MERISTEM CULTURE OF DENDROBIUM (ORCHIDACEAE)

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We certify that we have read this thesis and that in our opinion it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Horticulture.

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INTRODUCTION

The purpose of this study is to establish the most suitable source of explants for use in meristem culture of orchids and to define the most efficient methods for the induction of protocorm-like bodies.

Although the technique of meristem culture is currently utilized commercially for asexual clonal propagation and virus elimination in some orchids, investigations for increasing its efficiency as a tool in propagating orchids are still lacking.

The increased use of Dendrobium for cut flower production suggested the need for adapting the meristem culture technique for propagating this group of orchids.

LITERATURE REVIEW

The aseptic methods of micro-propagation have been classified by Hartman and Kester (1968) into three groups: embryo culture, tissue culture and shoot tip (meristem) culture. Embryo culture is used to nurture embryos which tend to abort during development, as in the Rosaceae (Kester and Hense, 1955) and Orchidaceae (Niimoto and Sagawa, 1961) or to circumvent seed dormancy (Lammerts, 1962). Callus tissue differentiation into shoots and roots has been successful in carrot (Steward et al., 1958; 1964; Halperin, 1964), tobacco (Vasil and Hildebrandt, 1966), endive (Vasil and Hildebrandt, 1966), aspen (Winton, 1968), Convolvulus (Hill, 1967), chrysanthemum (Hill, 1968), and asparagus (Takatori et al., 1968).

Using stem tip culture Ball (1946) demonstrated that explants of shoot apices of Lupinus and Tropaeolum which included three leaf primordia could be successfully grown into whole plants. Morel and Martin (1955) showed that potato apices isolated from virus infected plants and grown in culture could result in virus-free plants. Shoot tip culture has been subsequently used as a method for obtaining disease-free plants from infected ones in carnation (Hollings, 1959); Baker and Phillips, 1962; Phillips and Matthews, 1964; Hackett and Anderson, 1966), strawberry (Belkengren and Miller, 1962), red raspberry (Bolton and Turner, 1962), potato (Norris, 1954; Kassanis, 1957; Chapman, 1955; Manzer, 1959; Oshima and

Livingstone, 1961), dahlia (Holmes, 1955), chrysanthemum (Holmes, 1956), sweet potato (Nielson, 1960), and geranium (Pillai and Hildebrandt, 1969).

Although only single plantlets develop from most meristem cultures, callus formation has been occasionally observed, as on the wound surface of the Lupinus shoot apex (Ball 1960). The potential of clonal propagation of carnations by meristem culture was implied by Hackett and Anderson (1968) who obtained many plantlets from callus derived from carnation apical meristem. It was noted that adventitous buds did not seem to be involved, but instead there appeared to be a proliferation of tissue at the axillary bud position.

When meristem culture was applied to orchids by Morel (1960) to obtain plants free of Cymbidium mosaic virus, it was observed that each meristem produced bodies morphologically similar to the protocorms formed upon germination of orchid seeds; hence these bodies were later called protocorm-like bodies (plb). Upon sectioning and further subculture of this tissue, numerous plb's were derived; when left undisturbed, each body produced a plantlet. This process thus allowed for rapid clonal propagation (Morel 1965). It was reported that by agitating the liquid medium, the number of plb's could be greatly increased (Wimber 1963).

Clonal propagation by meristem culture has been applied on a commercial scale with Cymbidium and Cattleya. The name "mericlone" has been suggested for plants resulting from this

technique (Dillon, 1964). Other genera such as Lycaste, Miltonia, Odontoglossum and Phaius (Morel, 1965), Calanthe, Odontonia, and Vuylstekeara (Bertsch, 1966), and Dendrobium (Sagawa et al., 1967) can also be successfully propagated by meristem culture.

New shoots arising from backbulbs or from leadbulbs have been the sources of explants. These shoots were 1-10 cm in length for Cymbidium (Morel, 1960; Wimber, 1963; Morel, 1965; Isley, 1965; Sagawa et al., 1966), 1-10 cm for Cattleya (Morel, 1965; Scully, 1966), and approximately 7 cm for Dendrobium (Sagawa et al., 1967). Both apical and axillary buds from new shoots have been used successfully as explants (Morel, 1960; Isley, 1965; Sagawa et al., 1966, 1967; Scully, 1967).

Morel (1960, 1965) excised very small explants, about 0.1 mm in width, to free Cymbidium of virus. For clonal progapation, explants 0.5 - 1.0 mm in size and including two to four leaf primordia were used to prevent the early degeneration or slow development found with small explants (Morel, 1965). Sagawa et al. (1966) devised a simple method for excising explants 2-3 mm^3 and stated that explants may be as large as 5 mm^3 which can be made without use of a stereomicroscope. Also, with Cattleya, Morel (1965) suggested that the explant be about 2-4 mm by 5 mm.

Wimber (1963) described several paths of explant development: 1) may proliferate without shoot differentiation,

2) may produce shoots and plb's, 3) may develop branched stem-like organs with plb's at each node, 4) may develop chlorophylless and shootless tissue masses.

Sagawa et al. (1967) classified the developing Dendrobium explants into three types: 1) explants which swelled, resulting in a single shoot or several shoots directly, 2) explants which formed green masses, the masses being replaced by plb, and 3) explants which developed plb upon subsequent subdivision and transplanting after initial culture.

A growth of callus in the wounded leaf epidermis caused by counter friction against glass, and the continued proliferation of this tissue to form plb's was observed (Wimber, 1965) with Cymbidium explants. Sectioning the plb before differentiation into a plantlet resulted in large masses of callus tissue or development into well-defined plb's in Cattleya explants (Reinert and Mohr, 1967). If the callus was cut into several pieces, the tendency for plb development decreased, while without dividing, numerous plb's were produced in subculture.

MATERIALS AND METHODS

Shoots from a seedling population of a Dendrobium phalaenopsis hybrid growing in the Horticulture Department greenhouse were obtained between May 12, 1968 and November 12, 1968. This hybrid was selected since flowering normally occurs in the fall in Hawaii and is usually preceded by a flush of new shoots between May and July.

New shoots were collected from leadbulbs (Fig. 1), backbulbs (Fig. 2), new growths which had been previously cut (cut leadbulb) (Fig. 3), and 'keikis' (an asexual offshoot arising in the upper portion of the plant (Fig. 4). A total of 79 shoots were selected from the four sources as shown in Table I. Each shoot was measured and weighed. The shoots were classified into five classes by weight, and the range and average lengths in each class were computed as shown in Table II. Since the shape of shoots varied considerably, there was not a good correlation between shoot length and shoot weight.

The axillary buds in a detached shoot were numbered acropetally. Usually, three or four buds were present but in tall shoots, five or six buds were present, as in a rapidly elongating 'keiki'. Apical buds were classified separately from axillary buds.

Explants were excised from the shoot by the method described for Cymbidium by Sagawa et al. (1966), but modified

Fig. 1-4. SOURCES OF NEW SHOOTS FROM *DENDROBIUM PHALAENOPSIS* HYBRIDS

Fig. 1. Basal part of plant showing a new growth on leadbulb.

Fig. 2. A new growth arising from backbulb.

Fig. 3. A new shoot developing from rhizome after removal of new growth from leadbulb. Note that the basal internodes of previously cut new growth have elongated (arrow).

Fig. 4. 'Keiki' arising in leaf axil of leadbulb (left arrow) and on backbulb (right arrow).

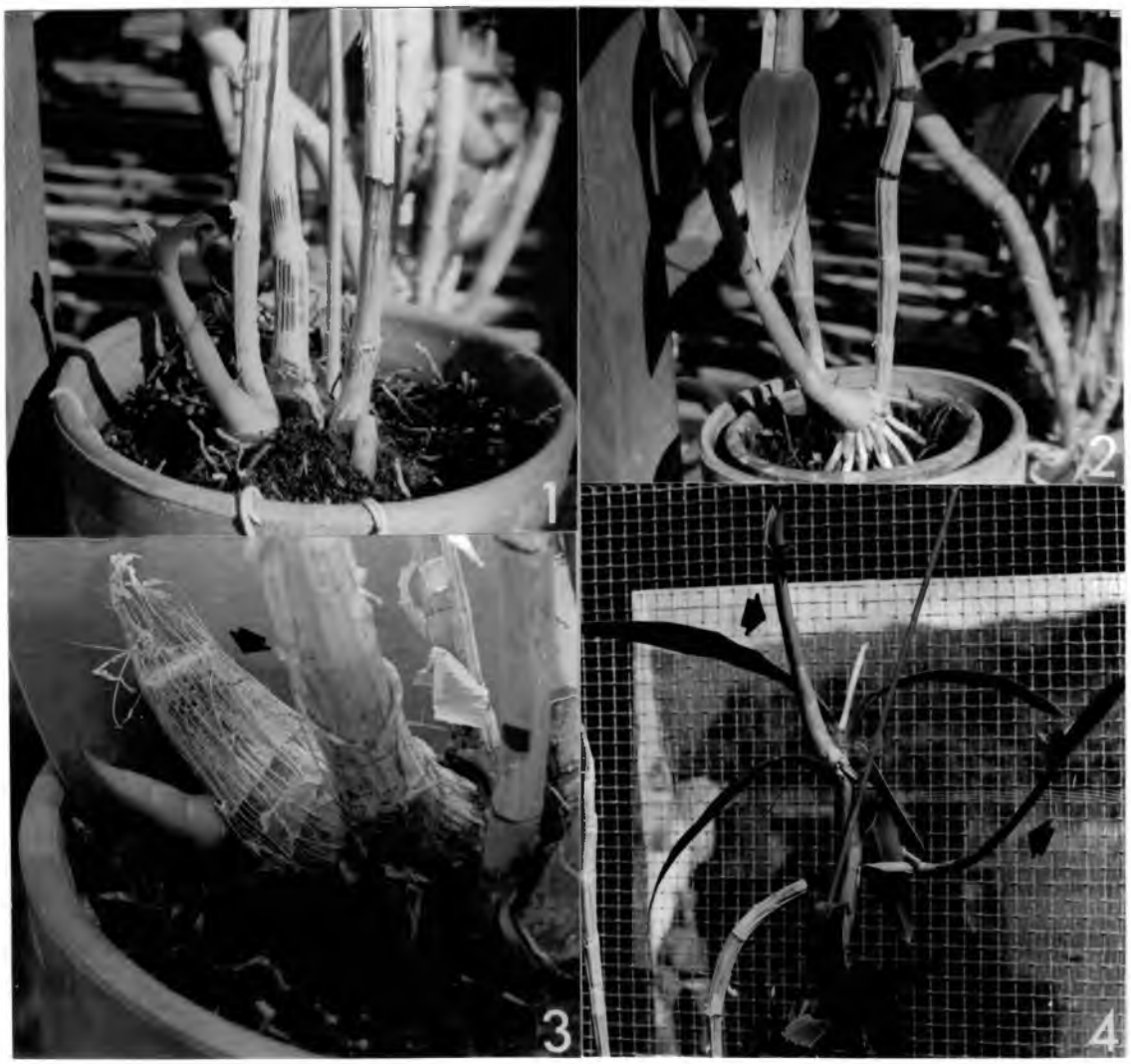
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TABLE I. NUMBERS AND SOURCES OF NEW SHOOTS

