the induction of whole plantlets of *C. kanran* from rhizomes cultured in vitro using a modified MS medium [16] without addition of plant growth regulators.

Materials and methods

Plant materials

Rhizomes

Immature seeds (10 months after pollination) of *C. kanran* were cultured on modified Kano's medium [9] containing 3 g l^{-1} Hyponex (GRACE), 2 g l^{-1} bacto-peptone (DIFCO), 20 g l^{-1} sucrose and 8 g l^{-1} Phytagar (GIBCO), pH 5.3 at 25°C under total darkness for the first 10 months. The culture was then maintained under continuous illumination of 1000 lux from a fluorescent light (Toshiba Biolight) to accelerate the growth of rhizomes developing from protocorms. After the development of chlorophyll, the rhizomes were used as explants.

In addition, white rhizomes induced directly from the axillary buds of pseudobulbs by topical application of NAA [23] were used.

Shoots

Vegetative shoots of *C. kanran* formed on apical segments of proliferated rhizomes cultured on modified Murashige and Skoog medium with the addition of 1 mg l^{-1} BAP were used for explants.

Culture media

MS media containing various concentrations of ammonium nitrate and potassium nitrate (modified MS, Table 1) containing 20 g l^{-1} sucrose and 7 g l^{-1} Phytagar, were used. Several concentrations of NAA, BAP, ammonium and potassium nitrate were examined. The pH was adjusted to 5.5 with either 0.1 N NaOH or 0.5 N HCl prior to sterilization of the medium. 200 ml Erlenmeyer flasks were used as culture vessels, each flask receiving 50 ml of medium.

Surface sterilization

Rhizomes emerging from pseudobulbs were excised, soaked in Wilson's solution [29] containing 0.01% Tween 20 for 5 to 10 minutes and then washed twice in sterilized distilled water.

Procedure

Rhizomes from immature seed cultures were rinsed twice in sterilized distilled water to remove the old

Table 1. Amounts of nitrogen-containing salts in the modified MS media.

Media	Amounts (mg l^{-1})	
	NH_4NO_3	KNO_3
1	0	0
2	0	237.5
3	0	475.0
4	0	950.0
5	0	1900.0
6	0	3800.0
7	206.25	0
8	206.25	237.5
9	206.25	475.0
10	206.25	950.0
11	206.25	1900.0
12	206.25	3800.0
13	412.5	0
14	412.5	237.5
15	412.5	475.0
16	412.5	950.0
17	412.5	1900.0
18	412.5	3800.0
19	825.0	0
20	825.0	237.5
21	825.0	475.0
22	825.0	950.0
23	825.0	1900.0
24	825.0	3800.0
25	1650.0	0
26	1650.0	237.5
27	1650.0	475.0
28	1650.0	950.0
29 (MS medium)	1650.0	1900.0
30	1650.0	3800.0
31	3300.0	0
32	3300.0	237.5
33	3300.0	475.0
34	3300.0	950.0
35	3300.0	1900.0
36	3300.0	3800.0

media. Approximately 5 mm apical segments of the rhizomes were excised, and placed into culture media.

Rhizomes emerging directly from pseudobulbs treated with NAA [23] were excised approximately 3 mm from the apical segments. Before sterilization of the rhizome, the cut surfaces of excised rhizomes were sealed with quick-drying silicon rubber (Toshiba silicon) to protect the explants from damage by the sterilizer percolating into rhizomes tissues.

After surface sterilization in Wilson's solution, rhizomes were transferred to culture vessels. All rhizome explants were horizontally inserted 3 mm into the culture media.

Vegetative shoots appearing on the apical segments of rhizomes cultured in vitro were excised approximately 5 mm in length and were vertically inserted 3 mm into the culture media.

Explants were maintained at 25°C with 24-hr photoperiods under 1000 lux fluorescent light illumination. Cultivation periods varied from 4 weeks to 9 weeks.

Results

Effects of auxin and cytokinin on the growth of explanted rhizomes and shoots

Effects of NAA and BAP added to MS media on the growth of underground rhizomes and vegetative shoot formation in *C. kanran* are shown in Table 2.

