Clonal Propagation of Cattleya Through Shoot Meristem Culture

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The application of a tissue culture method to the clonal propagation of Cattleya alliance is somewhat difficult now compared with that of Cymbidium. Morel (1960) and Wimber (1963) succeeded most typically in applying the idea of the tissue culture technique first to clonal propagation of Cymbidium. The difficulties found in the case of Cattleya may lie first in a high percentage of growth failure of explants when cultured in a nutrient medium, and second in a slow growth rate and growth failure of subsequent cultures. Many technical and physiological factors might be concerned with these problems, but only a few papers on these points (Reinert & Mohr 1967, Kako 1968b, 1970 and Ichihashi & Kako 1971) are available.

The purpose of this article is to introduce and discuss some of our recent views focusing on the above-mentioned problems.

Kinds of buds as the source of explants

There will be no objection to look at any vegetative buds as one of the best sources of explants. But it is often observed that explants differ from each other in the ability to grow into a protocorm-like body even if obtained from the same mother plant and cultured under the same conditions. This indicates that some physiological differences may exist among the buds before they are excised.

1) Terminal and lateral buds

It is evident from Fig. 1 that terminal and upper buds are inferior both in growth and surviving ability to the lower lateral buds. The buds used here were from newly growing shoots about 15 cm in length and classified from terminal to base as 0, 1, 2, 3, 4 in order. This result agrees with those obtained from Dendrobium (Sagawa, et al. 1967) and Cymbidium (Ueda, et al. 1968), but their buds were classified only as terminal and lateral.

2) Buds on new growths and back bulbs

As shown in Table 1, some dormant buds on old back bulbs are available, but the dominant buds on new growths are the best to use. When we grow Cattleya in a pot, usually only one or two buds on the newest bulbs

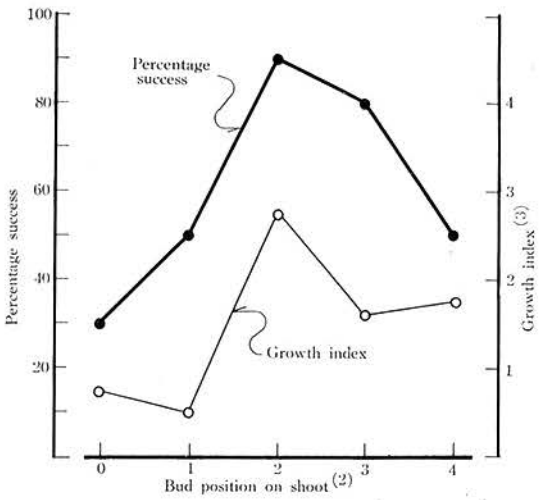

Fig. 1. Growth and surviving ability of the bud explants when classified by the position on a shoot[1)]

1) Cultured for one month in a liquid-standing medium of MS with 0.1 ppm NAA (see text)
2) See text
3) Growth index was indicated as (Final fresh) weight—Initial fresh weight)/(Initial fresh weight)

(From Kako, 1968 1969)

Table 1. Formation of protocorm-like bodies on the excised buds from new and old bulbs of LC. Excellency[1,2]

Age of bulb (year)	Location[3] of buds on a bulb	No. of buds used	No. of buds formed protocorm
1	a	12	12
	b	10	6
	c	3	1
2	b	10	6
	c	2	0
3	b	7	2
	c	4	1
4	b	5	3
	c	1	0
5	b	3	2
	c	3	1
6	b	3	1

1) Culture medium; MS (see text) + 1ppm NAA + 1% agar
2) Cultured for one month at 25℃ with continuous illumination of a fluorescent light
3) The buds on a bulb were classified from base to terminal as a, b, c

(From Kako, 1968b)

will grow into a shoot and the other buds, especially on back bulbs, remain at rest. This can be seen well after the plants were transplanted.