SOURCES	NUMBERS
LEADBULB	47
BACKBULB	9
CUT LEADBULB	8
'KEIKI'	15
TOTAL	79

TABLE II. CLASSIFICATION OF NEW SHOOTS BY WEIGHT

MEDIAN WEIGHT CLASS	WEIGHT LIMIT (gm)	LENGTH (cm)		NUMBERS OF SHOOTS
		RANGE	AVERAGE	
<1.0	- 1.0	2.5- 3.5	3.0	10
2.0	1.1 - 3.0	3.7-11.5	4.9	18
4.0	3.1 - 5.0	5.5-14.0	8.2	18
6.0	5.1 - 7.0	6.5-15.5	10.4	17
>7.0	7.1 -	9.5-20.0	15.5	16
TOTAL				79

by the elimination of sterilization with 1% Clorox. Explants were therefore transferred directly to the media after treatment with 5% Clorox. The modifications of excisions devised for morphological studies are presented in the section on results.

For culture, 20 ml of liquid Vacin and Went medium (Vacin and Went, 1949), modified by the addition of 15% by volume of coconut water was placed in 50 ml flasks. The same medium solidified with 0.9% agar was used for growing differentiated plantlets or for explants for special morphological observations. Liquid cultures were constantly agitated on a New Brunswick Model V Shaker operated at 160 rpm. All cultures were maintained at 26±3°C with continuous illumination of about 200 foot candles from G.E. white fluorescent lamps (Power Grove).

The numbers of explants made for different buds in each class are tabulated in Table III along with the numbers contaminated in each group. Most contaminations could be identified within 1 to 3 days by turbidity of the liquid media. In total, 51 of 326 explants or 15% were contaminated in initial culture. A slight increase in the percentage contamination occurred with the heavier shoots. The axillary buds which were at the base of large shoots (7.0< gm) showed 38.4% contamination since many were either completely exposed or covered by dried thin leaf sheath. There was no further difference between buds.

TABLE III. NUMBERS OF EXPLANTS MADE AND CONTAMINATED FOR DIFFERENT BUDS IN EACH WEIGHT CLASS

BUD POSITION		WEIGHT CLASS OF SHOOT (gm)					TOTAL
		1.0>	2.0	4.0	6.0	7.0<	%
LATERAL BUDS	1[a]	0/10[b]	1/18	3/16	3/16	5/13	12/73 (16.4)[c]
	2	1/10	1/18	3/17	4/15	3/12	12/72 (16.7)
	3	2/4	0/18	2/16	5/13	1/9	10/60 (16.7)
	4		2/6	2/9	0/10	0/7	4/32 (12.5)
	5		0/3	0/2	0/6	1/5	1/16 (6.3)
	6		1/1		0/1	0/2	1/4 (25.0)
	TOTAL	3/24	5/64	10/60	12/61	10/48	40/257 (15.6)
	APICAL BUD	2/10	1/17	3/16	2/14	3/12	11/69 (15,9)
TOTAL		5/34	6/81	13/76	14/75	13/60	51/326 (15.6)
	%	(14.7)	(7.4)	(17.1)	(18.7)	(21.7)	

[a]Axillary buds numbered acropetally in a detached shoot

[b]The denominator shows the number of explants made and the numerator the number of explants contaminated

[c]Percentage contamination in parenthesis

All samples for anatomical study were killed and fixed in Craf fixative, dehydrated in a series of tertiary butyl alcohol, and embedded in paraffin by an Autotechnicon. Sections were cut at 10μ or 12μ and stained with the rapid Safranin Fast Green technique (Shapiro, 1947). A Zeiss photomicroscope and Wild M-5 stereo-microscope with photo-tube were used for morphological and anatomical observations and photography. All dissections were performed within a White Roomette which was placed in an aseptic culture room serviced by a Westinghouse Precipitron.

RESULTS

The results have been organized into three major sections. The studies on success of culture as related to sources and weight of shoot, and position of bud (apical or axillary) are presented in Section I. The pattern of growth of explant is presented in Section II with some anatomical observations. Since axillary bud explants showed a general pattern of growth into shoots directly without forming plb, further experiments on induction of plb by variation of excision methods and by subdividing in subculture are presented in Section III.

I. Studies on success of culture as related to source and weight of shoot and position of bud

These studies were designed to establish the relative success of cultures of explants derived from different sources of shoots such as leadbulb, backbulb, cut new shoot, and 'keiki', from different weights of new shoot, and from buds located in different position on the shoot. These data were only concerned with survival of explants.

A. Success of explant cultures as related to source of shoot

The percentages of survival of explants derived from various sources of shoots are presented in Table IV. A high percentage (50.0%) of success was obtained from explants excised from shoots arising from backbulbs. Low percentages (32.5%, 31.8%) of success appeared in cultures of explants

TABLE IV. THE PERCENTAGES OF SURVIVAL OF EXPLANTS AS RELATED TO SOURCES OF NEW SHOOT

SOURCES	SURVIVAL (%)		
	LATERAL BUD	APICAL BUD	TOTAL
LEADBULB	38.8 (50/129)*	29.7 (11/37)	36.7 (61/166)
BACKBULB	60.0 (12/20)	25.0 (2/8)	50.0 (14/28)
CUT LEADBULB	30.0 (6/20)	50.0 (1/2)	31.8 (7/22)
'KEIKI'	30.3 (10/33)	42.9 (3/7)	32.5 (13/40)
TOTAL	38.6 (7/202)	31.5 (17/54)	37.1 (95/256)

*Number of successful cultures/total number cultured

from 'keiki's and cut leadbulb shoots, respectively. Explants from leadbulbs, which provided most of the materials used in this study, showed 36.7% success which is almost the same as the overall average of 37.1%.

B. Success of explant culture based upon shoot weight and position of bud

The percentages of successful culture related to different types of buds and different weights of shoots are tabulated in Table V. A total of 95 explants out of 256 (37.1%) grew to plantlets of produced plb's. The highest percentage (50.0%) was obtained from explants excised from shoots 2.0 gm in median weight. As shown in Fig. 5, the percentage of successful cultures decreased with increasing shoot weight. As for the success of explant culture with regard to bud position the percentage of survival of lateral bud explants was 38.6%--slightly higher than that of terminal bud explants, 31.5%. There was little difference in the success of culture from lateral and apical buds except for those from the largest shoots (>7.0 gm), where cultures from lateral buds were more successful than the terminal buds (Fig. 5). The dotted line in Fig. 5 shows the success of culture of terminal buds as related to shoot weight. The percentage of successful culture peaked with 2.0 gm shoots and decreased gradually with increase in shoot weight.

The percentages of successful culture of lateral buds as related to position in a shoot are shown in Fig. 6. There was

a decrease in percent of survival with increasing node number. The limited successes of No. 5 and No. 6 buds are significant showing 7.7% and 0.0% respectively.

TABLE V. THE PERCENTAGES OF SUCCESSFUL CULTURE AS RELATED TO BUD POSITION AND SHOOT WEIGHT

BUD POSITION		WEIGHT CLASS OF SHOOT (gm)					TOTAL
		<1.0	2.0	4.0	6.0	>7.0	
LATERAL BUDS	1[a]	20.0[b] (2/10)	46.6 (7/15)	50.0 (6/12)	58.3 (7/12)	40.0 (2/5	44.4 (24/54)
	2	11.1 (1/9)	70.6 (12/17	42.9 (6/14)	40.4 (4/10)	62.5 (5/8)	48.3 (28/58)
	3	0.0 (0/2)	38.9 (7/18)	21.4 (3/14)	37.5 (3/8)	57.1 (4/7)	34.5 (17/49)
	4		66.7 (2/3)	42.9 (3/7)	11.1 (1/9)	28.6 (2/7)	30.8 (8/26)
	5		0.0 (0/2)	50.0 (1/2)	0.0 (0/5)	0.0 (0/4)	7.7 (1/13)
	6				0.0 (0/1)	0.0 (0/1)	0.0 (0/2)
	SUB-TOTAL	14.3 (3/21)	50.9 (28/55)	38.8 (19/49)	33.3 (15/45)	40.6 (13/32)	38.6 (78/202)
	APICAL BUD	12.5 (1/8)	46.6 (7/15)	36.4 (4/11)	33.3 (4/12)	12.5 (1/8)	31.5 (17/54)
	TOTAL	13.8 (4/29)	50.0 (35/70)	38.3 (23/60)	33.3 (19/57)	35.0 (14/40)	37.1 (95/256)

[a]Lateral buds numbered acropetally in a detached shoot

[b]Each number represents percentage of successful culture; the numbers in parenthesis show numbers of successful culture/total number cultured