Addition of BAP to the culture media at concentrations of less than 0.1 mg l^{-1} was ineffective in the induction of vegetative shoots on the underground rhizomes. On the other hand, higher BAP levels (> 0.1 mg l^{-1}) in the culture media strongly inhibited the proliferation of *C. kanran* rhizomes and was highly effective for the induction of protocorm-like shoots (Fig. 1) from the rhizomes. The proliferation of *C. kanran* rhizomes was enhanced when higher NAA levels (> 1 mg l^{-1}) in the medium were employed (Fig. 2).

Lower NAA:BAP ratios in the culture medium resulted in the formation of many protocorm-like shoots on the rhizomes but these protocorm-like shoots never developed to plantlets. Under higher NAA:BAP ratios, the formation of abnormally slender shoots occurred in the rhizome cultures.

Rhizome of *C. kanran* cultured in vitro were highly sensitive to NAA and BAP. Root formation in the rhizome cultures was strongly inhibited when a plant growth regulator, either NAA or BAP, was added to the media. The combination of higher concentrations (> 10 mg l^{-1} each) of these plant growth regulators in the medium consequently killed the rhizomes.

Rhizome cultures started from the excised tips of underground rhizomes emerging from axillary buds of pseudobulbs showed similar results (data not shown). However, the initiation of rhizome greening followed by a rapid growth of the rhizome was considerably delayed compared to those of seed origin.

Effects of NAA and BAP on the growth of explanted shoots

The effects of NAA and BAP added to MS media on the growth of shoot explants were studied (Table 3). Emergence of rhizomes from the axillary

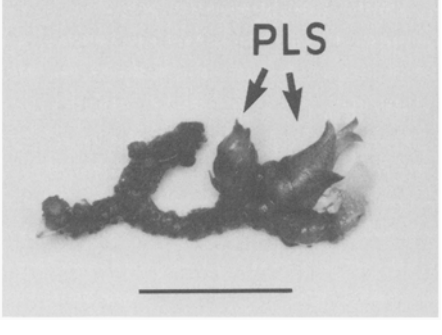

Fig. 1. Protocorm-like shoot differentiation from rhizome cultured in MS medium supplemented with BAP 1 mg l^{-1}. Explanation of symbol: PLS, protocorm-like shoot. Bar = 10 mm.

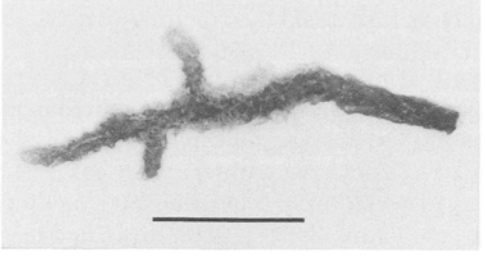

Fig. 2. Rhizome cultured in MS medium supplemented with NAA 10 mg l^{-1}. Bar = 10 mm.

Table 2. Effects of BAP and NAA on growth and differentiation in *C. kanran* rhizome apices after 4 weeks of culture.

Treatments ($mg l^{-1}$)		No. of branches		Length of branches (mm)	
BAP	NAA	Rhizome	Shoot	Rhizome	Shoot
0	0	2.3 ± 0.5[a]	0	3.2 ± 0.5	–
0	0.1	3.3 ± 0.6	0	5.2 ± 0.7	–
0	1	2.8 ± 0.6	0	7.8 ± 1.0**	–
0	10	4.3 ± 2.0[c]**	0	4.8 ± 1.3	–
0.1	0	5.3 ± 0.6**	0	5.1 ± 0.9	–
0.1	0.1	3.6 ± 0.6	0	6.1 ± 0.6[b]*	–
0.1	1	0.8 ± 0.3	0	8.0 ± 1.6**	–
0.1	10	2.3 ± 0.6	0	6.2 ± 0.9*	–
1	0	0	2.3 ± 0.2**	–	6.9 ± 0.1**
1	0.1	0	4.8 ± 0.4**	–	7.4 ± 1.7**
1	1	0	4.3 ± 0.2**	–	7.3 ± 0.7**
1	10	0	1.3 ± 0.2*	–	8.8 ± 2.0**
10	0	0	2.6 ± 0.6**	–	5.0 ± 0.4**
10	0.1	0	3.0 ± 0.4**	–	4.8 ± 0.7**
10	1	0	1.8 ± 0.6**	–	9.9 ± 1.0**
10	10	death	death	death	death

[a] Values are the means ± SE.
[b] The values followed by these marks (*) are significantly different to that of control treatment at the 5% level.
[c] The values followed by these (**) marks are significantly different to that of control treatment at the 1% level.

