PROPAGATION OF TROPICAL PLANTS BY TISSUE CULTURE

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ABSTRACT

Experiments were conducted to establish more efficient propagation systems for aroids and other monocot families by tissue culture. Scindapsis aureus (Lind. and André.) Engl was chosen as a model plant. Top 1st and 2nd nodes produced multiple shoots in Murashige-Skoog medium supplemented with 10 ppm BA which elongated when transferred to 1 ppm BA, and formed roots in 1 ppm NAA medium; the resulting plantlets were successfully cultured in pots. Internodal and leaf tissues also produced adventitious buds in medium supplemented with BA + NAA. From the results of antiauxin treatment, involvement of endogenous auxin in bud formation was suggested. Intercalary meristem in node tissue and marginal meristem in leaf tissue were suggested as sites of auxin production. A hypothesis of basipetal movement and accumulation of endogenous auxin was proposed to explain the location of bud formation in leaf segment culture in BA medium. Polyploidy was not found in the plantlets obtained by node culture. Propagation by node culture was successful in Philodendron oxycardium Schott, Philodendron lacerum (Jacq.) Schott, Spathiphyllum 'Clevelandii', and Alocasia cucullata (Lour.) Schott. From these results, a general procedure for propagation of aroids was proposed. Similar procedure can be applied to other plants in different families (Liliaceae, Asparagus myriocladus Hort, Zingiberaceae, Zingiber officinale Roscoe, and Orchidaceae, Doritaenopsis Dorette. However, Asparagus myriocladus failed to form roots. Neomarica caerulea (Ker-Gaw) Sprague in Iridales, which is phylogenetically

located between Orchidales and Liliales, formed multiple shoots by node culture. Woody monocots and dicots were not successfully propagated by this method. Anatomical study on node culture suggests that several epidermal cells and cells of some subepidermal layers are involved in forming a bud. Histochemical and micro-autoradiographic studies showed increases in DNA, RNA, proteins, reducing sugars, activities of respiration enzymes, and activity of peroxidase prior to bud formation. Anatomical study of leaf culture suggested that several cell layers were involved in forming a tumor-like tissue. Adventitious buds formed on the peripheral zone in the tumor.

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INTRODUCTION

In order to establish productive acreages of a number of food and ornamental crops which are highly heterozygous, rapid and efficient methods of asexual propagation are essential once desirable clones have been selected after breeding or plant exploration. Propagation by current methods of division, cutting, and grafting, are relatively slow and require substantial area.

Recently, tissue culture has been shown to be useful for rapid clonal propagation of some plants (Murashige, 1974). In propagation by tissue culture, there are two approaches: one is through induction of callus which, in turn, is induced to differentiate into plantlets and the other is through induction of adventitious shoots without callus formation. Rapid clonal propagation through shoot tip culture is highly successful with some orchid clones and is currently utilized commercially by some orchid nurseries. Recent efforts are shifting to induction of adventitious shoots without callus formation since some callus cultures have been found to vary in chromosome number which may result in plants with undesirable morphological changes (Shimada, 1967; Norstog et al., 1969). On the other hand, Hasegawa et al. (1973) and Yang and Clore (1973) have reported that plantlets obtained through shoot tip or lateral bud cultures have normal chromosome numbers.

Since a number of useful food and ornamental crops belong to Araceae, Liliaceae, and Zingiberaceae, the object of this dissertation was to investigate whether tissue culture could serve as an

efficient system for rapid asexual propagation in these families. In order to develop a model system, node and leaf cultures from Scindapsis aureus, an aroid available in abundance, were studied. Once adventitious buds were induced, physiological, morphological, anatomical, histochemical and microautoradiographic studies were used to elucidate the process.

MATERIALS AND METHODS

Specimens of 15 taxa in 10 families as listed below were obtained from Lyon Arboretum, Horticulture Department or on campus of the University of Hawaii.

Apocynaceae: *Plumeria obtusa* L.

Araceae: *Alocasia cucullata* (Lour.) Schott.

Monstera deliciosa Liebm.

Philodendron oxycardium Schott.

Philodendron lacerum (Jacq.) Schott.

Scindapsis aureus (Lind. and André.) Engl.

Spathiphyllum 'Clevelandii'.

Iridaceae: *Neomarica caerulea* (Ker-Gawl.) Sprague.

Leguminosae: *Erythrina crista-galli* L.

Liliaceae: *Asparagus myriocladus* Hort.

Orchidaceae: *Doritaenopsis* Dorette.

Palmae: *Chrysalidocarpus lutescens* (Bory) Wendl.

Proteaceae: *Macadamia integrifolia* Maiden and Betche.

Rubiaceae: *Coffea arabica* L.

Zingiberaceae: *Zingiber officinale* Roscoe.

Culture medium consisted of major elements (Murashige and Skoog, 1962), modified Nitsch-Nitsch minor elements and vitamins as listed below, 2% sucrose and 0.8% agar.

Minor elements		Organic supplements	
$MnSO_4 \cdot 4H_2O$	25.8[a]	glycine	2
H_3BO_4	10	myo-inositol	100
$ZnSO_4 \cdot 4H_2O$	10	nicotinic acid	5
KI	1	pyridoxine HCl	0.5
$Na\ MoO_4 \cdot 2H_2O$	0.25	thiamine HCl	0.5
$CuSO_4 \cdot 5H_2O$	0.025	biotin	0.05
$CoCl_2 \cdot 6H_2O$	0.025	folic acid	0.5

[a]Quantities are given in milligrams per liter.

N^6-benzylaminopurine (BA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-butyric acid (IBA) and gibberellin (GA) were used as supplements. GA was added to the autoclaved medium through sterilized millipore filter. Medium was adjusted to pH 6.0 with NaOH and autoclaved at 120°C at 15 psi for 15 minutes.

Plant materials were washed with detergent, soaked in 95% ethanol for several seconds, rinsed with sterile water, sterilized in 10-15% Clorox for 10 minutes, and finally rinsed twice with sterile water. Excised tissue was cultured in 11x2 cm vials, 16x2 cm test tubes or 50 ml Erlenmeyer flasks at 27±2°C under 250 ft candles of continuous illumination from white fluorescent lamps. Liquid cultures in 50 ml Erlenmeyer flasks were continuously agitated at approximately 120 rpm on a New Brunswick Model V shaker.

Cultures were studied morphologically and photographed with a Wild stereomicroscope with built-in photographic camera.

Tissue for anatomical studies was fixed in FAA (90 parts 60% ethanol : 5 parts glacial acetic acid : 5 parts 40% formaldehyde) under reduced pressure, dehydrated in a graded series of tertiary butyl alcohol (Johansen, 1940) and embedded in paraffin. Ten micron sections cut with a rotary microtome were stained with safranin and fast green (Jensen, 1962).

For histochemical studies, fresh hand sections or tissue fixed in FAA or modified Carnoy (6 parts 70% ethanol : 1 part glacial acetic acid : 5 parts chloroform) were used. The techniques with modifications and specifications are listed below. Sections were studied and photographed with a Zeiss photomicroscope.

Compounds	Techniques	Fixation	Specifications	Reaction
DNA	Feulgen (Jensen, 1962)	Carnoy	Hydrolysis in 1N HCl, 15 minutes. Staining in Schiff's reagent, 30 minutes.	reddish purple
DNA, RNA	Methyl Green-Pyronin Y (Jensen, 1962)	Carnoy	1% methyl green + 0.4% pyronin Y, 30 minutes.	blue (DNA) pink (RNA)
Total proteins	Ninhydrin-Schiff's (Jensen, 1962)	FAA	0.5% ninhydrin, 24 hours. Staining in Schiff's reagent 30 minutes	reddish purple
Histones	Fast Green (Jensen, 1962)	FAA	0.1% fast green FCF, 30 minutes.	green
Total carbohydrates of insoluble polysaccharides	PAS (Jensen, 1962)	FAA	0.5% periodic acid, 20 minutes. Schiff's reagent, 10 minutes.	reddish purple

Compounds	Techniques	Fixation	Specifications	Reaction
Reducing sugars	Fehling (Hawk et al. 1951)	fresh hand section	A few drops of reagent on the tissue and heat till it boils.	pink
Respiration enzyme, succinate dehydrogenase	Nitro-BT (Jensen, 1962)	fresh hand section	Incubation mixture at 37°C, 10 minutes.	deep blue
Respiration enzyme, cytochrome oxidase	Nadi (Jensen, 1962)	fresh hand section	Incubation mixture at room temp. 5 minutes.	blue
Respiration enzyme, glucose-6-phosphate dehydrogenase	Nitro-BT (Nachlas et al., 1958	fresh hand section	Incubation mixture at 37°C, 15 minutes.	blue
Peroxidase	Benzidine (Povvaia, 1973)	fresh hand section	1% H_2O_2+ 0.01 M benzidine-base, at room temp., 5 seconds.	brown
IAA and indole derivatives	Ehlrich[a] (Hawk et al., 1951)	fresh hand section	Incubation, 5 minutes.	pink

[a]Ehlrich reagent: 1 part of conc. HCl + 1 part of 5% dimethyl-aminobenzaldehyde in absolute ethanol.

For microautoradiographic study of RNA (Fig. 1), general procedure was that described by Herrmann and Abel (1962). Tissue was incubated in 5 cc of liquid culture medium containing 10μ Ci/ml of tritiated RNA precursor, cytidine-3 H (specific activity 36 Ci/mmole) for 4 and 12 hours, fixed in modified Carnoy, washed twice with 5% ice-cold trichloroacetic acid for 5 minutes, and rinsed with ice-cold

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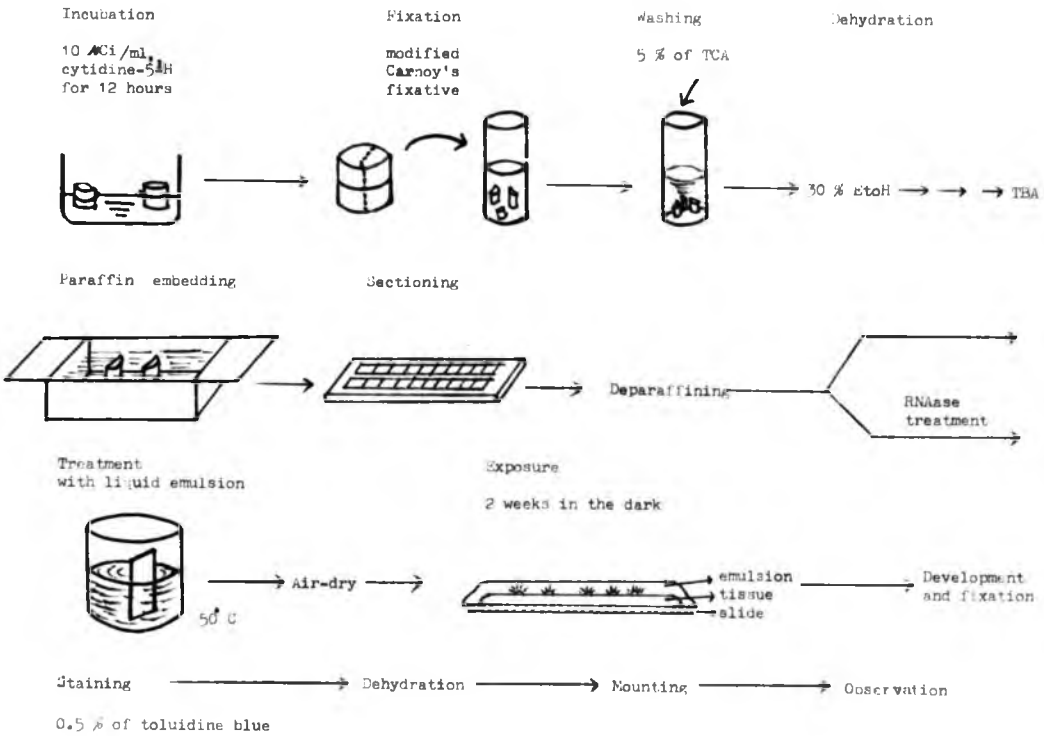
Procedure of microautoradiography



water to remove unincorporated cytidine. Four micron sections were made instead of 10 micron to decrease self-absorption by tissue. Paraffin sections were placed on slides coated with gelatin adhesive (0.5% gelatin + 0.05% chromium potassium sulfate) instead of Haupt's adhesive. After removal of paraffin, slides were dipped twice in a diluted liquid emulsion, Kodak NTB-2, at 50°C and dried in gentle airstream overnight. Then, they were put into a light-tight plastic box with silica gel, exposed for 2 weeks at 4°C, developed in D-19 for 4 minutes, and fixed in acid fixer for 8 minutes. For staining, 0.5% of toluidine blue was used. For control, several slides were treated with 0.2 mg/ml of RNA
dipping in liquid emulsion.