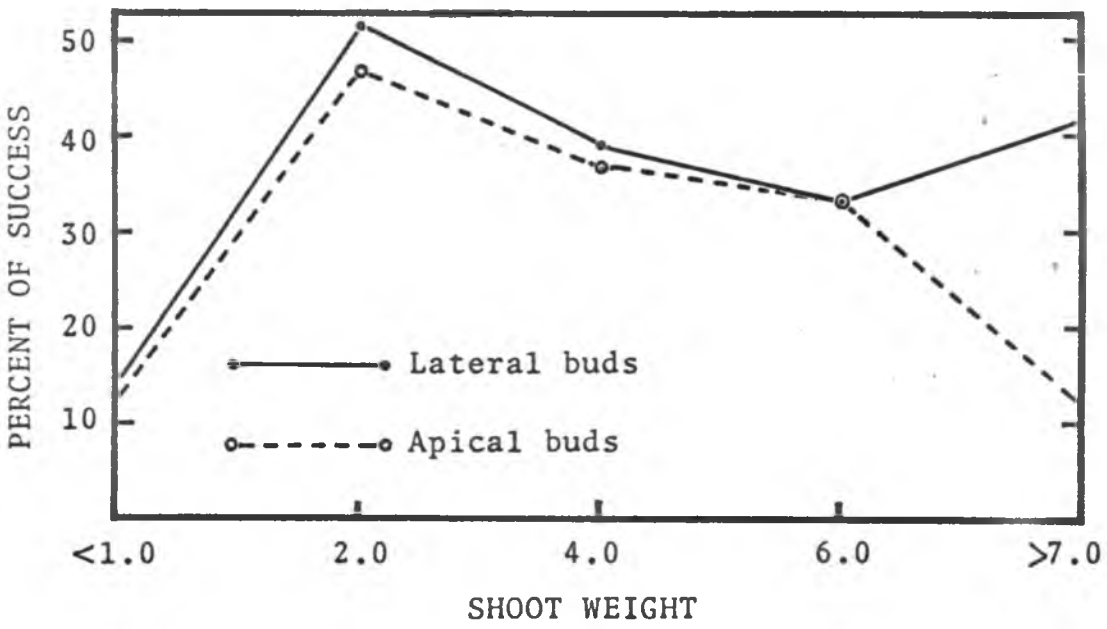


FIG. 5. PERCENTAGE OF SUCCESSFUL CULTURE IN EACH MEDIAN WEIGHT CLASS OF SHOOT

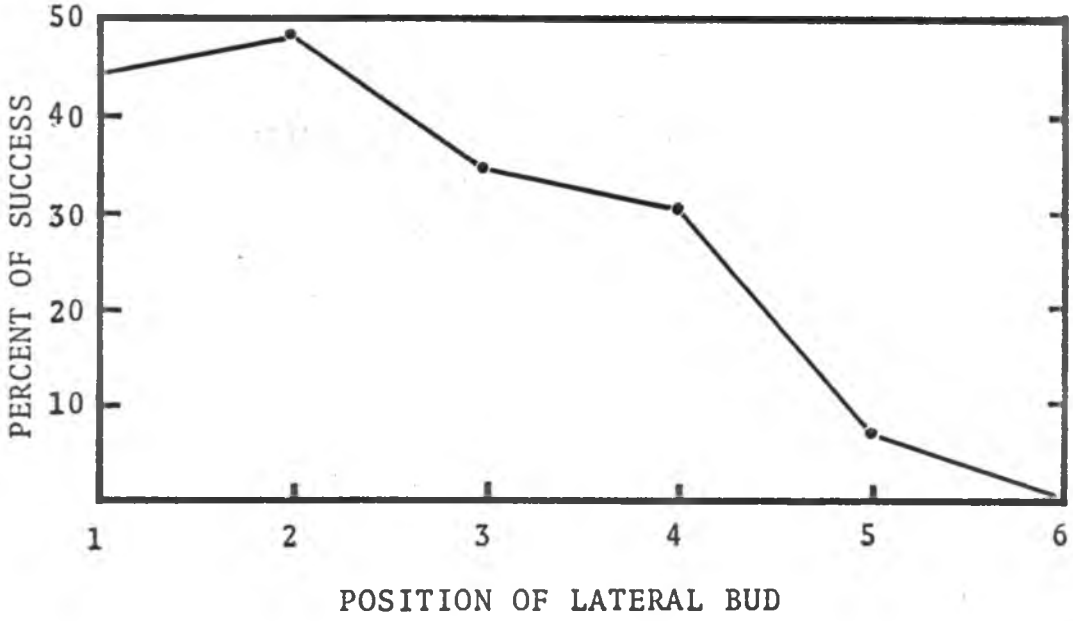


FIG. 6. PERCENTAGE OF SUCCESSFUL CULTURE IN EACH POSITION OF LATERAL BUDS

II. Observations on explant growth

Explants were observed for a period of 7 to 63 days. They were transferred to new medium within 30 to 60 days from the time of initiation. They showed wide variations in growth pattern. However, successful cultures can be classified into two major types: 1) explants which produce plb which later produce plantlets, and 2) explants which grow directly into a shoot or shoots without forming plb. Hence, the observations are presented in two subsections and with some anatomical studies, mainly on plb development, included in another subsection.

A. Standard pattern of explant development

The explant derived from an apical bud (apical bud explant) consisted of an apical meristem plus several leaf primordia whereas the axillary bud explant contained a lateral meristem with three to eight young leaves depending on the stage of bud development.

Explants which were colorless at first gradually turned green, with rather slow growth during five to ten days. The first sign of growth was the splitting of the outer leaves which originally enclosed the meristem when excised (Fig. 8). In 15 to 30 days, the explant consisted of a small ball with necrotic tissue on the basal end. This growth mostly resulted from the enlargement of leaf primordia and showed differences in appearance between apical bud (Fig. 7) and axillary bud explants (Fig. 8). In the apical bud explant, the juvenile

leaves which were yellowish and fragile were opposite each other; whereas in the axillary bud explant, the newly grown leaves completely surrounded the meristem, forming a hemisphere. The degeneration of the explant occurred very slowly after this stage. It started with blackening of the tissue or some necrosis from the outer part of the explant. It has been occasionally observed that upon removing the outer degenerated leaves, the inside tissue was still active.

The plb's were observed either when the explant had somewhat degenerated or after complete degeneration (Fig. 9, 10). Since it started as a very small round mass of tissue, it is difficult to determine the time of initiation of this tissue. Plb's originated in the region of lateral bud primordia. In few cases, plb's formed before degeneration of explants could be observed (Fig. 11). A change in the color of the media from clear to dark brown was a general phenomenon accompanying the degeneration of explants and formation of plb's.

The plb, attached to the degenerated explant, eventually separated without any dissection. The growth stage, at the time of separation varied from very small tissue to a fairly large ball beginning to develop shoot and root. From a small plb the proliferation of tissue was very rapid. Each plb grew protuberances in several directions (Fig. 14). Since both the new protuberances and the round mass from which they were derived sporadically multiplied and increased in size,

FIG. 7-12. STANDARD GROWTH PATTERN OF EXPLANT GROWTH

Fig. 7. An apical bud explant after 37 days of culture showing juvenile leaves and signs of degeneration and necrosis. 4x.

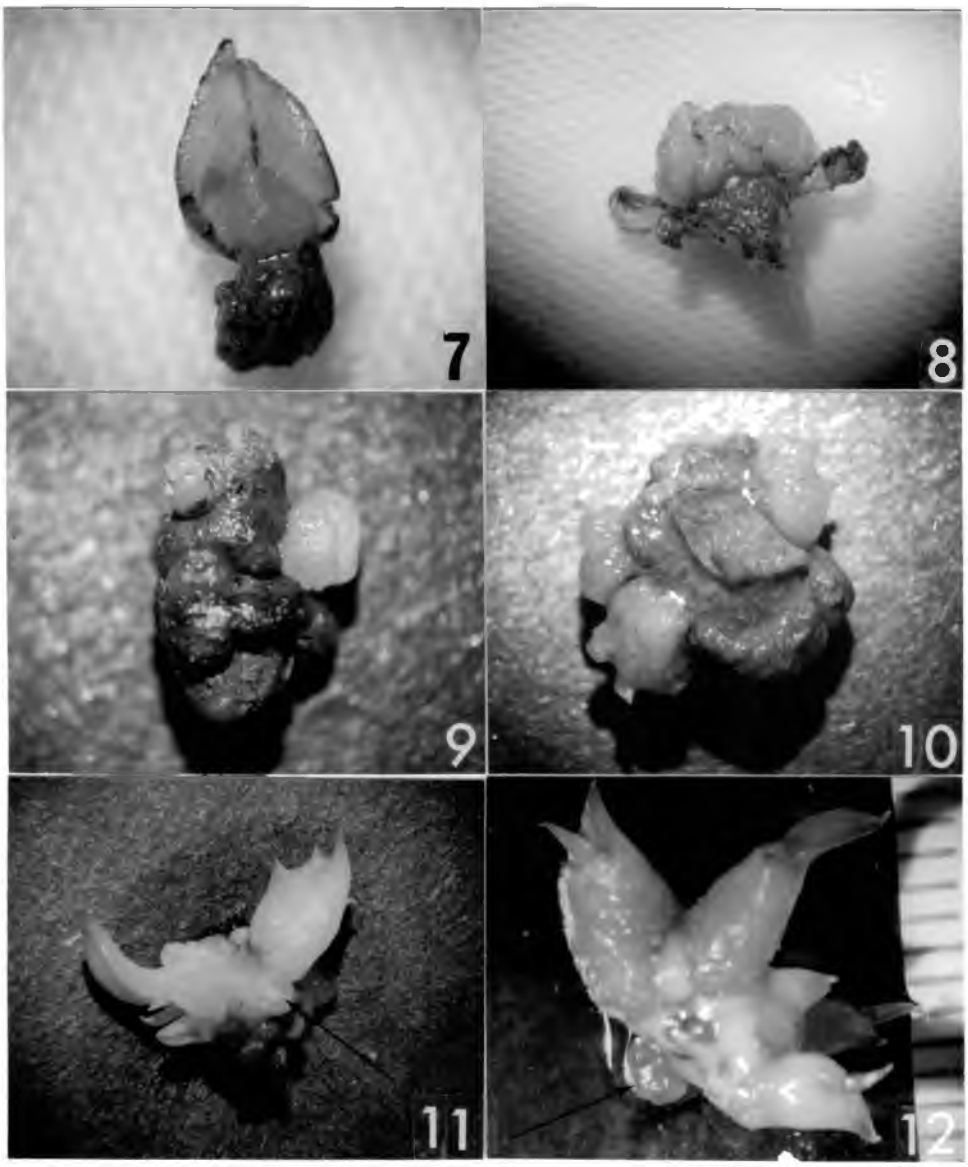
Fig. 8. An axillary-bud explant after 37 days of culture showing a small mass of tissue with necrotic tissue below. Note that the split outer leaves and beginning of degeneration which eventually leads to Fig. 10. 4x.