Table 3. Effects of BAP and NAA on growth and differentiation in *C. kanran* Makino shoot after 4 weeks of culture.

Treatments ($mg l^{-1}$)		No. of branches		Length of branches (mm)	
BAP	NAA	Rhizome	Shoot	Rhizome	Shoot
0	0	1.8 ± 0.5[a]	0	3.4 ± 0.8	–
0	0.1	2.1 ± 0.4	0	3.2 ± 0.4	–
0	1	2.2 ± 0.6	0	2.3 ± 0.3	–
0	10	0	0	–	–
0.1	0	3.0 ± 1.0	0	2.5 ± 0.1	–
0.1	0.1	4.5 ± 1.2[b]**	0	3.3 ± 0.5	–
0.1	1	2.3 ± 0.8	0	2.1 ± 0.1	–
0.1	10	0.4 ± 0.4	0	2.0 ± 0	–
1	0	1.1 ± 0.3	0	3.8 ± 0.4	–
1	0.1	1.8 ± 0.4	0.2 ± 0.2	4.2 ± 0.5	5.7 ± 1.4**
1	1	1.5 ± 0.3	5.0 ± 0	11.1 ± 0.8**	6.0 ± 1.5**
1	10	0	0	–	–
10	0	1.0 ± 0.6	0	5.6 ± 0.9	–
10	0.1	0	0	–	–
10	1	2.7 ± 0.4	2.6 ± 0.5**	3.3 ± 0.3	2.0 ± 0**
10	10	1.0 ± 0.4	0.8 ± 0.4**	1.3 ± 0.3	2.6 ± 0.3**

[a] Values are the means ± SE.
[b] The values followed by these marks (**) are significantly different to that of control treatment at the 1% level.

buds of explanted shoots (Fig. 3) occurred in media containing NAA. The maximum number of proliferated rhizomes was observed in cultures containing NAA at a concentration of $1 mg l^{-1}$. Few roots were induced from these explants except in one case. Vegetative shoot induction from rhizomes developing from the shoot cultures was enhanced when the cultures received both BAP and NAA at a concentration of 1 mg l^{-1}. Application of BAP 10 mg l^{-1} and NAA 1 mg l^{-1} into these rhizome cultures resulted in the formation of protocorm-like shoots (Fig. 4), but further development of the protocorm-like shoots was not observed under the condition used.

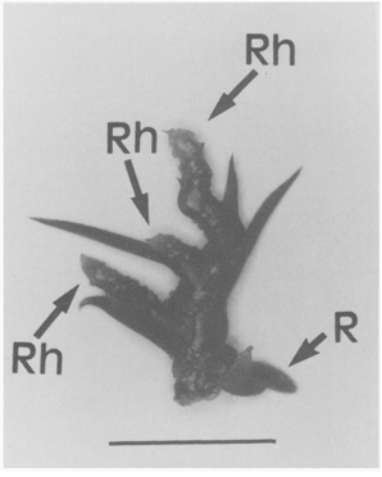

Fig. 3. Rhizome development from axillary buds of shoot cultured on MS medium supplemented with NAA 1 mg l^{-1}. Explanation of symbols: Rh, rhizome; R, root. Bar = 10 mm.

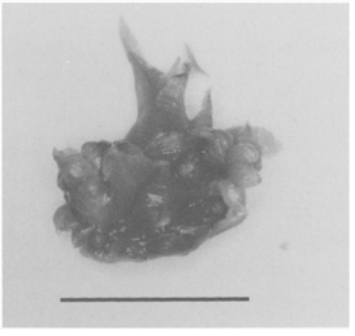

Fig. 4. Protocorm-like shoot differentiation from axillary buds of shoot cultured on MS medium supplemented with BAP 10 mg l^{-1} and NAA 1 mg l^{-1}. Bar = 10 mm.