Somatic chromosome counts were made from aceto-orcein smears of root tips excised from plantlets in culture. Tissue was pre-treated in a saturated p-dichlorobenzene solution for 90 minutes, fixed in Carnoy and hydrolyzed in 1-HCl at 60°C for 5 minutes.

RESULTS

The results will be presented in two major sections. Section I includes results on the various aspects of node and leaf cultures of Scindapsis aureus. Section II includes results on application and modifications of techniques developed in Section I to other aroids or other genera.

I. Scindapsis aureus

A. Node culture

1. Morphological and physiological studies

2. Anatomical study

3. Histochemical studies

4. Microautoradiographic study

B. Leaf culture

1. Morphological and physiological studies

2. Anatomical study

II. Other genera

A. Other aroids

B. Other genera

I. Scindapsis aureus

A. Node culture

1. Morphological and physiological studies

a. Effect of BA on shoot and root formation from node

This experiment was conducted to induce adventitious buds from nodal tissue. Stem sections of 3 cm length which included the 2nd node were sterilized, 6 mm sections were then excised and cultured in 0, 1

and 10 ppm BA medium for 75 days (Fig. 2). The results are shown in Table 1 and Figure 3. In 1 ppm BA medium, the axillary bud elongated and a few roots formed. In 10 ppm BA medium, small multiple shoots without roots were obtained on the nodal area. No roots or shoots were produced in basal medium (0 ppm BA). In Figures 4 and 5, early developmental stages of bud formation (40 days) are shown.

Table 1. Effect of BA on bud and root formation on node after 75 days in culture

BA conc. (ppm)	Avg. no. of buds induced[a]	Avg. height of bud (cm)	Avg. no. of roots[a]
0	0	0	0
1	1	4.5	2.0
10	8.0	1.0	0

[a]Mean of 10 sections.

b. Differences in adventitious bud formation on nodes from different positions

This experiment was conducted to determine whether differences in adventitious bud formation occur when nodes from different positions are cultured in 10 ppm BA medium. Stem sections with 1st (top), 2nd, 3rd and 4th nodes were cultured in 10 ppm BA medium. The results are shown in Table 2 and Figure 6. The 1st and 2nd nodes formed adventitious buds; on the 3rd and 4th nodes, the axillary bud elongated and branched. The 1st node produced more adventitious buds than the 2nd node. Buds in the 1st node culture emerged from

Shoot and root formation by node culture (Scindapsis aureus)

Figure 2. Sequence for node culture. 0.5X. Left: shoot axis, middle: excised node, right: cultured node.

Figure 3. 75-day culture in BA medium. 0.5X. Left: culture in basal medium showing no growth, middle: culture in 1 ppm BA showing elongation of axillary bud, right: culture in 10 ppm BA showing adventitious bud formation.

Figures 4 and 5. Adventitious buds on the nodal zone induced in 10 ppm BA 40 days after culture. 20X.

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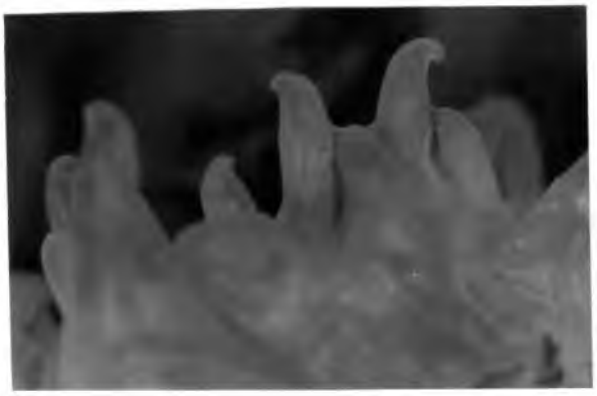

the basal part of stem section while those in the 2nd node culture emerged from the nodal area.

Table 2. Differences in adventitious bud formation on nodes from different positions, cultured for 60 days in 10 ppm BA

Node position	Avg. no. of buds/node
1st (top)	11
2nd	4.1
3rd	2.0
4th	2.0

c. Effect of BA and NAA on elongation and root formation of adventitious buds

Since in section a adventitious buds were obtained in 10 ppm BA medium, this experiment was conducted to promote shoot elongation and root formation. When adventitious buds, induced in 10 ppm BA medium, grew to about 5-10 mm in height, they were separated into clumps with a few buds and a small portion of the nodal tissue, and transferred to media with 2 ppm BA, 1 ppm NAA, or 1 ppm NAA + 2 ppm BA. The results are shown in Table 3 and Figure 7. Two ppm BA promoted elongation of bud, but no roots were formed during the culture period of 1 month. One ppm NAA induced roots, although bud elongation was slower than that in 2 ppm BA. In 1 ppm NAA + 2 ppm BA medium, both bud elongation and root formation were poor and some tissue produced callus. Shoots cultured in 2 ppm BA formed roots when transferred to 1 ppm NAA medium. The rooted plantlets were transferred to vermiculite in pots and covered

Shoot and root formation by node culture (Scindapsis aureus)

Figure 6. Node cultures from different positions showing different responses in 10 ppm BA. 0.5X. From left to right, 1st (top), 2nd, 3rd and 4th nodes, respectively. First node shows adventitious buds forming at the basal part of the stem section. Second node shows buds forming just on the nodal zone. Third and 4th nodes show elongation and branching of axillary bud.

Figure 7. Elongation and root formation of bud induced in 10 ppm BA. 1X. Left: culture in 2 ppm BA showing shoot elongation, middle: culture in 1 ppm NAA showing root formation, right: culture in 2 ppm BA + 1 ppm NAA showing poor growth of shoot and root.

Figure 8. Plantlets obtained by node culture (4 months). 0.5X.

Figure 9. Repeated node culture using plantlet obtained by node culture in 1 ppm BA. 0.5X. From left to right, 10th, 20th, 30th and 40th days after culture in 2 ppm BA. Buds emerged from nodal zone and basal part of the stem section.

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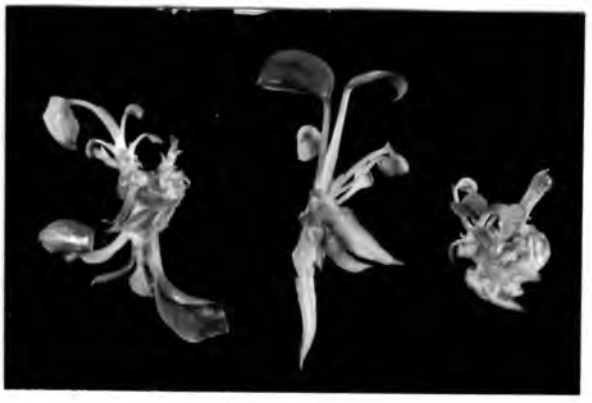

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with plastic bags to avoid desiccation. A week later, the bags were removed and the established plants were photographed (Fig. 8).

Table 3. Effect of BA and NAA on elongation of bud and formation of root after 1 month in culture

Treatment (ppm)	Avg. height of shoot (cm)	Avg. no. of roots
BA 2	1.6	0
NAA 1	1.2	10
BA 2 + NAA 1	0.9	0.3

Adventitious buds induced in 10 ppm BA medium were transferred to the above media.

d. Difference in bud formation of node induced by different BA concentrations

This experiment was conducted to evaluate the response of nodal tissues which were excised from shoots that were induced by different BA concentrations. Nodes were excised from the shoots which were induced by node culture in 1 ppm and 10 ppm BA medium. Then, they were cultured in 2 ppm BA medium. The result is shown in Table 4. The nodes, which were obtained from the plantlets in 10 ppm BA, produced more buds than those obtained in 1 ppm BA. The process of bud formation from nodes (pre-culture in 1 ppm BA) is shown in Figure 9. Adventitious buds formed from the base of the stem section as well as from the nodal area.

Table 4. Comparison of bud formation from node of plantlet induced in low and high BA concentrations (1 month old culture)

BA conc. of pre-culture (ppm)	Avg. no. of bud
1	3.0
10	10.1

Nodes which were excised from plantlets induced in 1 and 10 ppm BA, were cultured in 2 ppm BA medium.

e. Effect of BA and NAA on bud and root formation from internode

This experiment was conducted to induce adventitious buds from internode. The sterilized internode (between 2nd and 3rd node) was sectioned to pieces of 5 mm in length, and cultured in 0, 0.1 and 1 ppm NAA medium in combination with 1 and 10 ppm BA medium. The results are shown in Table 5. Both NAA and BA are necessary for bud formation. Ten ppm BA + 0.1 ppm NAA medium was most effective for bud formation. Roots formed in 1 ppm BA + 0.1 ppm NAA medium and 1 ppm BA + 1 ppm NAA medium. Ten ppm BA suppressed root formation. In the preliminary experiment, it was found that bud formation from below 3rd node was very poor.

Table 5. Effect of BA and NAA on bud and root formation from internode after 2 months in culture

Treatment		Avg. no. of bud	Avg. no. of root
NAA	BA (ppm)		
0	1	0	0
0	10	0.25	0
0.1	1	1.7	0.8
0.1	10	7.3	0
1	1	1.5	0.4
1	10	0.9	0

f. Effect of TIBA (tri-iodobenzoic acid) on bud formation on node

This experiment was conducted to see whether endogenous auxin is involved in bud formation at node by using an antiauxin (TIBA). Fifteen ppm TIBA was added to the culture medium supplemented with 1 ppm BA. Nodes for culture were obtained from plantlets in culture in 1 ppm BA. As shown in Table 6 and Figure 11, bud formation was completely suppressed by TIBA treatment.

Table 6. Effect of TIBA on bud formation at node after 3 months in culture

Treatment	Avg. no. of bud
1 ppm BA	2.6
1 ppm BA + 15 ppm TIBA	0

g. Effect of removal of axillary bud from node on bud formation

In section f, it was suggested that endogenous auxin is required for bud formation. In node culture, there are two possible sites of auxin production (Fig. 10). One is axillary bud, the other is intercalary meristem. This experiment was conducted to see which meristem is responsible for bud formation. Node was excised from the plantlet in test tube culture and cultured in 2 ppm BA medium. After 9 days, the axillary bud was removed. The results are shown in Table 7 and Figure 12. No difference was found in the number of adventitious buds between intact node and node after bud was removed, indicating that existence of intercalary meristem is enough for bud induction.

Table 7. Effect of removal of axillary bud from node on bud formation after 3 months in culture in 2 ppm BA

Treatment	Avg. no. of buds
intact	3.4
removed	4.8

h. Relationship between auxin content in node and adventitious bud formation

In section b, adventitious bud formation was not successful in the nodes below the 3rd; in section f, participation of endogenous auxin in bud formation was suggested. This experiment was conducted to compare auxin content in the nodes from different positions. Fresh stem sections with 1st, 2nd and 3rd nodes were incubated in Ehlrich

Figure 10. Effect of removal of axillary bud from node on multiple shoot formation.