From such an observation, when we use a bud on back bulbs as a source of explants, it might be proposed that we divide the bulb with buds just coming to use from the other newer bulbs, and leaving the pot for a while, we excise the buds just a little before or when their growth starts.

3) Differences of the surviving ability of explants among hybrids or clones

It is important from a practical point of view to know if surviving ability of explants differs among hybrids and clones. Although from Table 2, the difference seems to exist, it is still uncertain, as in this experiment, the buds were not classified as shown before and the number of the explants of some plants was not enough. In general, species of Cattleya alliance have a lower surviving ability and the hybrids a higher one which is probably due to hybrid vigor.

Table 2. Growth and surviving ability on the excised buds from species and hybrids of Cattleya alliance[1]

Materials	No. of buds used	No. of buds formed protocorm	Growth[2] index
Species			
C. aurantiaca	4	3	1.8
C. bowringiana	6	1	2.7
C. trianae	3	1	0.5
L. flava	3	2	0.7
L. pumila	4	3	2.9
S. grandiflora	3	0	—
Hybrids			
C. Fabingiana	7	7	3.3
C. Shirayuki	6	3	4.3
BC. Princess Patricia	12	10	1.8
LC. Momus var. Brindir	5	2	2.8
LC. Santa Claus	7	5	4.0

1) Cultured in a liquid-standing medium (MS + 0.1ppm NAA) for 4 weeks, at 25℃ under a continuous light illumination
2) See Fig. 1

(From Ichihashi & Kako, 1971)

Cultural conditions

1) Culture medium

The effect of inorganic and organic nutrients on the growth of explants was studied, together with the comparison between Knudson's C-medium (=KC-medium) (1946) and Murashige's and Skoog's RM-1962 medium (=MS-medium) (1962). The results are shown in Fig. 2.

No difference of the survival rate was seen between the media, but MS-medium gave a uniform growth to the hybrids at least used here. The hybrids differed in the growth. For example, C. Fabingiana required rich inorganics of MS, but no minor organics, while LC. Culminant required rich organics and inorganics of MS-medium.

2) Culturing explants in liquid or on solid medium

During the first culture of excised shoot

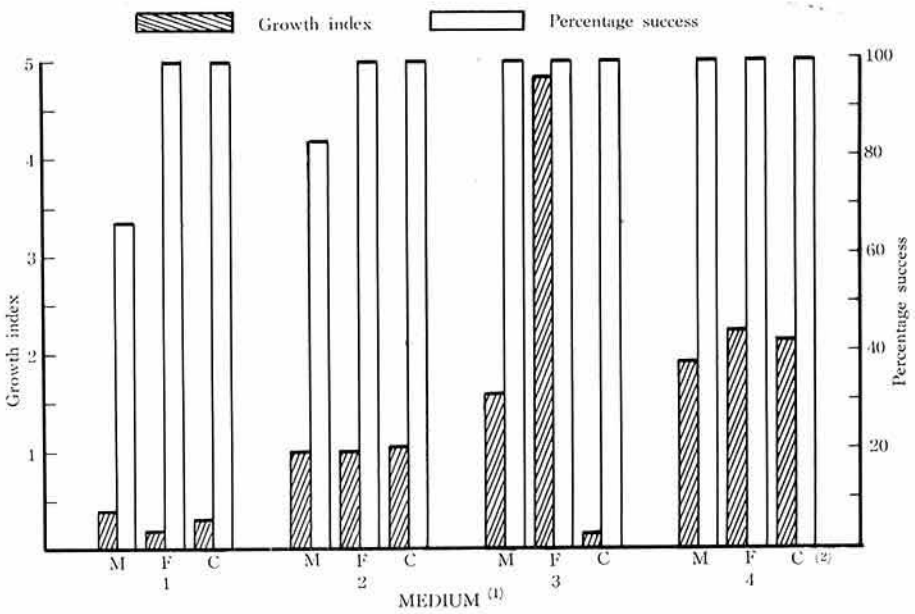

Fig. 2. Effect of inorganic and organic nutrients on growth and survival rate of the explants from three varieties of Cattleya[1)]