Effects of ammonium nitrate and potassium nitrate

Effects of concentration of ammonium and potassium nitrate on the differentiation of *C. kanran* rhizomes were investigated in the modified MS media without addition of plant growth regulators (Table 4, Fig. 5).

Vegetative shoot differentiation in rhizome cultures occurred when the amounts of ammonium and potassium nitrates in the modified MS medium were reduced. Apparently, shoot differentiation was inhibited by higher ammonium nitrate content in the media. Maximum differentiation of vegetative shoots from the rhizomes was observed when the amounts of ammonium nitrate and potassium nitrate in MS medium were reduced to 412.5 mg l^{-1} and 950 mg l^{-1}, respectively.

On the other hand, media containing 412.5 mg l^{-1} ammonium nitrate and 1900 mg l^{-1} of potassium nitrate of MS medium favored maximum growth of the rhizome.

Root formation on vegetative shoots developed from rhizomes promoted in media containing 412.5 mg l^{-1} and 950 mg l^{-1} of ammonium nitrate and potassium nitrate in MS medium (Fig. 6). The optimum concentrations of ammonium nitrate and potassium nitrate for the production of *C. kanran* plantlets from the rhizomes were 412.5 mg l^{-1} and 950 mg l^{-1} in MS medium, respectively.

Discussion

Underground rhizomes have been reported from several terrestrial *Cymbidium* species distributed in Eastern Asia [6, 12–15]. *C. kanran* produces two morphologically different rhizomes, a typical underground and a tuber-like rhizome in situ. The underground rhizomes of *C. kanran* could endure changes in environments such as total removal of surface vegetation in the living habitat. However, the importance and function of underground rhizomes in terrestrial *Cymbidium* species have attracted interest of a few orchid investigators [2, 3, 12–14, 23].

Terrestrial *Cymbidium* seeds formed protocorms after germination which develop into rhizomes in vitro [1, 8–10, 20–22]. Usually those rhizomes produced few plantlets but formed masses of underground rhizomes during extended cultivation.

Axillary buds of the pseudobulbs of temperate terrestrial *Cymbidium* species (*C. kanran* and *C. goeringii*) can differentiate into underground rhizomes or vegetative shoots in the presence of auxin or cytokinin [23]. Similarly, underground rhizomes and shoots were induced from the pseudobulbs of *C. sinense* (Shimasaki unpubl.). However underground rhizomes of terrestrial *Cymbidium* have attracted little attention from investigators as a plant material for micropropagation. We have demonstrated that the underground rhizomes of temperate terrestrial *Cymbidium* species are a highly useful plant material for micropropagation. Several investigators reported that

Table 4. Effects of nitrogen-containing salt amounts in modified MS media on organogenesis in *C. kanran* rhizome after 9 weeks of culture.