Fig. 9. An apical bud explant showing a white undifferentiated plb after 85 days of culture and complete degeneration of original explant. 4x.

Fig. 10. A 60-day old culture, showing several plb's attached at the axillary bud positions and degenerating original explant. 4x.

Fig. 11. A 60-day old culture showing rapid differentiation of plb's and proliferation of new plb's (arrow). The explant shown as dark tissue at the base. 4x.

Fig. 12. A mass of plb's showing individual plb's differentiating and a juvenile plb (arrow). 90 days of culture. 4x.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the flask was soon filled with various sizes of tissue masses (Fig. 13).

The differentiation into shoots occurred rather rapidly and it was very difficult to maintain these masses of tissue in the juvenile stage. Generally, a large older mass differentiated first and the attached smaller masses followed later. When a small mass was separated from a differentiating clump, it showed a tendency to keep proliferating without organ formation. Therefore, a compactly oriented clump often produced a clump of shoots (Fig. 17), whereas a mass in which new protuberances were continuously separating, developed into a population of various sizes of plb and plantlets (Fig. 16).

B. Growth pattern of explants that did not form plb

Most of the axillary bud explants grew directly into shoots without forming plb. The large axillary bud arising from the basal nodes of a shoot produced explants of this type almost without exception.

A distinctive character of this growth pattern was the vigorous growth of the explant. An explant formed a ball within 10 to 20 days. In 25-30 days, a small dense area appeared at the distal end of the ball and this area grew into a juvenile leaf (Fig. 31). Leaf development and shoot growth were rather rapid and in 45-60 days, a shoot of approximately 3.0 cm in length was formed (Fig. 32).

FIG. 13-18. DEVELOPMENT OF PROTOCORM-LIKE BODIES

Fig. 13. A plb mass showing rapid proliferation, 30 days after isolating a small plb. Total culture period 86 days.

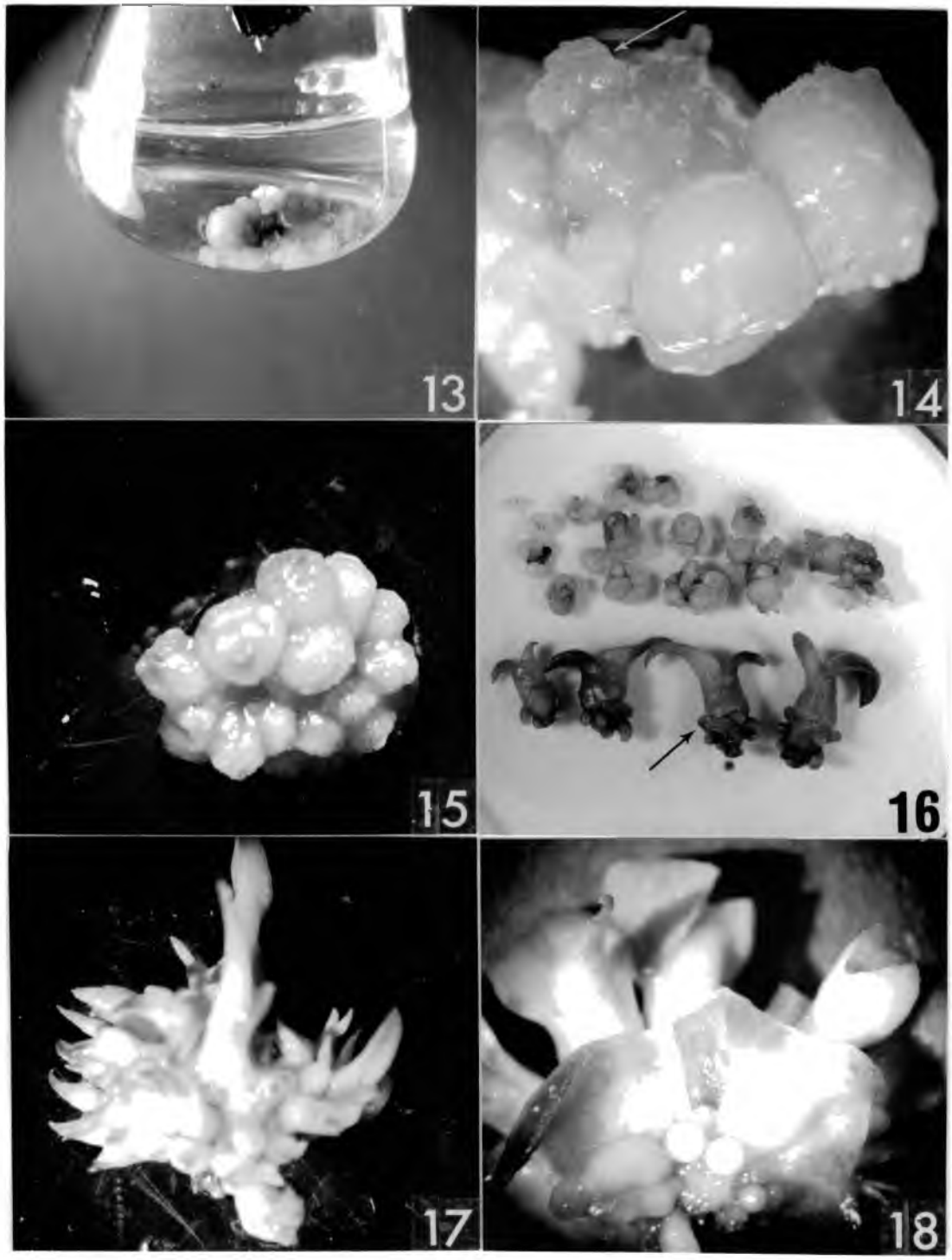
Fig. 14. Close up of Fig. 13. showing many bulges (arrow). 8x.

Fig. 15. A clump of plb which may develop into a clump of shoots or a population of various sizes of plb. Culture period 135 days. 4x.

Fig. 16. A population of plb cultured in a flask 180 days showing various stages of development. Arrow points to the remnant of basal tissue of plb.

Fig. 17. A clump of shoots derived from a mass of plb showing variation in development after 135 days of culture. 4x.

Fig. 18. A rosette of shoots in the axil of a fan-shaped leaf indicating bud proliferation of the plb at an early stage of development. 4x.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Axillary shoots usually emerged when a juvenile leaf of the explant started to grow. Axillary shoots generally arose at the base of the outer leaf of the explant and rapidly increased in thickness at the base. Hence, when an axillary shoot was 1-2 cm in size, the attachment was weak and could be separated by a light touch with an instrument. Occasionally, the axillary shoots arose in the axil of juvenile shoots and formed extremely thickened rosette leaves at the base of shoots. This thickened leaf which was developed from the outer young leaf of a ball-shaped explant dried out eventually when the explant was transferred to solid media. The time of root formation varied from 40-60 days from the initiation of culture.

C. Anatomical observation of explant development

The apical meristem of the shoot of the Dendrobium phalaenopsis hybrid examined showed a typical dome-shaped apex with three tunica layers. Primordial axillary buds were present in the second leaf axil and a well-defined shell zone (Esau, 1960) was prominent in the leaf axil further from the apex. The lateral meristem of shoots which were compactly covered with leaves without any internodal elongation, did not show any distinct axillary bud primordia. The larger axillary buds which arose at the basal nodes of shoots had 6 to 8 leaves, whereas smaller buds had 3 to 5 leaves.

The residual meristematic activity became prominent

after 30 days of culture (Fig. 19). Most of the growth was by development of new leaves and by expansion of leaves included in the explant. Considerable intercalary growth resulted in a loose structure from a formerly compact arrangement of leaves surrounding the dome of the apex.

The plb, upon isolation from the original explant, started to grow from a small white round mass of tissue. In this undifferentiated mass of tissue, several meristematic zones were distinguished from the parenchyma cells of ground tissue by small isodiametric cells with active nuclei and dense cytoplasm (Fig. 20). These superficially located zones were producing hemispherical protuberances. These zones gradually moved to the edge of new protuberances.

Because of the sunken position of the organized growing point, shoot differentiation seemed to be from a position deep in the mass of tissue (Fig. 21). The meristematic zone was reduced in area and at the same time increased in depth because of the immediate cell elongation of the other adjacent areas. The leaf-like structures which occurred because of the sunken position of the organized growing point eventually became pushed back to the base of the plantlet when a leaf emerged from the shoot apex by continued growth of the apex (Fig. 21).