1) Cultured for one month in liquid-standing medium
   1=KC+0.1 ppm NAA
   2=1+the minor organics of MS
   3=4−the minor organics of MS
   4=MS+0.1 ppm NAA
2) M=LC. Momus var. Brindir
   F=C. Fabingiana
   C=IC. Culminant

(From Ichihashi 1972)

tips, Cattleyas do not show a stable growth but die with the tissue in brown. This sometimes happens at a rate too high to ignore for a practical purpose. It is often said that the cause of tissue browning is due to the formation of polyphenols by air and enzyme oxidation.

But it has not been confirmed experimentally in respect of orchids and is discussed later in this paper. Scully (1967) and Reinert & Mohr (1967) and Lindemann (1967) recommended a liquid culture for the first culture, probably to avoid tissue browning.

Reinert, et al. states that a liquid culture will bring forth success of more than 75 per cent and a faster growth than an agar culture. The liquid medium is usually agitated by a shaker or a rotator.

Scully shook it at the rate of 160 rpm and Reinert, et al. and Lindemann rotated it at 1 rpm and 0.2 rpm, respectively. A proper agitating speed cannot be determined because the cultural conditions reportedly differ.

Then the effects of liquid and solid cultures on the survival rate and growth of explants were studied and shown in Table 3. The survival rate of the liquid-standing culture was the highest one month after the culture, but decreased two months after.

The liquid culture shaking at 90 rpm was the worst both for survival and growth, and the culture solution changed into brown. The cultures on the solid medium caused tissue browning starting from the cut surface of

Table 3. Effect of liquid-shaking, liquid-standing and solid culture on the survival rate and growth of the explants of LC. Dinah[1]

Culture condition	No. of explants used	After one month			After two months		
		No. of explants survived	Success (%)	Growth[2] index	No. of explants survived	Success (%)	Growth[2] index
Liquid-shaking	8	4	50	1.0 + 1.3	2	25	2.4 + 2.8
Liquid-standing	10	9	90	2.2 + 1.4	7	70	4.1 + 2.4
Solid (1% agar)	10	5	50	2.1 + 3.1	3	30	3.7 + 2.8

1) A MS-medium with 0.1ppm NAA was used and cultured at 25℃ under a continuously illuminated light

2) See the note of Fig. 1 (From Ichihashi & Kako, 1971)

the explants and developing into the upper side.

The agar below the cultures sometimes changed into brown. The cultures on agar and in lqiuid-shaking, caused only a small part of green tissues even if they are alive. But those in liquid-shaking caused the browning merely at the cut surface and did not develop into the other parts of tissues.

In the solution of liquid-standing a longer period after the culture, some amount of ethyl alcohol was detected by a gas-chromatography. The alcohol can be one of the causes of the decrease in the survival rate which was seen two months after the culture.

The paraffin section of the cultures was made to observe a morphological development. The explants swelled in a few days of culture. Two to four weeks after, larger cells were formed at the side of the cut surface to heal the wound and new vascular bundles were developed from the inner side of larger cells (Fig. 3).

3) Growth regulating-substances

The explants require some proper concentrations of plant hormones. From Fig. 4, the addition of 0.1 ppm of α-naphthalene acetic acid (=NAA) or 2,4-dichlorophenoxy acetic acid (=2,4-D) is recommended as an optimum concentration.

No evident effect of kinetin and its combination with other hormones was observed by Ichihashi, et al. (1970) and Lindemann (1967). But it may be worth to note here the observation by Reinert, et al. (1967) that the addition of 1 ppm of kinetin and 100 ppm of inositol to a liquid medium, caused a rapid tissue browning and a slower growth to some Cattleyas.