Media	No. of branches		Length of branches (mm)	
	Rhizome	Shoot	Rhizome	Shoot
1	4.1 ± 0.2[a]**	0.4 ± 0.2*	4.5 ± 0.5**	3.0 ± 0.3**
2	4.4 ± 0.4[c]**	1.4 ± 0.2**	6.8 ± 0.7	3.0 ± 0.3**
3	4.4 ± 0.6**	2.9 ± 0.8**	7.1 ± 1.1	4.0 ± 0.8**
4	5.2 ± 0.6[b]*	2.8 ± 0.3**	8.2 ± 0.8	2.4 ± 0.1**
5	6.8 ± 0.7*	2.9 ± 0.3**	8.3 ± 0.7	2.3 ± 0.1**
6	10.3 ± 1.4	1.4 ± 0.6**	9.1 ± 0.7	1.8 ± 0.0**
7	8.9 ± 1.2*	1.2 ± 0.4**	10.6 ± 1.3*	2.8 ± 0.3**
8	11.4 ± 1.4	4.7 ± 1.0**	9.2 ± 1.1	2.3 ± 0.2**
9	13.2 ± 1.0	4.9 ± 0.6**	10.4 ± 0.5*	2.5 ± 0.2**
10	11.5 ± 1.2	4.3 ± 0.9**	10.9 ± 1.1*	2.8 ± 0.2**
11	9.6 ± 1.0	4.2 ± 0.5**	8.2 ± 0.6	2.2 ± 0.2**
12	5.7 ± 1.2*	1.0 ± 0.4**	5.7 ± 2.3	5.3 ± 1.7**
13	11.4 ± 1.0	2.6 ± 0.5**	9.3 ± 0.9	2.6 ± 0.2**
14	13.2 ± 1.6	3.5 ± 0.5**	10.0 ± 1.0*	3.1 ± 0.3**
15	13.0 ± 1.6	6.2 ± 1.2**	9.7 ± 1.2	2.9 ± 0.6**
16	13.6 ± 1.4	7.5 ± 1.1**	11.2 ± 1.1*	2.8 ± 0.1**
17	14.8 ± 1.9	4.2 ± 0.5**	14.0 ± 1.2**	3.8 ± 0.4**
18	15.7 ± 2.6	1.7 ± 0.6**	10.4 ± 1.8	3.4 ± 0.8**
19	13.6 ± 2.3	1.4 ± 0.8**	11.0 ± 1.7*	2.8 ± 0.0**
20	14.2 ± 3.2	2.5 ± 0.7**	11.3 ± 1.3*	2.6 ± 0.2**
21	15.8 ± 2.2	3.2 ± 0.5**	9.2 ± 0.9	2.9 ± 0.2**
22	11.1 ± 2.3	2.9 ± 1.2**	13.2 ± 1.5*	2.7 ± 0.2**
23	11.4 ± 2.0	3.4 ± 0.7**	11.1 ± 1.3*	3.6 ± 0.6**
24	16.7 ± 2.0	2.5 ± 1.0**	5.9 ± 1.5	3.1 ± 0.5**
25	7.3 ± 1.0*	0	6.6 ± 0.4	–
26	11.6 ± 1.4	0	8.5 ± 0.6	–
27	13.3 ± 1.6	0	8.4 ± 0.8	–
28	15.3 ± 3.9	0	6.5 ± 1.3	–
29 (Cont: MS medium)	15.7 ± 1.6	0	6.8 ± 0.5	–
30	11.6 ± 1.3	0	10.0 ± 0.9*	–
31	13.3 ± 3.1	0	5.8 ± 0.6	–
32	14.1 ± 3.3	0	6.4 ± 1.1	–
33	14.3 ± 2.9	0	5.8 ± 0.7	–
34	14.4 ± 2.6	0	7.2 ± 0.6	–
35	16.0 ± 3.3	0	8.0 ± 0.4	–
36	11.0 ± 6.2	0	4.7 ± 2.4	–

[a] Values are the means ± SE.
[b] The values followed by these marks (*) are significantly different to that of control treatment at the 5% level.
[c] The values followed by these marks (**) are significantly different to that of control treatment at the 1% level.

shoot differentiation in rhizome cultures was inhibited when the cultures were maintained under dark condition [1, 24, 25]. In our experiments, all rhizomes were cultured under 24-hr illumination photoperiods. Continuous illumination of rhizome cultures resulted in not only the stimulation of shoot development, but also in promotion of rhizome proliferation. Chlorophyll synthesis in rhizome cultures seemed to be involved with these growth habits. Auxin and cytokinin supplemented in culture media resulted in protocorm-like shoot differentiation at the apex segments of *C. kanran* rhizome. However, both auxin and cytokinin had little or no effect on root formation in shoots developing from rhizome cultures, hence, plantlet multiplication using auxin and cytokinin have been considered rather difficult. We found that concentrations and ammonium:nitrate ratios appeared to greatly affect organogenesis in *C. kanran* rhizome. Root differentiation from shoots developed in rhizome apices was observed when the rhizomes were cultured on modified MS media