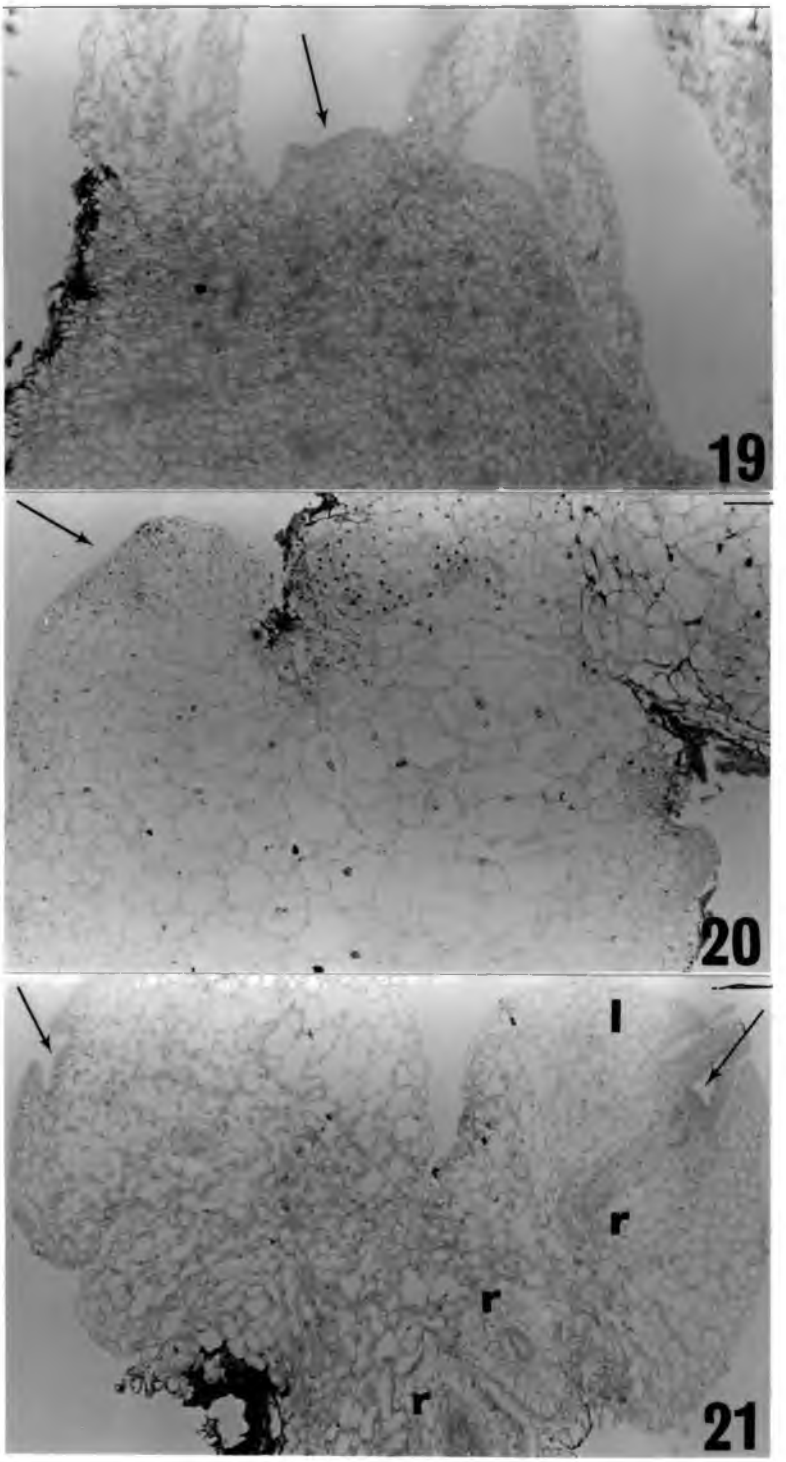
The root initials appeared to develop very early and more rapidly than the shoot apex. However, the development of root initials without any connection to the shoot apex

FIG. 19-21. ANATOMICAL OBSERVATIONS OF EXPLANT DEVELOPMENT

Fig. 19. An explant 38 days after excision, showing an activated axillary bud primordium (arrow). The intercalary growth and cell enlargement within leaves resulted in a loose structure of the explant. 48x.

Fig. 20. A small undifferentiated plb showing several regions of superficial meristematic activity (arrow). 48x.

Fig. 21. A plb showing two growing points (arrow) and three well-developed root initials (r). The shoot apex (right arrow) is surrounded by leaf-like structures (l). 48x.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was never observed. Since these two growing points (shoot apex and root initial) showed a direct connection from the early stages of differentiation, they appeared as the axial development of an embryo (Fig. 21, 22).

When a new mass of plb's proliferated from an old undifferentiated plb mass which was made up of large chlorenchyma cells and a distinct vascular system, a sharp dividing line was noted between the two tissues (Fig. 22). This indicated that only one or two superficial layers were included in the proliferation. Many sections of plb's of early differentiating stage showed outstanding lateral meristematic activity. This phenomenon occasionally led to rosette leaves or shoots (Fig. 18).

During the time a plb proliferated rapidly, a light-yellowish soft tissue occurred occasionally. This tissue was different from that of the usual plb which was firm white or green. This yellow tissue contained large parenchyma cells and some highly confined and localized meristematic areas without any superficial meristem (Fig. 23). This area was derived from a single or a group of cells. These dedifferentiated cells with dense cytoplasm, comparatively large nuclei, and small cell size were similar to Torrey's meristemoids (Torrey, 1966). This group of cells further resulted in a small bulge at the surface of the tissue (Fig. 23) and immediately formed a superficial meristem which developed as protuberances as in the plb proliferation

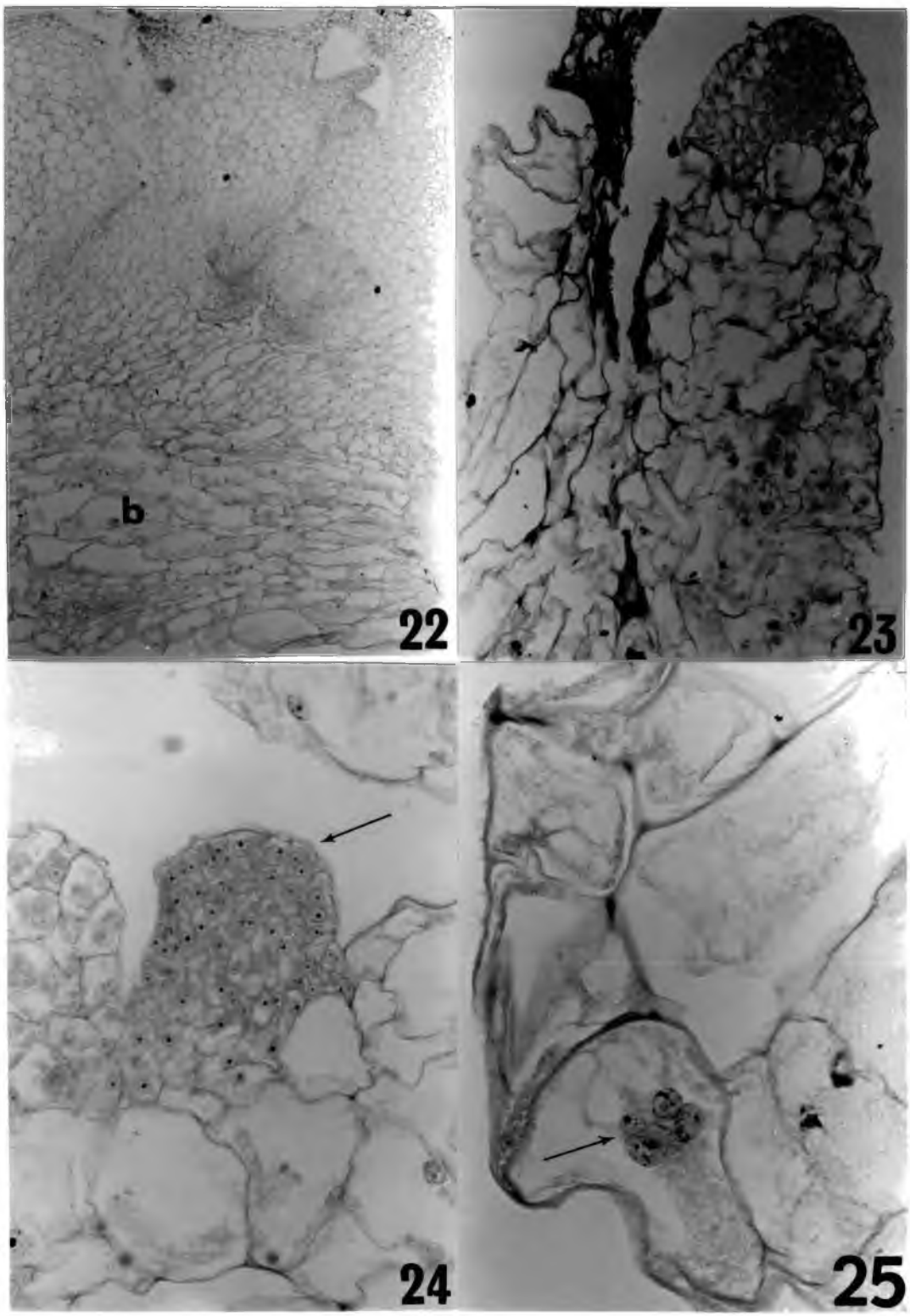
FIG. 22-25. ANATOMICAL OBSERVATIONS OF PROTOCORM-LIKE BODIES

Fig. 22. A plantlet differentiated from an old undifferentiated plb mass. The basal tissue (b) can be distinguished from newly proliferated tissue by its large cell size. 48x.

Fig. 23. A bulge consisting of rapidly dividing small cells which may be the result of constant activity of a single cell or a group of cells in the ground tissue of the plb. 61.4x

Fig. 24. Further development of dedifferentiated area into a plb structure showing a superficial meristem (arrow). 300x

Fig. 25. An abnormal somatic cell showing four nuclei (arrow) in the soft tissue of plb. 300x.


b
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(Fig. 24).

Some multinucleate cells were observed among the large parenchyma cells of the soft mass of tissue which was previously described. It was not possible to establish whether these cells were included among the meristematic cells of plb.

III. Experiments on the induction of plb

Since axillary bud explants showed a general pattern of developing into shoots without forming plb, it became apparent that further steps were necessary to induce plb's. These have been done in two ways: 1) by excising explants from axillary buds in several ways to study whether the condition of the explant at the time of excision controls the pattern of explant growth, 2) by subdividing and subculturing an explant which is growing into a shoot to study the effect of subdividing during culture on the modification of explant growth pattern.

A. Behavior of axillary bud explants in culture after various types of excision

Since the vigorous growth of explants into shoots mostly occurred in explants of axillary buds arising from the lower nodes of shoots, several different types of excision were made by 1) slicing a bud cube horizontally (Fig. 26), 2) dividing a bud cube into several pieces (Fig. 27), or 3) cubing the apical dome tissue (Fig. 28).

1. Horizontal slicing of bud cube

A large axillary bud from the basal node of a shoot (bud No. 2) was removed by four incisions and a cut 8.0 mm in depth, and sliced thinly. Each slice was numbered from 1 to 8 from the outer surface to the tissue below the bud respectively.

Slice No. 2, which possibly included the apical dome of the bud degenerated except for a small portion of the center of the slice. The medium became brown. In 10 days, two bulges were observed at the center of the piece. These grew to form leaves and a shoot was produced. Slice No. 3 first degenerated in the same manner as Slice No. 2 up to 30 days. Two small round plb were observed around the center of the explant, which were separated from each other. After 55 days, each plb produced two or three small plb's. All the rest of the slices degenerated without regeneration.

A slight modification of the above method of excision was made to a large axillary bud. After removing four outer leaves from the bud, the cube was removed and sliced. Among the slices, only slice No. 1 grew to produce some plb's, following the same manner of growth as the previous slice No. 3. Because of discoloration of the medium a transfer to new medium was made in 18 days.