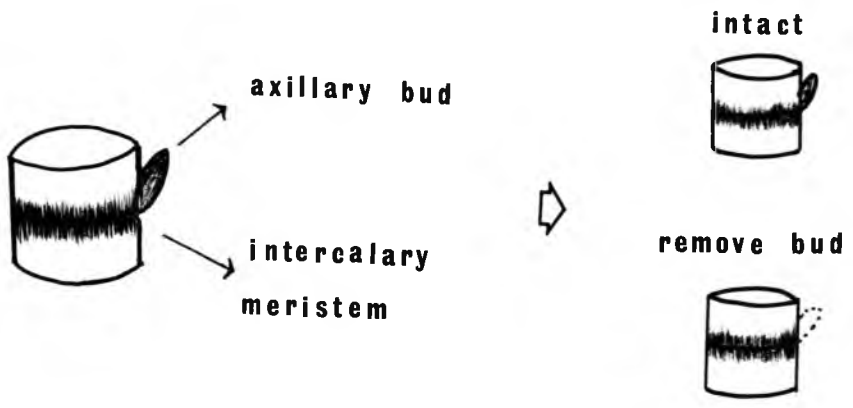
axillary bud
intercalary
meristem
intact
remove bud
Possible site
of auxin production
Culture (BA medium)

Figure 11. Effect of TIBA on bud formation from node (Scindapsis aureus). Left: node culture in 1 ppm BA showing bud formation, right: node culture in 1 ppm BA + 15 ppm TIBA showing no bud formation. 1X.

Figure 12. Effect of removal of axillary bud on bud formation (Scindapsis aureus). Intact node (left) and bud-removed node (right) cultured in 2 ppm BA showing almost the same number of adventitious buds. 1X.

Figure 13. Auxin content in node from different position (Scindapsis aureus). Node was incubated in Ehrlich reagent for 5 minutes. First node (left) and 2nd node (middle) showing the same intensity of color reaction, while 3rd node (right) showing lower intensity. 1.2X.

Figure 14. Chromosome number of the plantlets obtained by node culture (Scindapsis aureus). Chromosomes of root cell showing the number, $2N = 52 \pm 2$. 800X.

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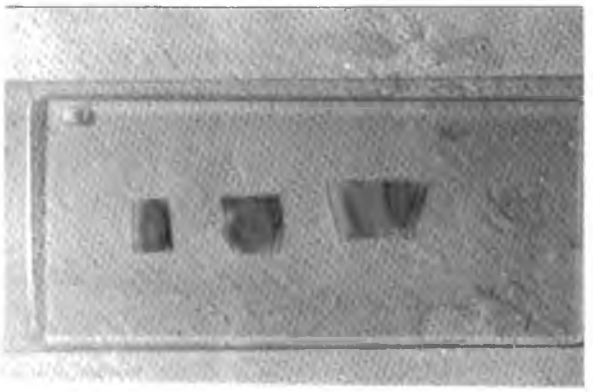

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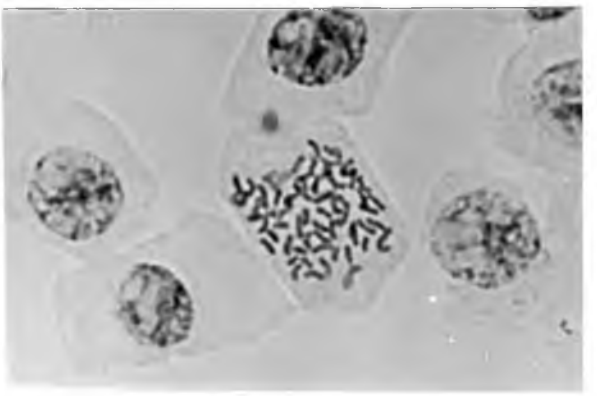

reagent. The results are shown in Figure 13. The 1st and 2nd nodes showed pink color, while 3rd node showed only a slight coloration. Although Ehlrich reagent is not highly specific for IAA, the reaction seems to favor presence of IAA, judging from the change of reaction color (pink $\longrightarrow$ blue). In the preliminary experiment, 3rd node produced adventitious bud only when NAA was added into the medium containing BA. This result also suggests that the level of endogenous auxin in the nodes below the 3rd is too low for bud formation.

i. Chromosome number of the plantlets obtained by node culture

Figure 14 shows the somatic chromosomes from root tip smears of plantlets from node culture. The number was $2N = 52 \pm 2$ in 15 plantlets examined.

2. Anatomical study on bud formation (Scindapsis aureus)

Anatomical changes of nodal tissue in bud formation are shown in longitudinal stem sections of 0, 15, and 30 day cultures in 10 ppm BA in Figures 15-17. Prior to culturing (0 day), all the cells within node, stained uniformly (Fig. 15), but some nuclei in the epidermis of stem (arrow) are more intensely stained by safranin than those in other tissues. On the 15th day (Fig. 16), the nuclei in several epidermal and subepidermal cells at the node (arrow, meristematic zone) are more heavily stained by safranin and the nuclear volume is greater as compared to the surrounding tissues even though the cell volume is smaller. By the 30th day (Fig. 17), a shoot tip is apparent. The nuclei in the meristematic cells are heavily stained by safranin.

Anatomical study of bud formation from node (*Scindapsis aureus*)

Figure 15. Longitudinal section through node cultured 0 day showing uniform staining in all cells within node and no evidence of meristematic activity (safranin and fast green stain). 125X. arrow: stem epidermis showing a layer of small cells.

Figure 16. Fifteen day culture in 10 ppm BA showing heavy staining by safranin in enlarged nuclei and aggregate of small cells recently divided in the meristematic zone (arrow). 300X.

Figure 17. Thirty day culture in 10 ppm BA showing shoot tip.

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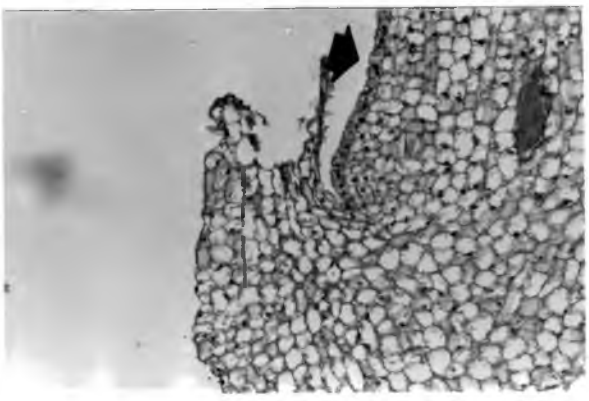

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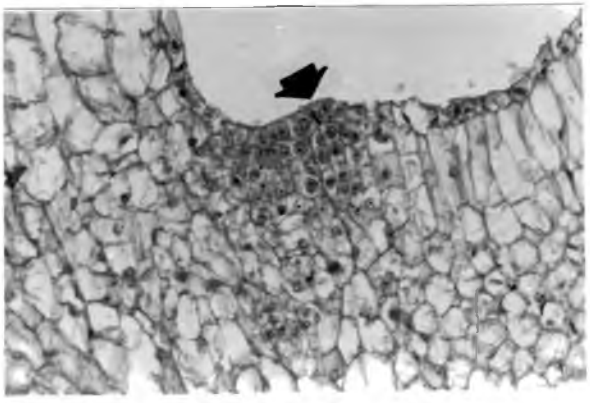

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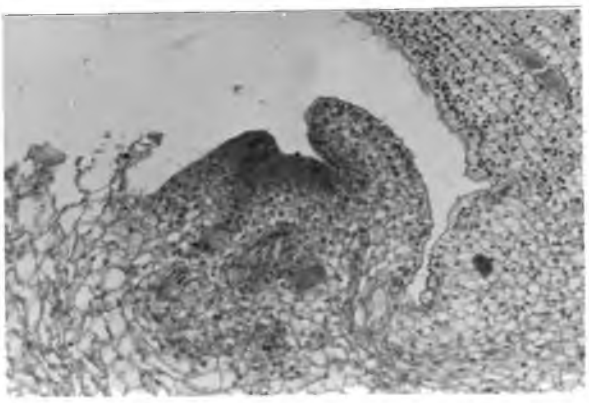

3. Histochemical studies on bud formation (Scindapsis aureus)

Histochemical studies were conducted to elucidate the metabolic changes of nodal tissue in bud formation.

a. DNA by Feulgen method (Jensen, 1962)

Longitudinal sections through nodes of 0, 15 and 30 day cultures in 10 ppm BA stained with Feulgen reaction are shown in Figures 18-20. Before culture (0 day) (Fig. 18), stained nuclei in cells of node section are scattered and show the same staining pattern (reddish purple). The stem epidermis (arrow) is evident because of row of deeper stained cells. On the 15th day (Fig. 19), a meristematic zone is apparent because of a cluster of densely stained nuclei, some of them more intensely stained than those of surrounding cells. On the 30th day (Fig. 20), leaf primordium and procambium in the shoot apex are apparent owing to densely stained nuclei.

b. DNA and RNA by modified Methyl Green and Pyronin Y method (Jensen, 1962)

Longitudinal sections through nodes of 0, 15 and 30 day cultures in 10 ppm BA, stained with methyl green and pyronin Y, are shown in Figures 21-23. At 0 day (Fig. 21), no difference in staining by methyl green (blue color) was observed in nuclei in cells of node, although some nuclei in cells of stem epidermis are more deeply stained. Staining by pyronin Y was very poor in all tissues. On the 15th day (Fig. 22), nuclei of epidermal and subepidermal cells in meristematic zone were more densely stained by methyl green than those in other areas. The stain by pyronin Y was so slight that the

Histochemical studies on bud formation (Scindapsis aureus)

Figures 18-20. DNA by Feulgen

Figure 18. Longitudinal section through node at 0 day showing uniform staining in nuclei within nodal cells. A row of nuclei shows stem epidermis as indicated with an arrow. 160X.

Figure 19. Fifteen day culture in 10 ppm BA showing accumulation of densely stained nuclei in the meristematic zone. 200X.

Figure 20. Thirty day culture showing dense staining of shoot tip. 160X.

Figures 21-23. DNA and RNA by modified Methyl Green and Pyronin Y

Figure 21. Lontitudinal section through node at 0 day showing uniform distribution of stained nuclei (methyl green) within node and no staining by pyronin Y. 200X.

Figure 22. Fifteen day culture in 10 ppm BA showing heavy stain by methyl green in nuclei in meristematic zone, but no staining by pyronin Y. 200X.

Figure 23. Thirty day culture showing heavy staining by methyl green in shoot apex, but no staining by pyronin Y. 160X.

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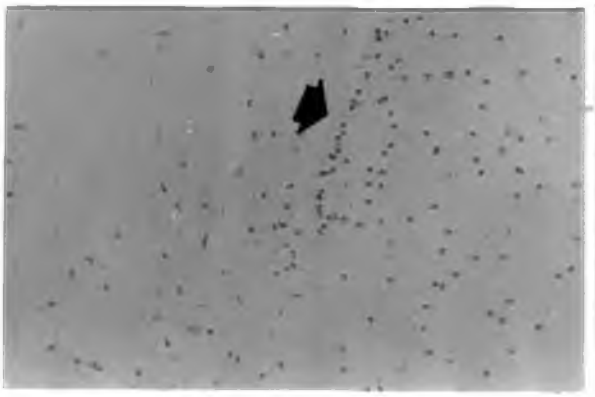

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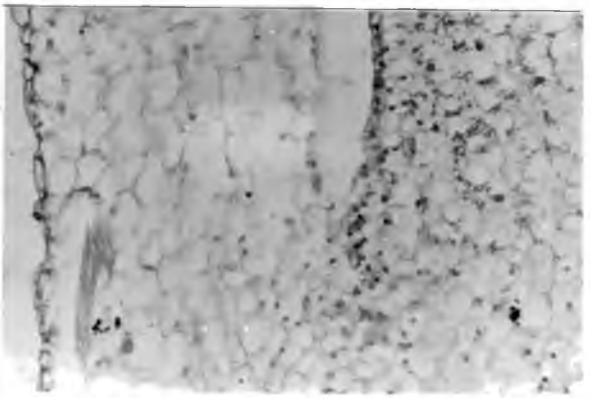