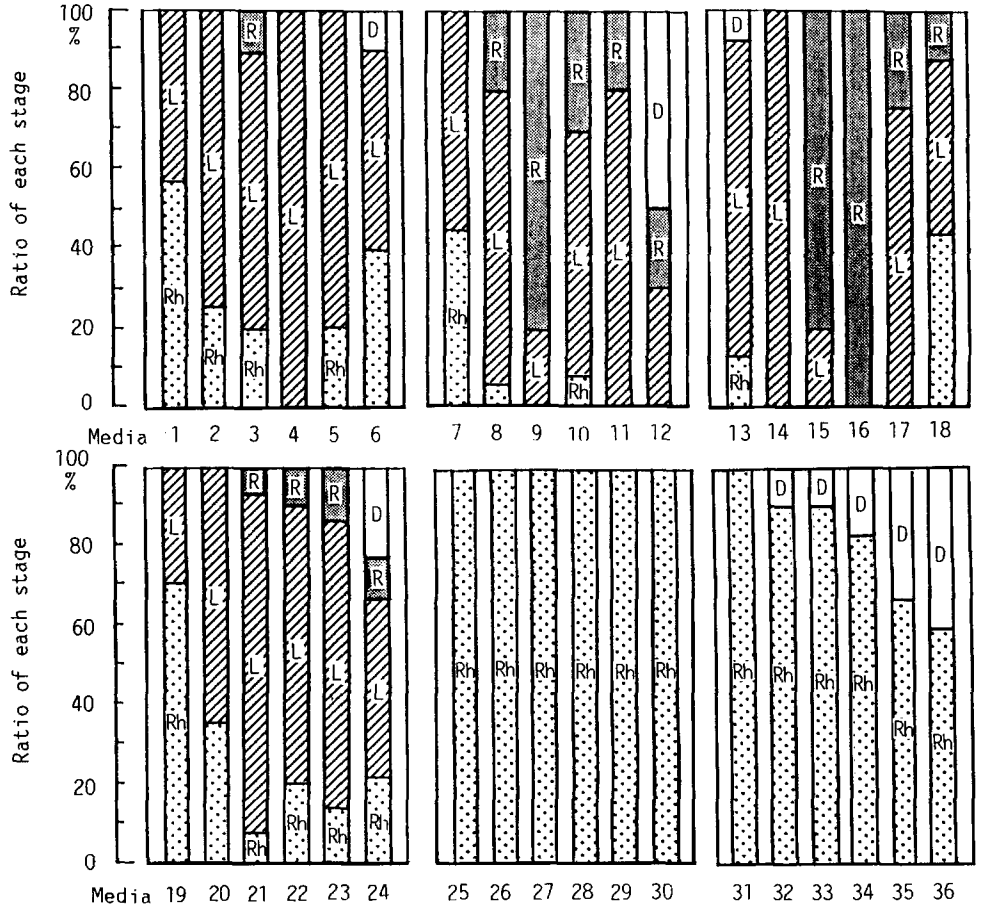

Fig. 5. Effects of nitrogen-containing salt amounts in modified MS media on organogenesis in *C. kanran* rhizome Explanation of symbols: Rh, rhizome stage; L, leafing stage; R, rooting stage; D, death.

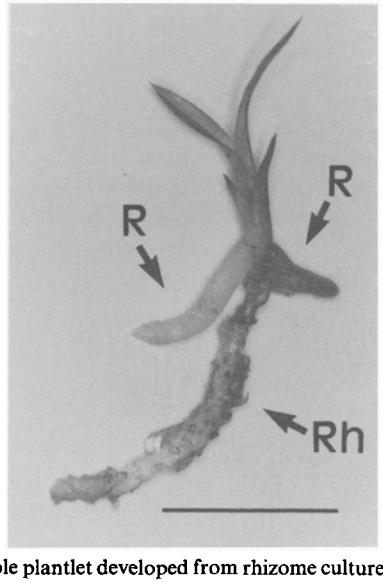

Fig. 6. Whole plantlet developed from rhizome cultured in modified MS medium. Explanation of symbols: Rh, rhizome; R, root. Bar = 10 mm.

containing lesser amounts of ammonium nitrate and potassium nitrate without addition of auxin and cytokinin. This observation suggested that application of exogenous plant growth regulators was not indispensable for normal plantlet production consisting of shoots and roots.

We believe these results have opened a new horizon of breeding and conservation of the little known terrestrial *Cymbidium* species growing in temperate evergreen forests of Eastern Asia.

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