2. Division of the bud into several pieces

a) Dividing a bud cube at right angles to the stem axis (Fig. 27-a)

A bud cube was cut into two or three pieces by one or

FIG. 26-28. VARIATIONS OF EXCISION

Fig. 26. Slicing bud cube. Axillary buds were bounded by 4 incisions adjacent to the bud and removed by a cut 8.0 mm behind the bud resulting in a cube (Inset A). This cube was sliced horizontally making 8 thin slices (Inset B).

Fig. 27. Dividing bud cube.

a. Dividing a bud cube at right angles to the stem axis. A bud cube which was bounded the same way as in Fig. 26 was cut 3.0 mm behind the bud followed by two vertical incisions at right angles to the stem axis making 3 pieces--upper, middle, and lower piece of bud.

b. A bud cube was divided by a vertical incision at right angles to the stem axis making two pieces--upper and lower pieces. The lower half was sectioned again into three pieces by two vertical incisions parallel to the stem axis.

c. A bud cube was divided into upper and lower halves by a vertical incision. A bud cube was sectioned into 8 pieces by a vertical incision at right angles to the stem axis and three incisions parallel to the stem axis.

Fig. 28. Cubing the apical dome. Apical dome of axillary bud was exposed by removing five leaves and a $2mm^3$ including the apex areas and 4 separated $2mm^3$ were excised.

FIG. 26. SLICING BUD CUBE

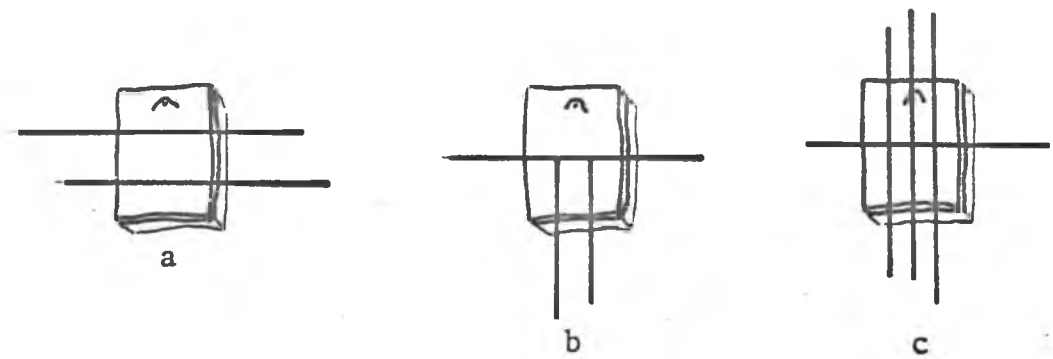


FIG. 27. DIVIDING BUD CUBE

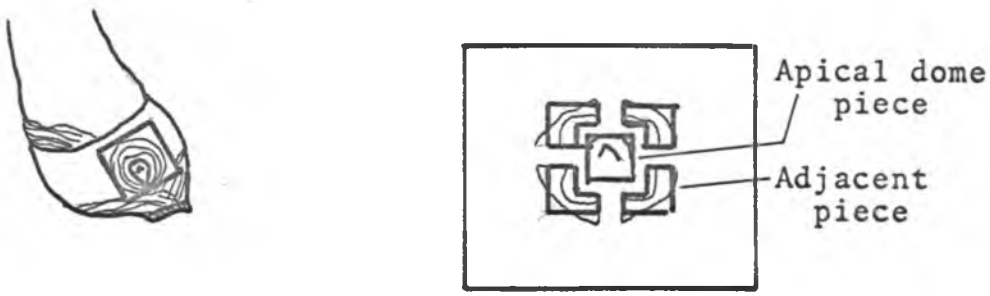


FIG. 28. CUBING THE APICAL DOME

two incisions. When the bud cube was cut into two pieces, the upper half showed an initial growth which resulted in leaf elongation, then gradually degenerated. The lower half grew into a ball of tissue after 40 days. The degeneration of surface tissue was followed by plb formation.

When a bud was divided into three pieces, each piece grew in one of the following ways: 1) direct growth into a shoot, 2) initial growth of explant into a ball shape followed by degeneration of outside tissue and plb production, 3) initial growth of leaf piece and eventual termination of growth. In several trials, the middle pieces usually grew while the upper and lower pieces occasionally grew.

b) Dividing a bud cube parallel to the stem axis (Fig. 27-b, 27-c)

After dividing a bud cube into two pieces by an incision at right angles to the stem axis, the lower half, was sectioned again into 3 pieces by cutting parallel to the stem axis (Fig. 27-b). The upper half grew into a shoot with 2 axillary shoots, while of the 3 pieces of the lower half, only the middle piece grew to form plb (Fig. 33).

A bud cube which was divided into two pieces was sectioned into eight pieces by three incisions parallel to the stem axis (Fig. 27-c). All of the 8 pieces were cultured in the same flask and had to be transferred to new media every 10 days because of discoloration of the medium. Four explants terminated growth after 20 days and after 50

days only one survived to form plb.

3. Cubing the apical dome tissue

Five leaves were removed from a bud, including the outer prophyll, resulting in the exposure of the green primodial leaf. A cube of $5mm^3$ was excised from the axillary bud. A $1\text{-}2mm^3$ including the apical dome was then excised. The remaining cube tissue was divided into 4 equal pieces (Fig. 28). The apical dome explant produced plb. The medium became discolored after 30 days even when the volume of explant was comparably small. The explants from the adjacent area degenerated.

B. Growth of subcultures after fractionation

Explants showing shoot growth in 30-60 days after initial culture were subdivided or re-excised to study the regeneration potential of explants or the induction of plb in subsequent subcultures.

1. Subdivision of explants (Fig. 29)

a) Longitudinal cut

A longitudinal cut was made when an explant formed a ball. In most cases, only one half regenerated or produced plb. Before regeneration or production of plb, the surface of the half piece usually degenerated. When the regeneration or plb production was obvious, the pieces had completely degenerated (Fig. 34). Regeneration or plb formation from both halves was infrequent.

b) Transverse cut

Several explants which grew to shoots 1 and 2 cm in

length were divided by a transverse cut. In one shoot, in which the cut was made just below the middle of the shoot, both halves grew. In two other plantlets, only the proximal pieces grew to shoots or produced plb. A proliferation of the axillary bud was observed in one of the proximal pieces. The proximal half at first formed one or two axillary buds which continued to proliferate. There was some difference in morphology since the bud-shaped form was present early in development in contrast to the approximately round mass normally found from other proliferating plb's. The sequential development of the shoot pieces is diagrammed in Fig. 29.

2. Re-excisions from plantlets grown in initial culture (Fig. 30)

The plantlets which reached 2.5-3.0cm, after 55-88 days in initial culture were used for re-excision of buds. Since the internodes of these plantlets had not elongated, they were spherical after removal of 2 or 3 leaves. The apical buds were removed by the same method as in initial culture. One or 2 axillary buds, or sometimes 4 buds, were excised from the plantlets. The plantlets remaining after excision were also cultured.

The secondary explants of apical buds grew in the same pattern as the standard growth pattern of initial cultures, but the proliferation of plb was faster. The plantlets from which apical buds were excised produced small white tissues

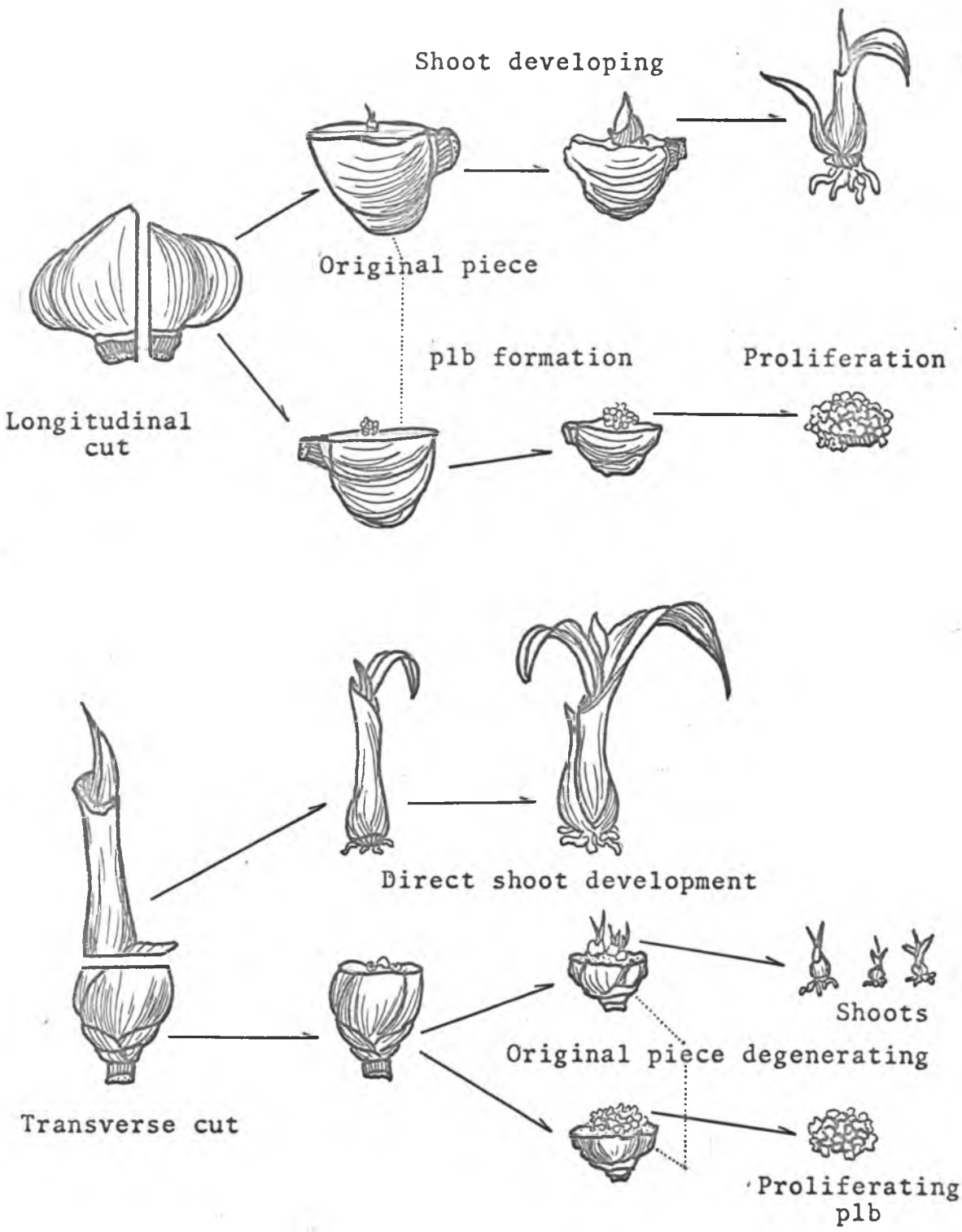


FIG. 29. SEQUENTIAL DEVELOPMENT IN SUBCULTURE OF CUT SHOOT

in two sites of the primordial axillary bud, after degeneration of most of the original plantlets. These small mass of tissue proliferated into a plb mass upon subculture.