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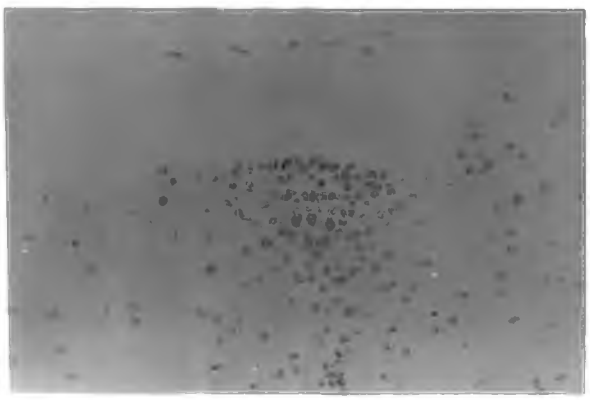

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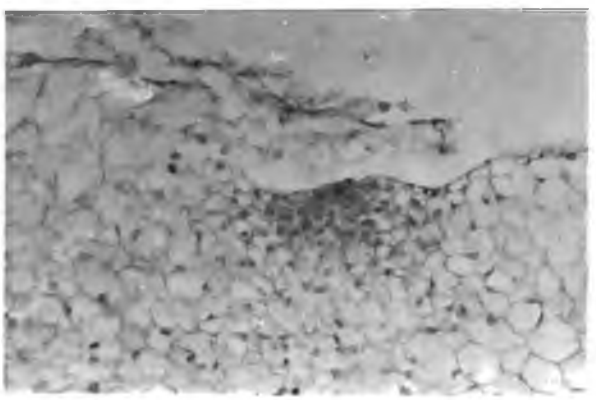

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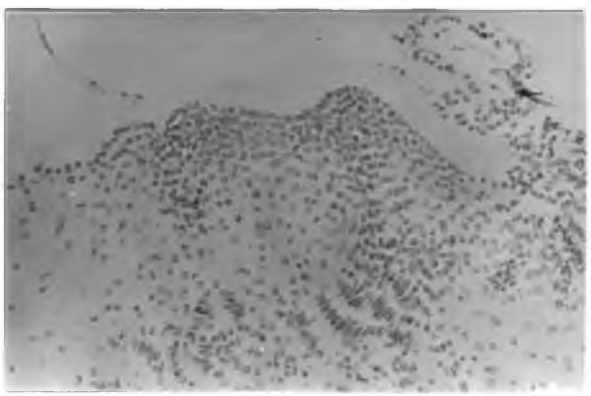

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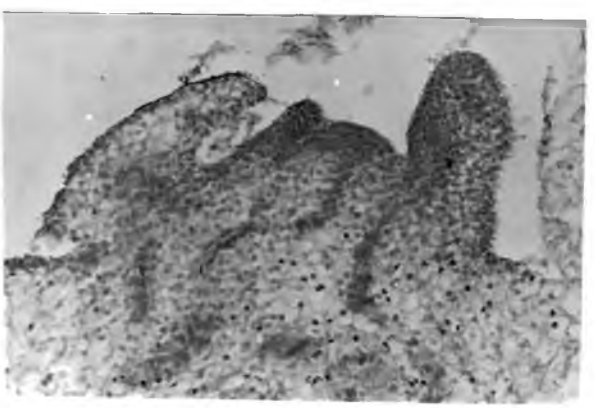

difference between meristematic zone and the surrounding area was not clear. On the 30th day (Fig. 23), apical meristem and leaf primordia were highly stained by methyl green, but the stain by pyronin Y was again very poor.

c. Total proteins by Ninhydrin-Schiff's reaction (Jensen, 1962)

Longitudinal sections through nodes of 0, 15 and 30 day cultures in 10 ppm BA stained with ninhydrin-Schiff's reagent are shown in Figures 24-26. At culture (0 day) (Fig. 24), nuclei are slightly stained in all cells, but cytoplasm is not appreciably stained. No difference in distribution of the stained nuclei was found within node. On the 15th day (Fig. 25), meristematic zone was more densely stained. The cytoplasm in some cells in this zone was stained as well as the nuclei. However, the degree of stain in cytoplasm was not as intense as that in nuclei. On the 30th day (Fig. 26), cells of apical meristem and leaf primordia were highly stained in both nuclei and cytoplasm.

d. Histones by Fast Green method (Jensen, 1962)

Longitudinal sections through nodes of 0, 15 and 30 day cultures in 10 ppm BA stained by fast green, are shown in Figures 27-29. At culture (0 day) (Fig. 27), nuclei were uniformly stained (bluish green) in all the tissues, although the degree of stain appeared slightly more intense in the stem epidermal cells. No difference in the distribution of the stained nuclei was found within the node. On the 15th day (Fig. 28), the meristematic zone appeared densely stained. On the 30th day, apical meristem was more densely stained (Fig. 29).

Histochemical studies on bud formation (*Scindapsis aureus*)

Figures 24-26. Total proteins by Ninhydrin-Schiff's reaction

Figure 24. Longitudinal section through node at 0 day showing slight staining in nuclei in all cells. 160X.

Figure 25. Fifteen day culture in 10 ppm BA showing stain both in nuclei and cytoplasm in meristematic zone. 400X.

Figure 26. Thirty day culture showing intense stain in nuclei and cytoplasm in apical meristem. 160X.

Figures 27-29. Histones by Fast Green method

Figure 27. Longitudinal section through node at 0 day showing no difference in distribution of stained nuclei within the node. 160X.

Figure 28. Fifteen day culture in 10 ppm BA showing denser stain of nuclei in meristematic zone. 200X.

Figure 29. Thirty day culture showing high density of stain of nuclei in apical meristem. 160X.

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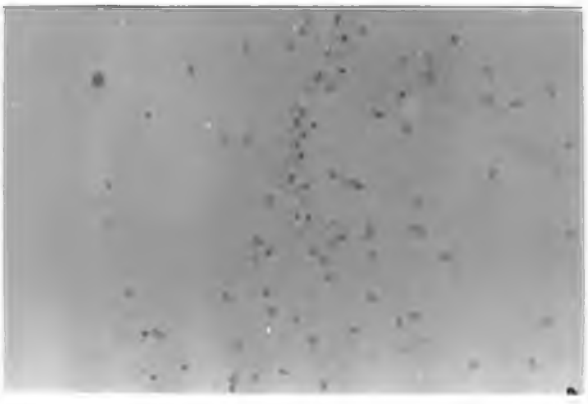

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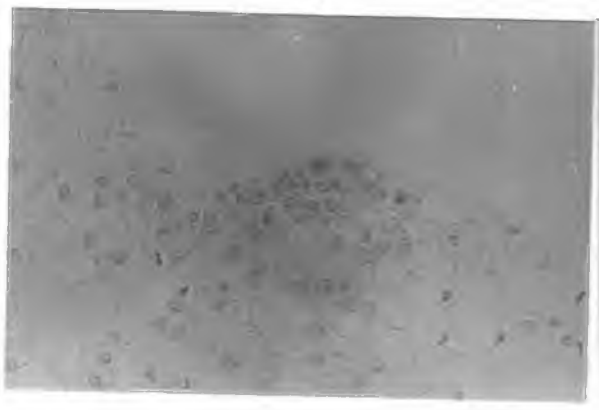

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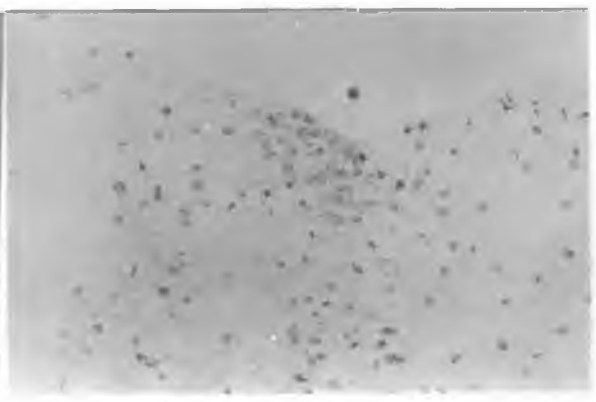

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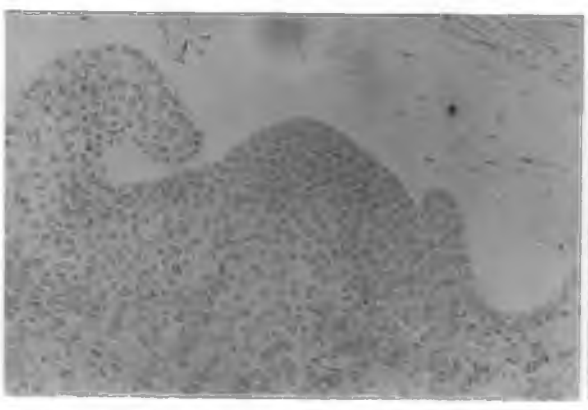

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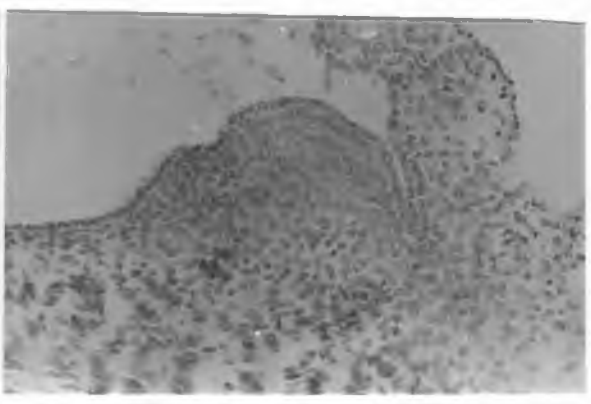

e. Total carbohydrates of insoluble polysaccharides by PAS reaction (Jensen, 1962)

Longitudinal sections through nodes of 0, 15 and 30 day cultures in 10 ppm BA stained with PAS reaction are shown in Figures 30-32. At culture (0 day) (Fig. 30), the wall structures are highly stained (pink color), but the nuclei only slightly. No stained carbohydrate grains were found in the cytoplasm. There was no difference in staining pattern within the node. On the 15th day (Fig. 31), the nuclei in several epidermal and subepidermal cells in the meristematic zone, as well as the wall structures, are clearly stained. However, no appreciable carbohydrate grains was seen in the cytoplasm of all the cells. On the 30th day (Fig. 32), apical meristem is highly stained in the nuclei as well as the wall structures. No stained carbohydrate grains were again observed in all the cells. In the preliminary experiment, no starch was detected by IKI reaction in any stage and any tissue in node section.

f. Reducing sugars by Fehling reaction

Since most of water soluble carbohydrates are lost in the process of fixation and dehydration, it is difficult to detect reducing sugars (water soluble). Therefore, fresh hand section was used instead of chemically fixed tissue. The results are shown in Figures 33-35. At 0 day culture (Fig. 33), stem epidermal cells are slightly stained (pink color). No appreciable stain was found within node. On the 15th day (Fig. 34), meristematic zone is clearly stained in cytoplasm as well as nuclei. On the 30th day (Fig. 35), apical meristem is highly stained.

Histochemical studies on bud formation (Scindapsis aureus)

Figures 30-32. Total carbohydrates of insoluble polysaccharides by PAS reaction

Figure 30. Longitudinal section through node at 0 day showing staining of wall structures but no other particular staining within node. 200X.

Figure 31. Fifteen day culture in 10 ppm BA showing slight staining of nuclei as well as wall compounds in meristematic zone but no stained compounds in cytoplasm. 200X.

Figure 32. Thirty day culture showing staining of nuclei and wall compounds but no stained compounds in the cytoplasm in apical meristem. 160X.

Figures 33-35. Reducing sugars by Fehling reaction

Figure 33. Longitudinal section through node at 0 day showing no stain within node. 125X.

Figure 34. Fifteen day culture in 10 ppm BA showing stain in meristematic zone. 200X.

Figure 35. Thirty day culture showing intense stain in apical meristem. 125X.