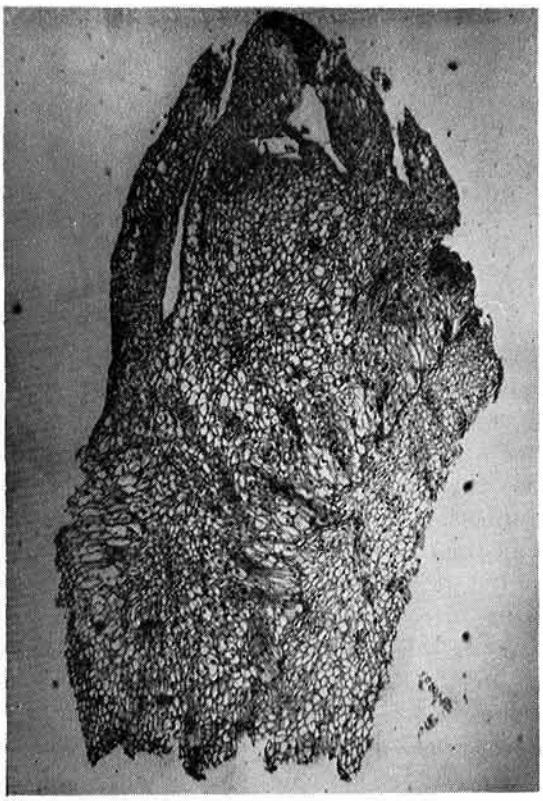

Fig. 3. Longitudinal section of an explant of Cattleya cultured on an agar part of tissues (From Kako, 1969, 1970)

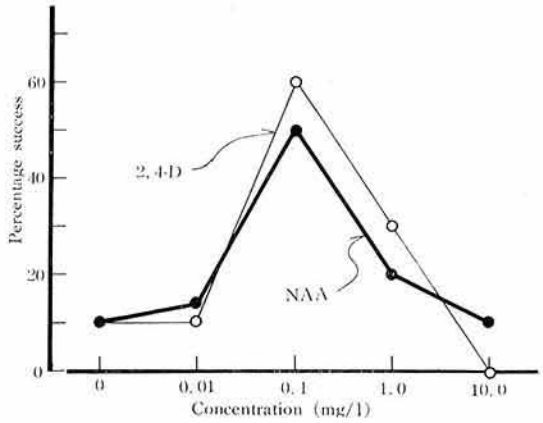

Fig. 4. Effect of concentration of NAA and 2,4-D on the survival of explants[1]
1) Cultured for one month in a liquid-standing medium of MS (see text)
(Ichihashi & Kako, 1971)

4) Conditions for successive subculturing

The longer the explants stay in the same culture solution, the lower the survival rate becomes. Therefore, the effect of transplanting on survival and growth was studied. One month after the culture of the explants in liquid-standing, the cultures were cut in half and the cut pieces were cultured in a new liquid-standing or on a solid medium. The results in Table 4 indicate that the growth failure of the halved pieces occurred at a high rate on agar and low in liquid-standing one month after the culture, but that this relation was reversed three months after the culture. When a whole body was transplanted into new media, a solid medium is preferable for survival and growth rather than liquid-standing.

Callus induction

Callus formation from the cultures has been attributed to chance. Callus may be obtained more frequently in a liquid medium than on agar (Reinert, et al. 1967).

The protocorms grown from seeds develop well into calli when cultured in liquid, but more developed protocorms do less (Kako, 1968a, 1969). Cell division of the callus is active on its epidermal cell layer, especially at the deep caved side (Fig. 5). The inner cells of callus which are larger, vacuolated and full of starch grains ceased cell division.

The addition of 2,4-D and Kinetin in an agar medium, each at 10 ppm, has been found effective for the induction of callus from the cultured explants (Ichihashi & Kako 1972).

The callus is different in its shape and quality from the "callus" or callus-like tissues that can be obtained by chance without 2,4-D. The callus will be stably formed in about four or three months.

Phenolics in Cattleya as a browning agent

The formation of a brown pigment is well said to be one of the factors in the unstable growth of Cattleya explants (Scully 1967, Reinert, et al. 1967 and Lindemann 1967), but the chemical nature of the pigment is not yet determined.