In the case of 6 plantlets, 4 axillary buds were sufficiently differentiated to be identified macroscopically. These buds, upon subculture, grew directly into shoots or produced plb's, whereas the remaining plantlet bodies produced plb masses after degeneration of the original plantlet bodies. The sequential development of each re-excised explant is diagrammed in Fig. 30.

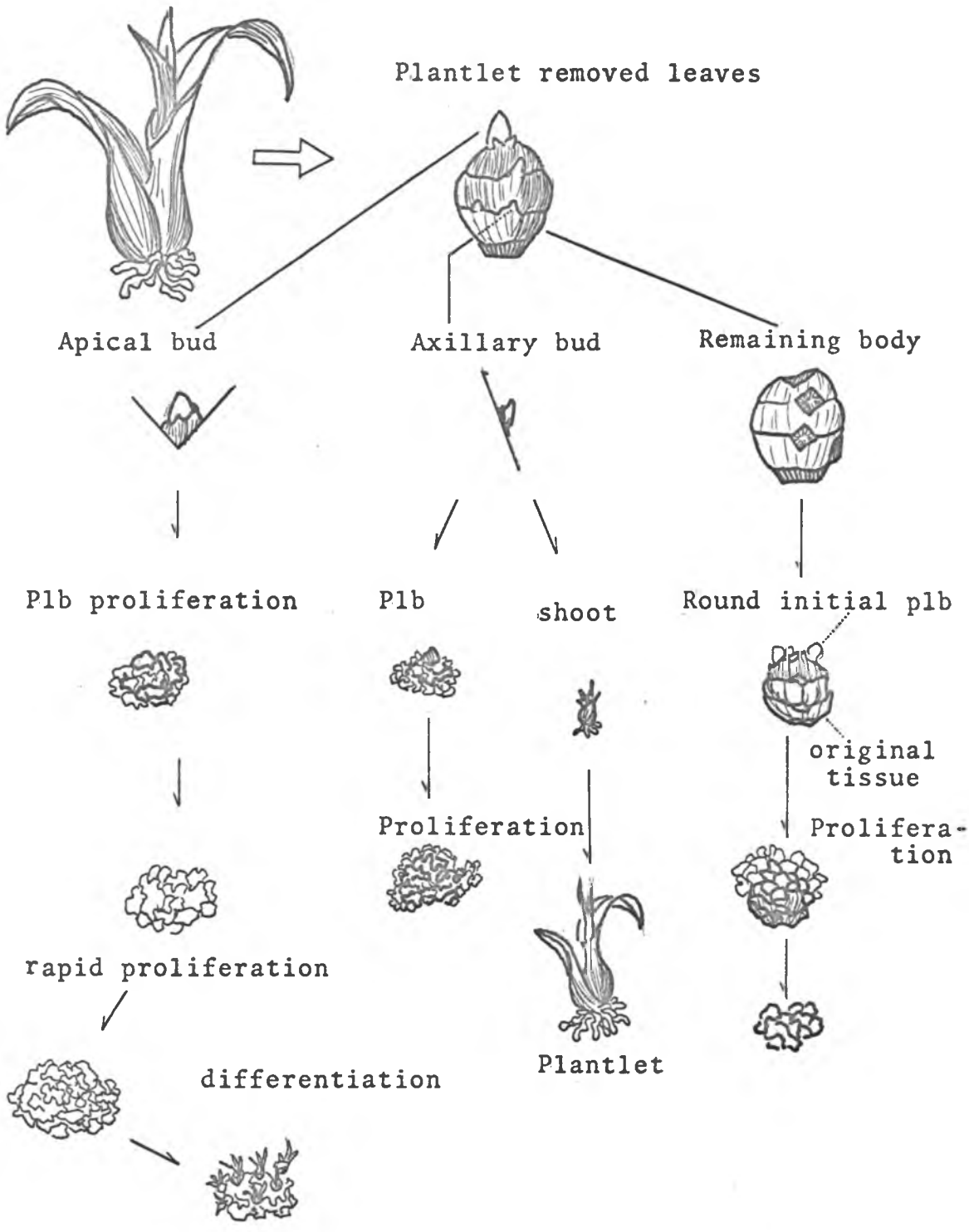


FIG. 30. SEQUENTIAL DEVELOPMENT OF VARIOUS SUBCULTURE EXPLANTS AFTER RE-EXCISION

FIG. 31-32. A TYPICAL PATTERN OF AN AXILLARY BUD EXPLANT

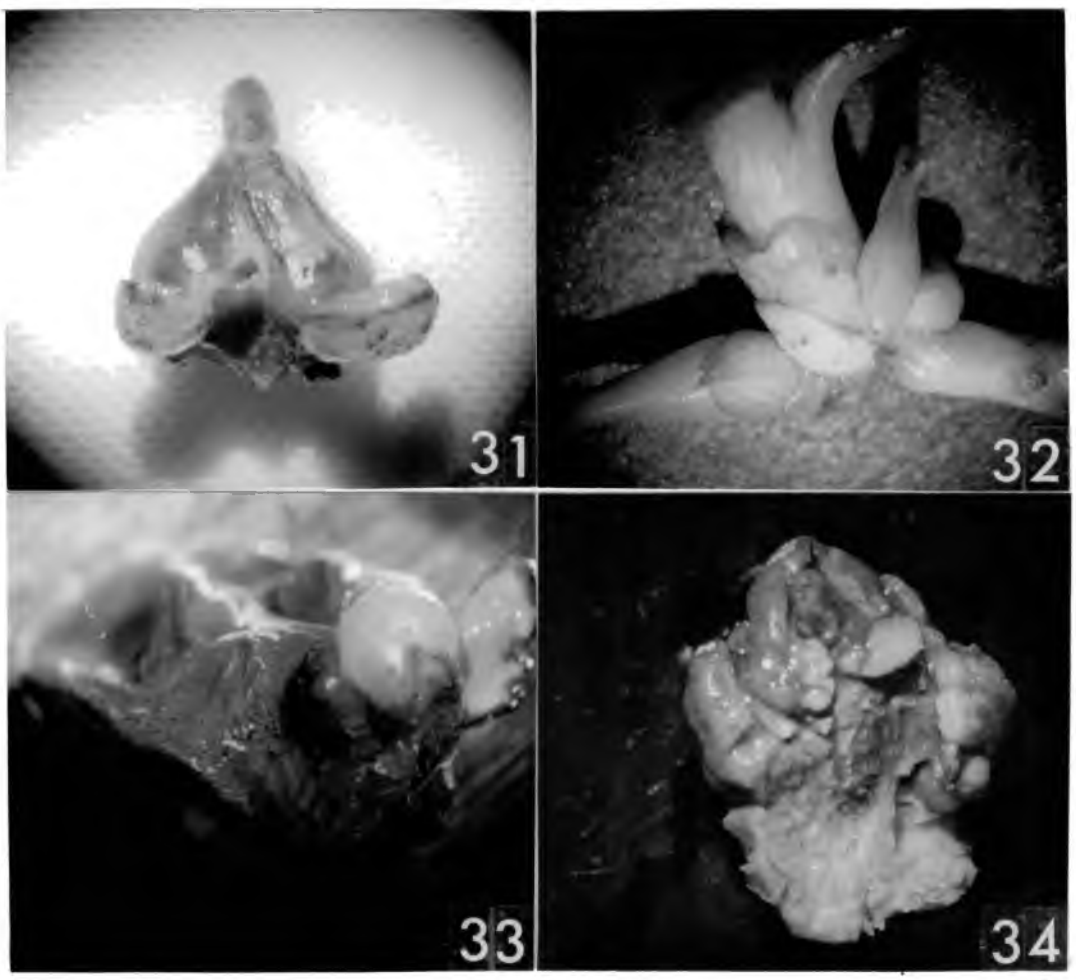
FIG. 31. Explant from a large axillary bud showing rapid and vigorous growth after 37 days with juvenile leaf development at the top. 4x.

Fig. 32. Explant showing direct shoot growth and three axillary shoots after 60 days of culture. 4x.

FIG. 33-34. INDUCTION OF PLB FROM AXILLARY BUD EXPLANT

Fig. 33. A middle section slice of lower half of axillary bud after 30 days of culture showing plb formation from the degenerating explant. Compare with Fig. 31 which is the same age. 8x.

Fig. 34. A half section of a shoot grown in initial culture showing plb formation from degenerating tissue 52 days after fractioning. Total culture period of 128 days. 4x.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DISCUSSION

A total of 95 successful cultures were obtained from 256 uncontaminated explants from new shoots of Dendrobium phalaenopsis hybrids resulting in 37.1% success. Upon isolation from the shoot, apical bud explants with median shoot weight of 2.0 gm have the highest percentage of survival. This may be related to the maximum in rate of growth attained by the new shoots. This in turn, may be related to the developmental stage of the shoot with the maximum number of nodes and the highest potential for intercalary meristematic activity. Axillary buds showed acropetally gradual decrease from 50% to 0% in success of culture. The proximal bud had 6-8 leaves and the distal bud 3-5 leaves. The success of culture may again be related to the number of nodes present per bud and the greater potential of the intercalary meristem. A further consideration may be the decreasing size of explant.