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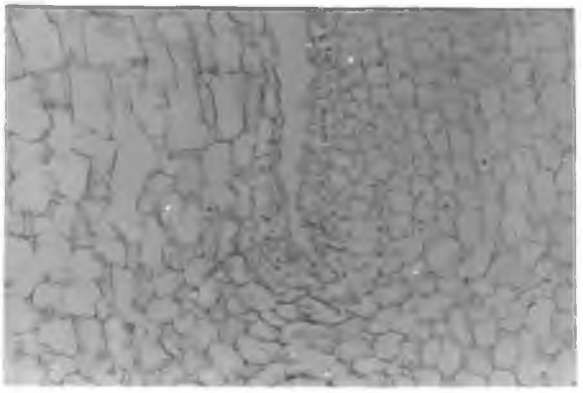

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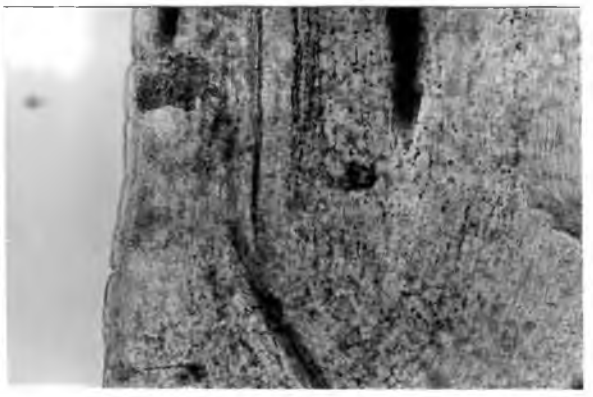

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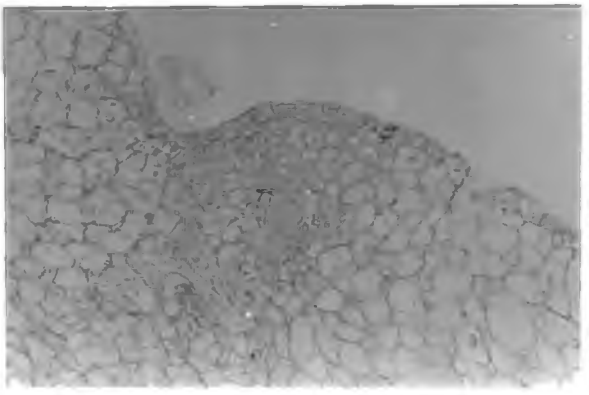

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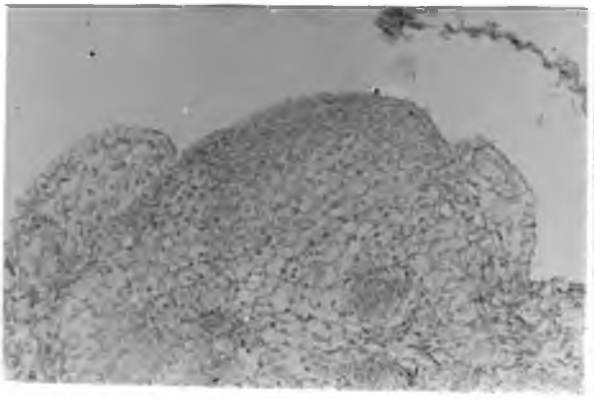

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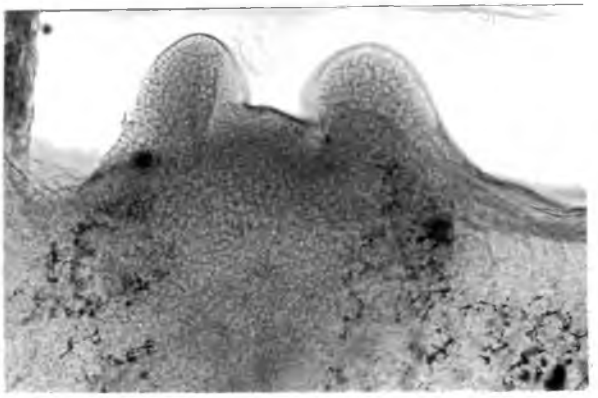

g. Respiration enzyme, succinate dehydrogenase, by Nitro-BT reduction (Jensen, 1962)

Longitudinal sections through nodes of 0 and 15 day cultures in 10 ppm BA stained with nitro-BT are shown in Figs. 36 and 37. At 0 day culture (Fig. 38), epidermal cells of stem are stained (purple) and cells in nodal zone, slightly. On the 15th day (Fig. 37), the meristematic zone is highly stained. No stain was found in heat-killed tissue, but slight stain was observed in tissue treated with inhibitor of this enzyme, p-phenylenediamine (10^{-3}M).

h. Respiration enzyme, cytochrome oxidase by Nadi reaction (Jensen, 1962)

Longitudinal sections through nodes of 0 and 15 day cultures in 10 ppm BA stained with Nadi reaction are shown in Figures 38 and 39. At culture (0 day) (Fig. 38), epidermal and subepidermal cells of leaf sheath (arrow) are stained (blue), and cells in nodal zone, slightly. On the 15th day (Fig. 39), staining is lacking in the meristematic zone, while other areas are stained. There was no stain in heat-killed tissue.

i. Respiration enzyme, glucose-6-phosphate dehydrogenase by Nitro-BT reduction (Nachlas et al., 1958)

Longitudinal sections through nodes of 0 and 15 day cultures in 10 ppm BA stained with nitro-BT are shown in Figures 40 and 41. At culture (0 day) (Fig. 40), no appreciable stain was found within node, although epidermal cells of stem are slightly stained (purple color). On the 15th day (Fig. 41), bud forming zone is highly stained.

Histochemical studies on bud formation (*Scindapsis aureus*)

Figures 36-37. Succinate dehydrogenase by Nitro-BT reduction

Figure 36. Longitudinal section through node at 0 day showing slight stain within node. 125X.

Figure 37. Fifteen day culture in 10 ppm BA showing intense stain in meristematic zone. 200X.

Figures 38-39. Cytochrome oxidase by Nadi reaction

Figure 38. Longitudinal section through node at 0 day showing intense stain of leaf sheath (arrow) and slight stain within node. 125X.

Figure 39. Fifteen day culture in 10 ppm BA showing no appreciable stain in meristematic zone. 200X.

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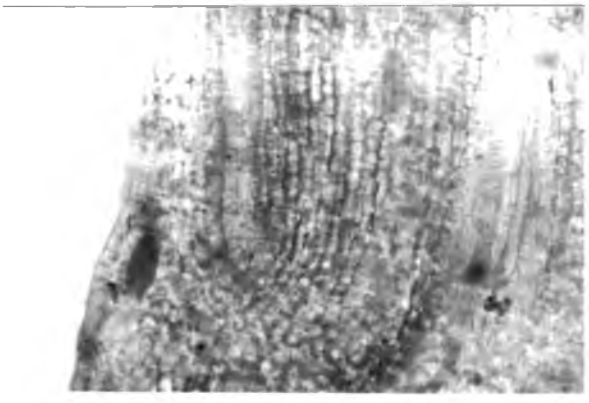

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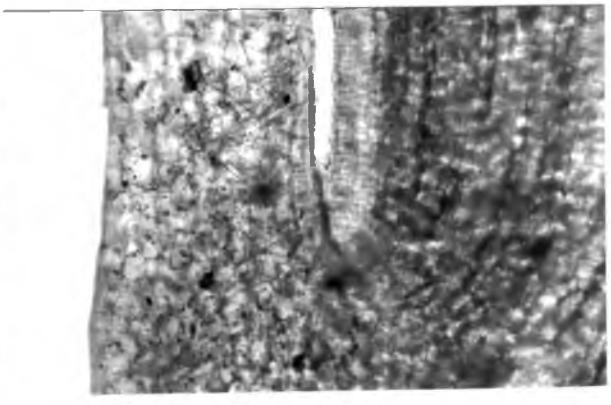

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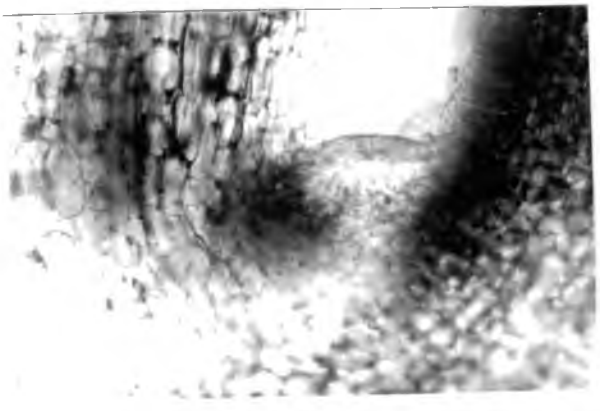

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j. Peroxidase by Benzidine reaction (Povvaia et al., 1973)

Longitudinal sections through nodes of 0 and 15 day cultures in 10 ppm BA stained with benzidine are shown in Figures 42 and 43. At culture (0 day) (Fig. 42), wall compounds in all the cells, vascular tissue and epidermis of stem, are highly stained (brown), but cells within node, moderately. On the 15th day (Fig. 43), meristematic zone is more intensely stained than the surrounding tissue. The vascular tissue is again highly stained.

4. Microautoradiographic study (Scindapsis aureus)

Since staining by pyronin Y was not clear, microautoradiographic technique was used for RNA study. Longitudinal sections through nodes of 0, 15 and 20 day cultures in 10 ppm BA are shown in Figures 44 and 45 (12 hour incubation in 10 Ci/ml cytidine-^{3}H, stained with toluidine blue). At culture (0 day) (Fig. 44), the density of silver grains is almost the same as background in all the cells. But, the nuclei show more grains. No difference in density of grains in nuclei was found within the node. On the 15th day (Fig. 45), the meristematic zone shows more grains than other areas, although no effort was made to quantify the grains per cell. On the 20th day (Fig. 46), the meristematic zone shows high density of grains. In sections of 20 day culture treated with RNAse (Fig. 47), most of the grains are absent, indicating that cytidine-^{3}H was incorporated into RNA. Appreciable grains were not found in any tissue of 4 hour incubation.

All the results of histochemistry and microautoradiography are listed in Table 8. The result of DNA and RNA stain by methyl green

Histochemical studies on bud formation (*Scindapsis aureus*)

Figures 40-41. Glucose-6-phosphate dehydrogenase by Nitro-BT reduction

Figure 40. Longitudinal section through node at 0 day showing no stain within node. 160X.

Figure 41. Fifteen day culture in 10 ppm BA showing intense stain in meristematic zone. 160X.

Figures 42-43. Peroxidase by Benzidine reaction

Figure 42. Longitudinal section through node at 0 day showing staining of stem epidermis and walls of all cells, and slight stain of cells within node. 125X.

Figure 43. Fifteen day culture in 10 ppm BA showing intense stain in meristematic zone and vascular tissue. 160X.