Table 4. Growth and surviving ability of the cultures when subcultured cut in half[1]

	Culture condition	No. of pieces used	After one month		After three months
			No. of pieces survived	Growth[2] index	No. of pieces survived
Whole pieces	Liquid-standing	6	4	0.37	1
	Solid	6	4	0.61	3
Halved pieces	Liquid-standing	10	7	0.36	1
	Solid	10	4	0.50	4

1) MS-medium with 0.1ppm NAA was used and 1% agar was added to the solid medium
2) See the footnote of Fig. 1

(From Ichihashi, 1972)

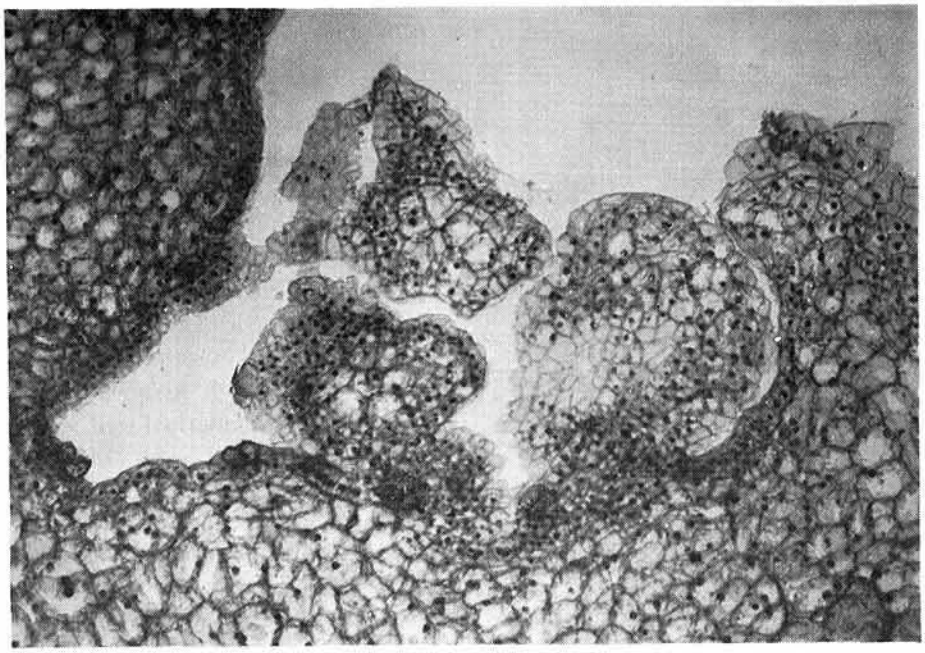

Fig. 5. Longitudinal section of Cattleya callus obtained by a liquid culture of protocorn, illustrating an active cell division of epidermal cell layers, especially at the deeply caved part (From Kako, 1968a, 1969)

In general it is known that the wound tissues show a browning phenomenon due to the biosybthesis of polyphenols. Thus, we conducted some experiments in the extraction of phenols from Cattleya leaves, in the chemical natures of the phenols and in the activity of polyphenol oxidase in the leaves (Ichihashi, 1972).

About five phenols were detected in the methanol extracts of leaves by a paper chromatography of the acidic fraction. One of the phenols, dominated in quantity and called S-2, has a molecular weight of 238 and a molecular formula of $C_{11}H_{10}O_6$. This is one of the para-hydroxy phenolic dicarboxylic acids.

Polyphenol oxidase extracted from leaves with cold acetone has its optimum pH at 6.5 in a phosphate buffer using 10/M catechol as substrate, and showed a high activity with chlorogenic acid, but low with S-2 alone and yet an increased activity when both S-2 and catechol were used as substrate.

This study is now under progress in our laboratory and we are going to clarify if the tissue browning of explants has any effects on their growth and survival.

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