The explants which grew either developed plb's which eventually differentiated into plantlets or developed into single or multiple shoots. Apical bud explants and small axillary bud explants from nodes 4-5 showed a high frequency of production of plb's preceded by degeneration of the apical meristem. On the other hand, large axillary bud explants from nodes 1-3 produced shoots. Meristem culture has been successful only when some leaf primordia were included on the explant (Morel and Martin, 1955; Phillips and Matthews, 1964;

Morel, 1960). The isolated dome itself, without any leaf primordia, did not develop into a whole plant because this dome of meristematic tissue was biochemically differentiated to the point of loss of synthetic ability for certain unknown essential substances (Ball, 1960). Hence, tissues associated with and inlcuding adjacent leaf primordia initially excised with the apical dome retain an essential capacity which allows the development of the whole organism (Torrey, 1966). The large axillary bud explants fall into this category. In the smaller explants, insufficient associated tissues were preserved, resulting in a decline of the apical dome and the development of a next of meristematic cells into the formation of plb. Anatomical studies revealed axillary primordial activity shortly after explanting. Experiments on bud slices have generally resulted in plb formation on only one slice per bud. In two cases, plb formation occurred at two independent sites on the slice rather than along the node as observed in Cymbidium. Hence in Dendrobium plb formation may be the result of activity of axillary bud primordia.

As plb formation was generally preceded by the degeneration of the apical meristem and necrosis of the outer surfaces of the explant, this resulted in the isolation of axillary bud primordia which were then induced to form plb rather than axillary shoots. Cell proliferation was taking place rapidly when tissue cells were physically isolated from each other by spontaneous separation in an elaborate nutrient culture

medium (Steward et al., 1964). Hence 'physiological isolation' of a cell within a tissue system may produce an essentially similar effect in releasing the cell from inhibitions imposed upon it by its neighbors. This allows an expression of its full potentialities, including the formation of a whole new plant (Torrey, 1966).

New shoots 2.0 gm in median weight with an average length of 4.9 cm provide the optimum source for explants for meristem culture. Large axillary buds, in which strong apical dominance prevails, grew mostly into single shoot or multiple shoots. However, plb's can be induced by eliminating the apical dominance of the bud apex by mutilating the apex when excising or by isolating meristematic tissues from juvenile shoots in culture. Small axillary buds, as a source of explants, are not effective because of the poor survival percentage although they are much more likely to form plb's upon survival. Hence the apical bud as well as axillary buds of the lower three nodes of a shoot are the best source of materials for explants. Rapid propagation through meristem culture can be accomplished by continuously separating newly developed plb from juvenile plb mass.

SUMMARY

Meristem explants from new shoots of Dendrobium phalaenopsis were cultured in Vacin and Went media modified by the addition of 15% coconut milk by volume to establish the most suitable source of explants and to define the most efficient methods for the induction of protocorm-like bodies.

The percentages of survival of explants both from apical bud and axillary bud were studied in relation to the weight of detached shoot. It was determined that comparatively small shoots weighing about 2.0 gm and 4.9 cm in length were the best sources of explants. Axillary buds showed an acropetal decrease in success of culture, hence the basal three buds were found to be the best source of explants.

The explants which grew either developed protocorm-like bodies which eventually differentiated into plantlets or developed into single or multiple shoots. Apical bud and small axillary bud explants showed a high frequency of production of protocorm-like bodies.

These studies suggest that protocorm-like bodies originated in lateral bud primordia. Physiological isolation resulting from the decline of surrounding tissues was essential for the induction of protocorm-like bodies rather than axillary shoots. Protocorm-like bodies can be induced from large axillary bud explants by mutilating the bud apex or from apical or axillary bud explants from juvenile shoots in culture.

LITERATURE CITED

Baker, R. and D. J. Phillips. 1962. Obtaining pathogen-free stock by shoot tip culture. Phytopathology 52:1242-1244.

Ball, E. 1946. Development in sterile culture of stem tips and subjacent regions of Tropaeolum majus L. and of Lupinus albus L. Am. J. Botany 33:301-318.

_____. 1960. Sterile culture of the shoot apex of Lupinus albus. Growth 24:91-110.

Belkengren, R. O., and P. W. Miller. 1962. Culture of apical meristem of Fragaria vesca, strawberry, plants as a method of excluding latent A virus. Plant Disease Reporter 46:119-121.

Bertsch, W. 1967. A new frontier: Orchid propagation by meristem tissue culture. Am. Orchid Soc. Bull. 36:32-37.

Bolton, A. T., and L. H. Turner. 1962. Note on obtaining virus-free plants of red raspberry through the use of tip cutting. Canadian J. Plant Sci. 42:210-211.

Chapman, H. W. 1955. Potato tissue cultures. Am. Potato J. 32:207-210.

Curtis, J. T., and M. A. Nichol. 1948. Culture of proliferating orchid embryo in vitro. Bull. Torrey Botan. Club 75:358-373.

Dillon, G. W. 1964. Speaking orchidwise. The meristem Merry-Go-Round. Am. Orchid Soc. Bull. 33:1023-1024.

Esau, K. 1960. Anatomy of seed plants. John Wiley and Sons, New York. 376 p.

Hackett, W. P., and J. M. Anderson. 1966. Aseptic multiplication and maintenance of differentiated carnation shoot tissue derived from shoot apices. Proc. Am. Soc. Hort. Sci. 90:365-369.

Halperin, W. 1964. Morphogenetic studies with partially synchronized cultures of carrot embryo. Science 146:408-410.

Hartman, H. T., and D. E. Kester. 1968 Plant Propagation, Principles, and Practices. Prentice-Hall, Inc., New Jersey. 702 p.

Hill, G. P. 1967. Morphogenesis in stem-callus cultures of Convolvulus arvensis L. Ann. of Botany 31:437-447.

_____. 1968. Shoot formation in tissue cultures of chrysanthemum 'Bronze Pride'. Physiol. Plantarum 21:386-389.

Hollings, M. 1959. Virology. Pp. 66-67 in Rept. Glasshouse Crops Res. Inst.

Holmes, F. O. 1955. Elimination of spotted wilt from dahlias by propagation of tip cuttings. Phytopathology 45:224-226.

_____. 1956. Elimination of aspermy virus from the Nightingale chrysanthemum. Phytopathology 46:599-600.

Ilsley, P. 1965. Meristem tissue propagation techniques and potentials. Orchid Rev. 73:371-376.

Kassanis, B. 1957. The use of tissue culture to produce virus free clones from infected potato varieties. Ann Appl. Biol. 45:422-427.

Kester, D. E., and C. O. Hesse. 1955. Embryo culture of peach varieties in relation to season ripening. Proc. Am. Soc. Hort. Sci. 65:265-273.

Lammerts, W. E. 1962. Practical values of embryo culture in nursery practice. Proc. Int. Plant Prop. Soc. 12:204-207.

Manzer, F. E. 1959. Potato meristem culture and virus X. Am. Potato J. 36:191-195.

Morel, G. M. 1960. Producing virus-free Cymbidium. Am. Orchid Soc. Bull. 29:495-497.

_____. 1964. Tissue culture: a new means of clonal propagation of orchids. Am. Orchid Soc. Bull. 33:473-478.

_____. 1965. Clonal propagation of orchids by meristem culture. Cymbidium Soc. News 20:3-11.

_____., and C. Martin. 1955. Guerison des plantes atteintes de maladies a virus; par culture de meristemes apicaux. Report of XIV Intern. Hort. Cong., Netherlands. Pp. 303-310.

Nielson, L. W. 1960. Elimination of the internal cork virus by culturing apical meristems of infected sweet potatoes. Phytopathology 50:840-841.

Niimoto, D. H., and Y. Sagawa. 1961. Ovule development in Dendrobium. Am. Orchid Soc. Bull. 30:813-819.

Norris, D. O. 1954. Development of virus-free stock of Green Mountain potato by treatment with malachite green. Australian J. Agr. Res. 5:658-663.

Oshima, N., and C. H. Livingston. 1961. The effects of antivral chemicals on potato virus X-1. Am. Potato J. 38:294-299.

Phillips, D. J., and G. J. Matthews. 1964. Growth and development of carnation shoot tips in vitro. Botan. Gaz. 125:7-12.

Pillai, S. K., and A. C. Hildebrandt. 1968. Geranium plants differentiated in vitro from stem tip and callus cultures. Plant Disease Reporter. 52:600-601.

Reinert R. A. and H. C. Mohr. 1967. Propagation of Cattleya by tissue cultures of lateral bud meristems. Proc. Am. Soc. Hort. Sci. 91:664-671.

Sagawa, Y., T. Shoji, and T. Shoji. 1966. Clonal propagation of Cymbidium through shoot meristem culture. Am. Orchid Soc. Bull. 35:118-122.

_____, _____, and _____. 1967. Clonal propagation of Dendrobiums through shoot meristem culture. Am. Orchid Soc. Bull. 36:856-859.

Scully, R. M. Jr. 1967. Aspects of meristem culture in the Cattleya alliance. Am. Orchid Soc. Bull. 36:103-108.

Shapiro, S. 1947. Warm Safranin for plant tissues. Science 105:50.

Steward, F. C., M. O. Mapes, and K. Mears. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am. J. Botany 45:795-708.

_____, _____, A. E. Kent, and R. D. Holstein. 1964. Growth and development of cultured plant cells. Science 143:20-27.

Takatori, F. H., T. Murashige, and J. I. Stillman. 1968. Vegetative propagation of asparagus through tissue culture. Hortscience 3:20-23.

Torrey, J. G. 1966. The initiation of organized development in plants. Advances in Morphogenesis 5:39-91.

Vacin, E., and F. Went. 1949. Some pH changes in nutrient solutions. Botan. Gaz. 110:605-619.

Vasil, I. K., and A. C. Hildebrandt. 1966. Variations of morphogenetic behavior in plant tissue cultures. I. Cinchorium endivia. Am. J. Botany 53:860-869.

_____, and _____. 1966. Variations of morphogenetic behavior in plant tissue cultures. II. Petroselinum hortense. Am. J. Botany 53:869-874.

_____, and _____. 1966. Differentiation of tobacco plants from single, isolated cells in microculture. Science 150:889-892.

Wimber, D. E. 1963. Clonal multiplication of Cymbidium through tissue culture of the shoot meristem Am. Orchid Soc. Bull. 32:105-107.

_____. 1965. Additional observations on clonal multiplication of Cymbidium through culture of shoot meristem. Cymbidium Soc. News 20:7-10.

Winton, L. L. 1968. The rooting of liquid-grown aspen callus. Am. J. Botany 55:195-167.

Withner, C. L. 1959. The Orchids. Ronald Press Company, New York. 647 p.