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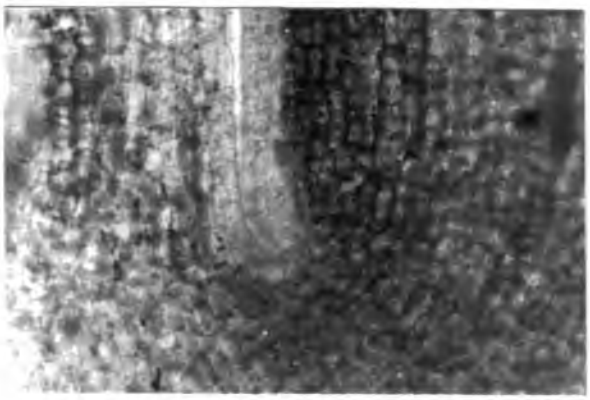

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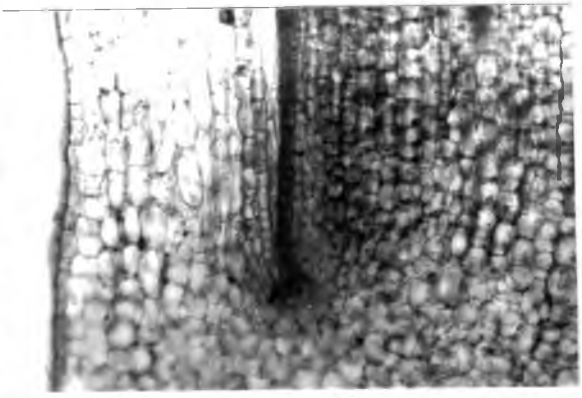

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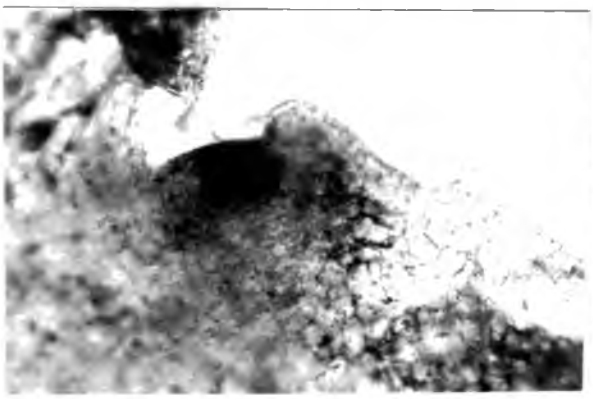

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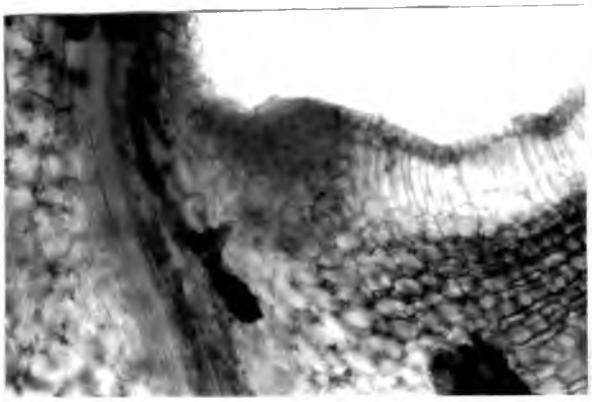

Microautoradiographic study of bud formation (Scindapsis aureus)

Figure 44. Longitudinal section through node at 0 day blue stain). 300X. Tissue was incubated in 10μ Ci/ml of cytidine-^{3}H for 12 hours. Most of the silver grains are in nuclei, but no difference in distribution pattern was found within node.

Figure 45. Fifteen day culture in 10 ppm BA showing accumulation of silver grains in meristematic zone. 300X.

Figure 46. Twenty day culture showing accumulation of silver grains in meristematic zone. 200X.

Figure 47. Tissue of 20 day culture treated with RNase showing no accumulation of silver grains. 200X

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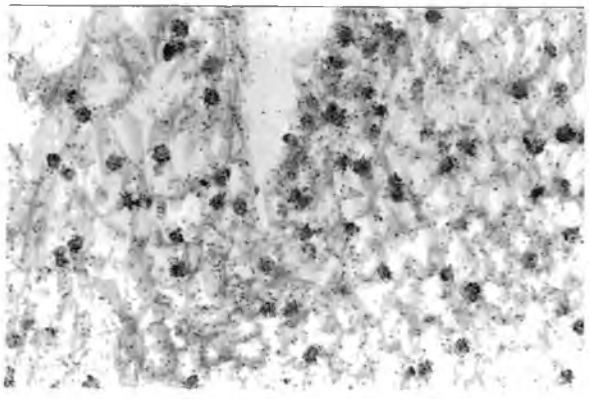

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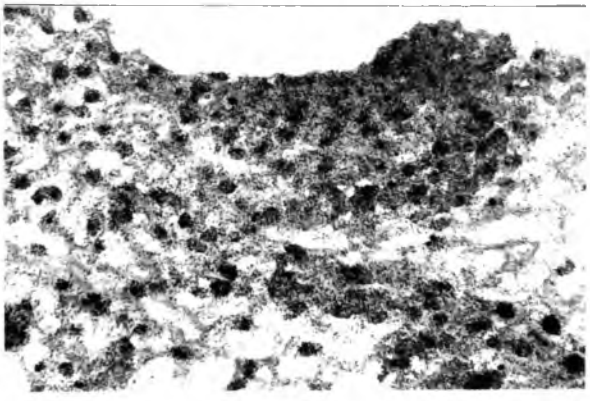

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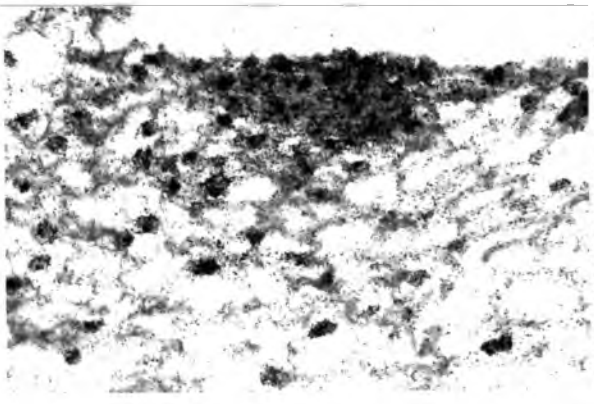

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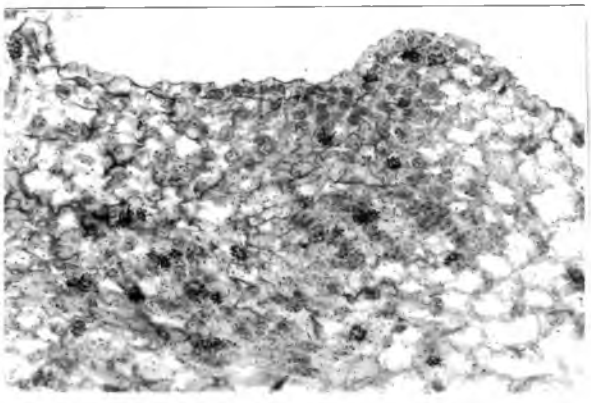

and pyronin Y is not shown because the stain of pyronin Y was poor and erratic.

Table 8. Histochemical and microautoradiographic studies in bud forming zone

Compound	Method		Time in culture (days) 0	15	30
DNA	Feulgen reaction	N	+[a]	++	++
RNA	Microauto-radiography		+	++	
proteins.	Ninhydrin-Schiff's reaction	N	+	++	++
		C	-	+	++
histone	Fast green	N	+	++	++
insoluble carbohydrates	PAS reaction	N	±	++	++
		C	-	-	-
reducing sugars	Fehling reaction		-	+	++
succinate dehydrogenase	Nitro-BT reduction		±	++	++
cytochrome oxidase	Nadi reaction		±	-	
G-6-P dehydrogenase	Nitro-BT reduction		-	++	
peroxidase	Benzidine reaction		+	++	

[a]Degree of stain per unit area: - none, ± slight, + intermediate, ++ high.

N nucleus, C cytoplasm

B. Leaf culture

1. Morphological and physiological studies

a. Effect of BA and NAA on bud and root formation from leaf tissue

Leaf blade + petiole (whole leaf), leaf blade, and petiole were excised from plantlets obtained by node culture in 1 ppm BA and cultured in 0, 0.1 or 1 ppm NAA medium containing 1 ppm BA. The results are shown in Table 9 and Figures 48-53. In cultures of blade with petiole and blade only, adventitious buds were induced in BA media without NAA, although more buds were obtained by addition of NAA. In blade culture, buds formed only at the base of the midvein in single treatment with BA whereas buds formed from the entire length of the cut surface in BA + NAA. In petiole culture, both BA and NAA were required for bud formation. The number of roots increased with NAA concentration.

In the preliminary experiment, it was found that single treatment with NAA induced only roots in whole leaf and petiole culture (Figs. 54 and 55). No organogenesis was observed in the basal medium. Morphological observations of blade and petiole in 1 ppm NAA + 1 ppm BA medium are shown in Figures 56-60. The tissue at the cut surface enlarged and tumor-like bumps were formed (Figs. 56 and 58). Later, green spots appeared on bumps (Fig. 59, arrow). Finally, buds emerged (Figs. 57 and 60).

Table 9. Effect of BA and NAA on bud and root formation from leaf cultures after 75 days in culture

Leaf tissue[a]	Treatment BA	Treatment NAA (ppm)	Avg. no. of bud	Avg. no. of root
blade + petiole		0	2.0	1.0
	1	0.1	2.4	1.4
	1	1	2.5	5.0
blade	1	0	0.3	0.2
	1	0.1	1.0	0.2
	1	1	1.2	0.4
petiole	1	0	0	0
	1	0.1	1.0	0.7
	1	1	1.8	0.4

[a]Leaf tissues were obtained from plantlets cultured in 1 ppm BA medium.

b. Effect of TIBA on bud formation on leaf

Leaf with petiole from plantlet which grew in 1 ppm BA was excised and lanolin paste containing 1000 ppm TIBA was applied around petiole. The treated leaf was cultured in 1 ppm BA medium. As shown in Table 10 and Figure 61, no bud was formed when treated with TIBA.

Table 10. Effect of TIBA on bud formation on leaf after 3 months in culture

Treatment (ppm)	Avg. no. of bud
BA 1	4.2
BA 1 + TIBA 1000	0

Shoot and root formation by leaf culture (Scindapsis aureus)

Figure 48. Culture of leaf with petiole. 1X.

Figure 49. Forty day culture in 1 ppm BA showing appearance of leaf primordia at the cut end of petiole. 1.5X.

Figure 50. Sixty day culture in 1 ppm BA showing developing shoots at the cut end of petiole. 1.3X.

Figure 51. Seventy-five day culture of leaf with petiole. 1X. Left: 1 ppm BA, middle: 1 ppm BA + 0.1 ppm NAA, right: 1 ppm BA + 1 ppm NAA. Adventitious buds were formed in all the treatments.

Figure 52. Seventy-five day culture of leaf blade. 1.2X. Left: culture in 1 ppm BA showing bud formation only at bottom of midvein, middle: culture in 1 ppm BA + 0.1 ppm NAA showing bud formation mostly at bottom of midvein, right: culture in 1 ppm BA + 1 ppm NAA showing bud formation along entire length of cut surface.

Figure 53. Seventy-five day culture of petiole. 1X. Left: culture in 1 ppm BA showing no bud formation, middle: culture in 1 ppm BA + 0.1 ppm NAA showing a few buds, right: culture in 1 ppm BA + 1 ppm NAA showing multiple shoot formation.

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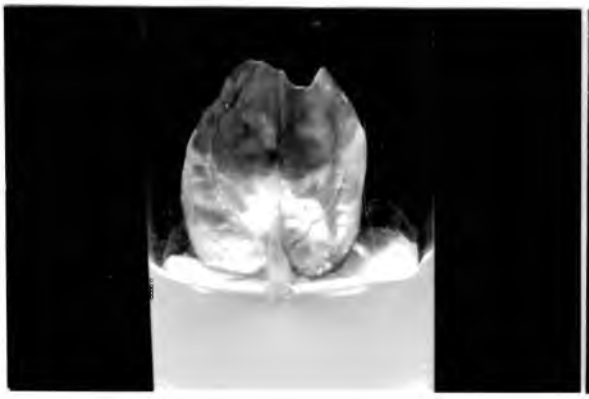

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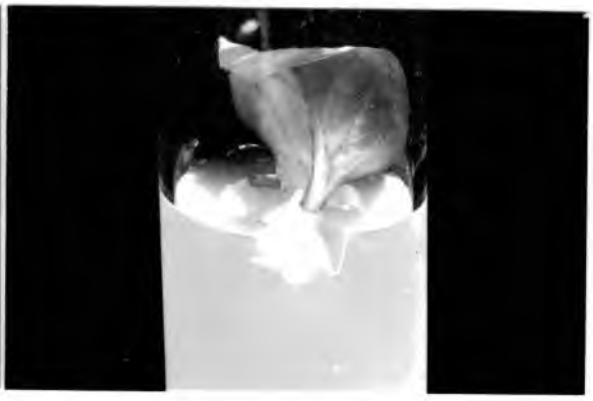

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Shoot and root formation by leaf culture (Scindapsis aureus)

Figure 54. Leaf with petiole grown in 1 ppm NAA showing formation of roots only. 1.2X.

Figure 55. Petiole culture in 1 ppm NAA showing formation of roots only. 1.2X.

Figure 56. Leaf blade culture in 1 ppm BA + 1 ppm NAA showing bumps on the cut surface. 10X.

Figure 57. Leaf blade culture in the above medium (Fig. 103) showing formation of adventitious buds. 10X.

Figure 58. Petiole culture in 1 ppm BA + 1 ppm NAA showing swollen tissue at the cut end. 10X.

Figure 59. Petiole culture in the above medium showing green spots on the bumps (arrow). 10X.

Figure 60. Petiole culture in the above medium showing shoot formation on the bumps. 5X.

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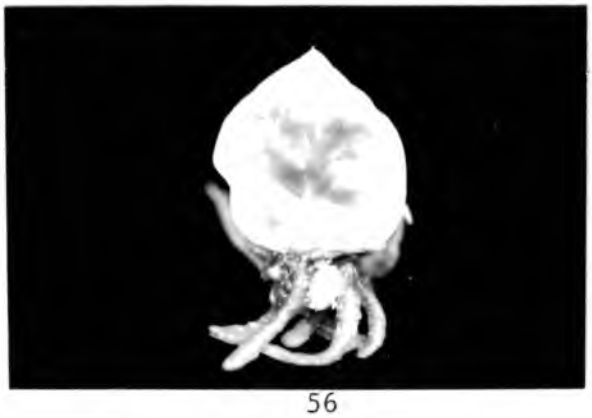

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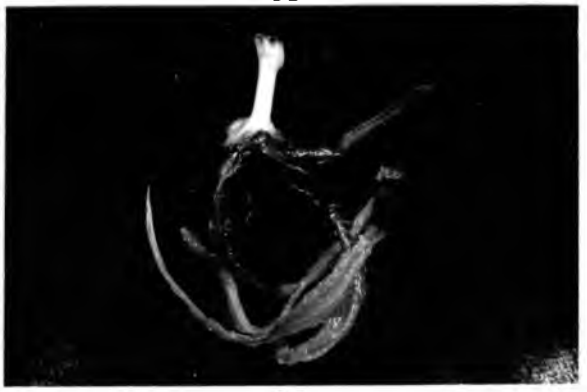

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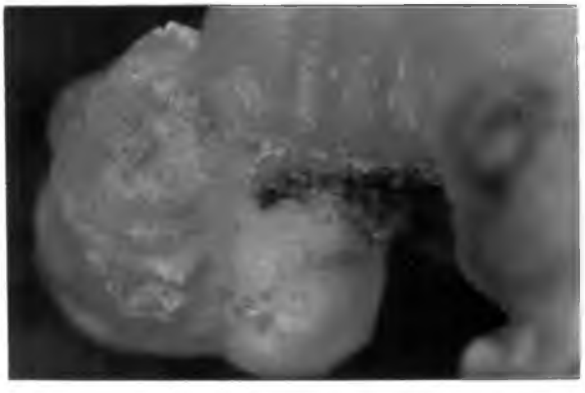

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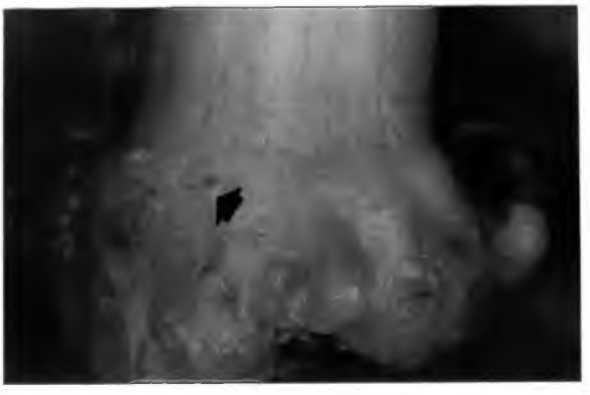

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c. Further studies on bud formation from leaf segment

This experiment was conducted to see whether it is possible to induce buds from both the proximal and distal cut surfaces of leaf segments. Leaf blades were excised from the plantlet which was obtained by node culture (1 ppm BA). Proximal and distal parts of the blade were discarded, and only the middle part was cultured in 1 ppm BA or 1 ppm BA + 1 ppm NAA medium. The results are shown in Table 11 and Figure 62. In single treatment with BA, buds formed only at the proximal end of midvein of cut surface. In BA + NAA treatment, buds were formed from entire length of both cut surfaces, although more buds were formed on the proximal side.

Table 11. Effect of BA and NAA on bud formation from leaf blade segment after 3 months in culture

	Avg. no. of bud	
	distal	proximal
BA 1	0	0.43
BA 1 + NAA 1	0.9	1.7

Leaves were obtained from plantlet cultured in 1 ppm BA medium. Proximal and distal parts were discarded and only the middle parts were cultured.

d. Relationship between auxin accumulation and location of adventitious bud formation in leaf blade culture

This experiment was conducted to see whether auxin is accumulated prior to bud formation in leaf blade culture. The bottom of leaf blade in culture in 1 ppm BA was incubated in Ehrlich reagent. The results are shown in Table 12. In the leaf blade prior to culture, no reaction

was observed in midvein and mesophyll. On the 15th day, the reaction was noticed with higher degree of staining in the midvein, suggesting auxin (probably IAA) accumulation at the proximal end of midvein.

Table 12. Reaction of leaf blade in Ehrlich reagent

Culture period (day) of leaf blade (1 ppm BA)	Tissue	
	Midvein	Mesophyll
0	-[a]	-
15	++	+

[a]Degree of staining: - none, + slight, ++ fair
The bottom of midvein and mesophyll were incubated in Ehrlich reagent.

e. Comparison between solid and liquid media on elongation of shoot obtained by leaf culture

Since growth of buds obtained by leaf culture was very slow, they were sub-cultured into liquid medium. Buds induced in 1 ppm BA + 1 ppm NAA medium were transferred to solid and liquid 1 ppm BA media with small portion of leaf blade tissue. The results are shown in Table 13 and Figure 63. Liquid medium clearly promoted elongation of buds.

Table 13. Effect of liquid and solid media on elongation of bud obtained by leaf blade culture (2 month old culture)

Treatment[a] (ppm)	Avg. height of shoot (cm)
solid	0.9
liquid	2.0

[a]Buds induced in 1 ppm BA solid medium were transferred to solid and liquid 1 ppm BA media.

Figure 61. Effect of TIBA on bud formation in leaf with petiole culture. Left: whole leaf (blade + petiole) culture in 1 ppm BA showing bud formation at cut end of petiole, right: culture of whole leaf treated with 1000 ppm TIBA in lanolin paste around petiole showing no bud formation. 1X.

Figure 62. Culture of leaf blade segment. Left: culture in 1 ppm BA showing bud formation only at the proximal end of mid-vein of cut surface, right: culture in 1 ppm BA + 1 ppm NAA showing bud formation from both proximal and distal cut surfaces. 1X.

Figure 63. Comparison between solid and liquid medium on elongation of buds obtained by leaf blade culture. Left: slow growth on solid 1 ppm BA medium, right: elongated shoot in liquid 1 ppm BA medium. 0.6X.

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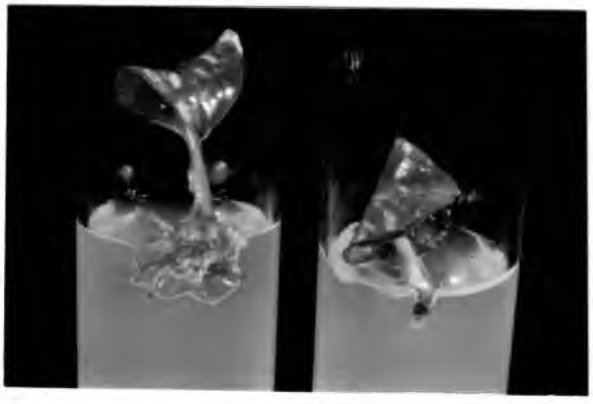

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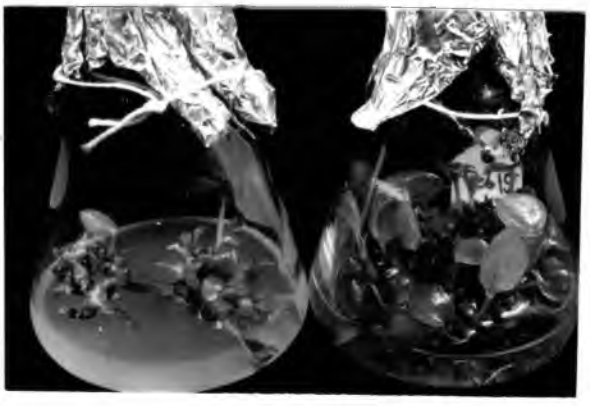

2. Anatomical study on bud formation from leaf blade and petiole (Scindapsis aureus)

These experiments were conducted to observe the process of bud formation from leaf tissue.

a. Study on leaf blade

Longitudinal sections of leaf blade cultured in 1 ppm BA medium were examined on 0, 10, 20 and 30 days after culture. The results are shown in Figures 64-70. On the 10th day, the nuclei in several cell layers above the cut surface are well stained with safranin (Figs. 65 and 66). The midvein (Fig. 65) is more intensely stained than mesophyll (Fig. 67). The stained cells are smaller in size, compared with the cells of 0 day culture (Fig. 64). On the 20th day (Fig. 68), cells on the midvein continue dividing. On the 30th day, a protruded tissue showing apical meristem is seen in the peripheral zone on the midvein (Fig. 69), whereas cells of the mesophyll have shrunken (Fig. 70).

b. Study on petiole

Longitudinal sections of petioles cultured in 1 ppm BA + 1 ppm NAA medium were examined on 0, 10, 20 and 30 days after culture. The results are shown in Figures 71-74. On the 10th day (Fig. 72), the nuclei in the several cell layers above the cut surface are well stained with safranin, and the cells showed cellular activity and are smaller in size than the cells prior to culture (Fig. 71). On the 20th day (Fig. 73), dividing cells are highly stained, and on the 30th day (Fig. 74), a shoot tip was observed.

Anatomical study on bud formation from leaf tissue (*Scindapsis aureus*)

Figure 64. Longitudinal section of midvein of 0 day leaf blade (safranin and fast green stain). 125X.

Figure 65. Midvein after 10 day culture in 1 ppm BA showing showing layers of divided cells above cut surface. 125X.

Figure 66. Enlargement of a portion of the above tissue showing enlarged nuclei in divided cells. 250X.

Figure 67. Mesophyll after 10 day culture in 1 ppm BA showing several layers of divided cells above cut surface. 125X.

Figure 68. Midvein after 20 days in culture in 1 ppm BA showing continuing cell division on the peripheral zone. 80X.

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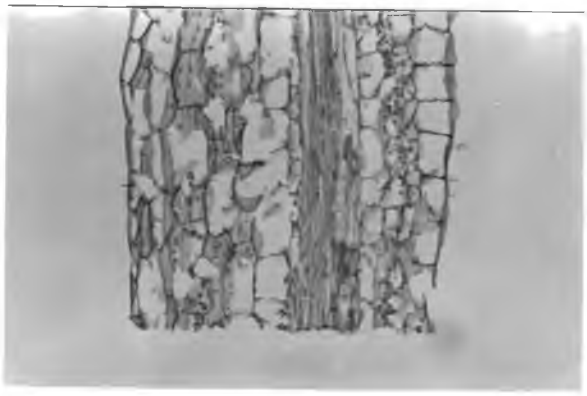

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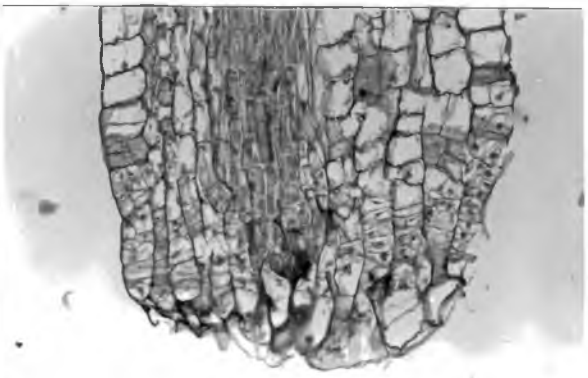

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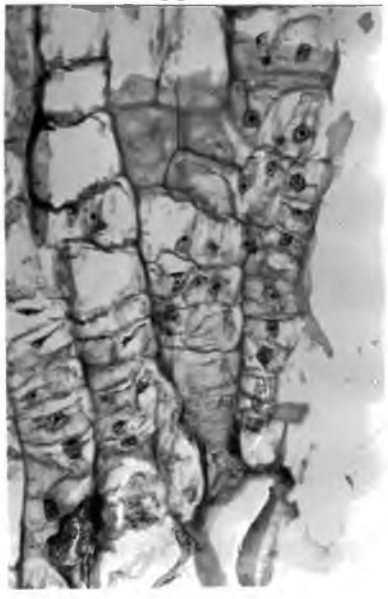

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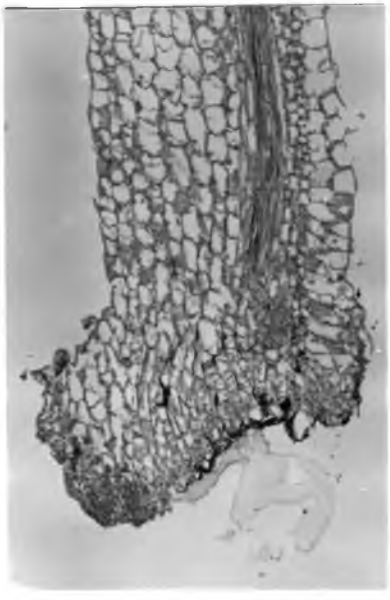

Anatomical study on bud formation from leaf tissue (Scindapsis aureus)

Figure 69. Midvein after 30 days in culture in 1 ppm BA showing shoot tip with apical meristem. 60X.

Figure 70. Mesophyll after 30 days in culture in 1 ppm BA showing shrunken cells and lignified cut surface. 125X.

Figure 71. Longitudinal section of 0 day petiole tissue (safranin and fast green stain). 125X.

Figure 72. Petiole 10 days in culture in 1 ppm BA and 1 ppm NAA showing several layers of divided cells above the cut surface. 125X.

Figure 73. Petiole 20 days in culture in the above medium showing continuing cell division on the peripheral zone. 80X.

Figure 74. Petiole 30 days in culture in the above medium showing a shoot tip. 80X.

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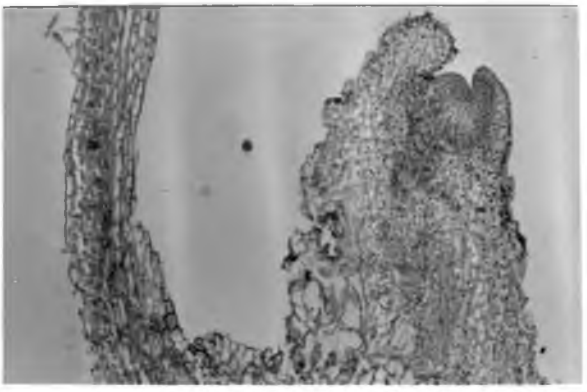

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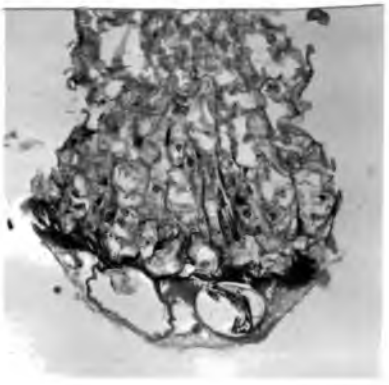

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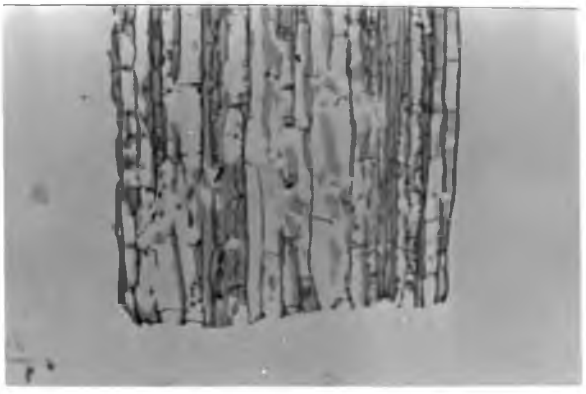

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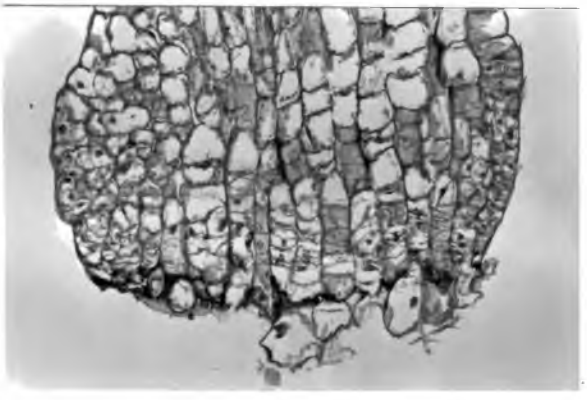

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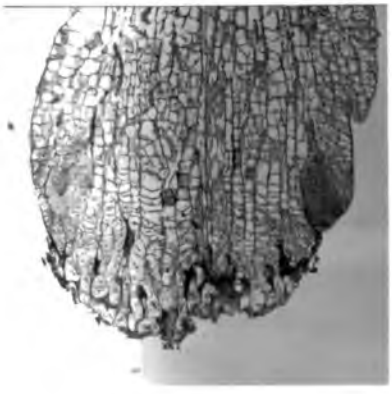

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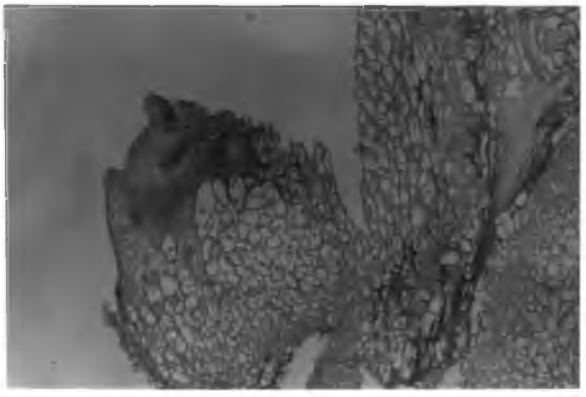

II. Other genera

A. Other aroids

Since the experiments with Scindapsis aureus demonstrated that node culture is very effective for multiple shoot formation, the usefulness of this technique to related plants in Araceae was examined.

1. Philodendron oxycardium Schott

Nodes were excised and cultured in 1 and 10 ppm BA medium. The results are shown in Table 14 and Figure 76. In 1 ppm BA, no adventitious buds were formed although elongation of the axillary bud was promoted. In 10 ppm BA, branching of axillary bud was observed. Many adventitious buds (Fig. 76, arrow) formed from the node of axillary shoot. These adventitious buds were transferred to 1 ppm BA solid and liquid medium with a small portion of nodal tissue. After one month, all the buds elongated in both media (Figs. 77 and 78). They were then transferred to 1 ppm NAA medium to obtain roots (Fig. 79). Forty days later, the rooted plantlets were transferred to pots and covered with plastic bags to avoid desiccation. Once plantlets are obtained by node culture, it is possible to multiply them by repeating the node culture procedure in 1 or 10 ppm BA medium.

Table 14. Effect of BA on bud formation from node after 50 days in culture

BA conc. (ppm)	Avg. no. of bud	Avg. height of shoot (cm)
0	0	0
1	1	3.0
10	2.5[a] + 12[b]	1.5[a]

[a]Branched shoot.

[b]Adventitious bud from node of axillary shoot.

Propagation of Philodendron oxycardium Schott

Figure 75. Philodendron oxycardium Schott used for culture. 0.3X.

Figure 76. Left: culture in 1 ppm BA showing elongation of axillary bud, right: culture in 10 ppm BA showing elongation and branching of axillary bud, and adventitious bud formation from node of axillary shoot (arrow). 1.2X.

Figure 77. Elongation of adventitious bud in 1 ppm BA solid medium. 2X. Adventitious buds were transferred from 10 ppm BA medium.

Figure 78. Elongation of adventitious bud in 1 ppm BA liquid medium. 1.2X. Adventitious buds were transferred from 10 ppm BA medium.

Figure 79. Root formation in 1 ppm NAA. 1X.

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2. Philodendron lacerum (Jacq.) Schott

Top node was excised and cultured in 5 and 10 ppm BA medium (Figs. 83 and 84). Later, the node with buds were transferred to 1 ppm BA solid medium for further growth. After one month, they were divided into a few individuals with a small portion of nodal tissue and cultured in the same medium. Other adventitious buds formed at the basal part (Fig. 85, arrow). When shoots reached 1.5 or 2 cm in height, they were transferred to 0.1 ppm NAA medium to induce roots. Forty days later, rooted plantlets (Fig. 86) were transferred to pots as described before. Once plantlets are obtained, it is possible to induce buds again by culturing the top of the shoot axis (Fig. 87).

3. Spathiphyllum 'Clevelandii'

Shoot axis was trimmed as shown in Fig. 89 and sterilized. Then, leaves were removed and the top several nodes including shoot tip were excised (Fig. 90). The explants were cultured in 1 and 5 ppm BA medium. Fifty percent of explants was lost by contamination. The results are shown in Table 15 and Fig. 91. Adventitious buds formed from nodal tissue in 1 ppm BA medium. A few buds formed in 5 ppm BA medium. Elongation of buds was very slow in both media. In order to promote growth, the main shoot was removed and cultured for another month. The results are shown in Table 18 and Figure 92. Removal of the main shoot clearly promoted elongation of buds. The removed shoot was used for production of other buds (Fig. 93). Later, buds were divided individually and cultured in 1 ppm BA medium until they reached 2-3 cm in height (Fig. 94). Then, they were transferred to 1 and 5 ppm NAA or 1 ppm 2,4-D medium to obtain roots. The results

Propagation of Philodendron lacerum (Jacq.) Schott

Figure 80. Leaf of Philodendron lacerum. 4X.

Figure 81. Excised shoot axis. 1X.

Figure 82. Top node used for culture (left) and 2 month culture in 5 ppm BA showing only elongation of axillary bud (right). 1X.

Figure 83. Node culture in 5 ppm BA solid medium showing adventitious bud formation. 1.2X. Node was excised from axillary shoot obtained by top node culture.

Figure 84. Node culture in 5 ppm BA liquid medium showing adventitious bud formation. 1.2X. Node was excised from axillary shoot obtained by top node culture.

Figure 85. Sub-culture in 1 ppm BA showing additional bud formation at the base of the node (arrow). Buds induced in 5 ppm BA were transferred with a small portion of nodal tissue to 1 ppm BA. 2X.

Figure 86. Root formation in 1 ppm NAA. 1X.

Figure 87. Adventitious bud formation from the basal cut end of shoot axis. 2X. The shoot axis was obtained from the plantlet induced by node culture.

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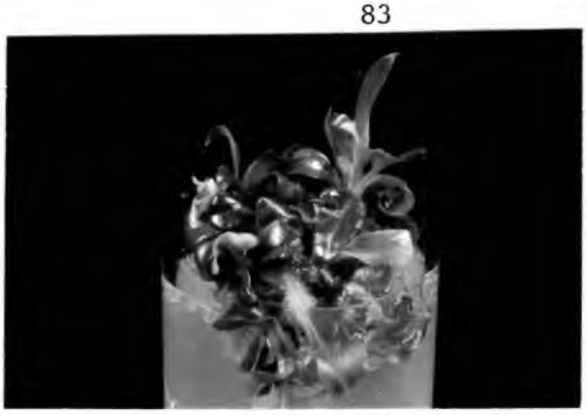

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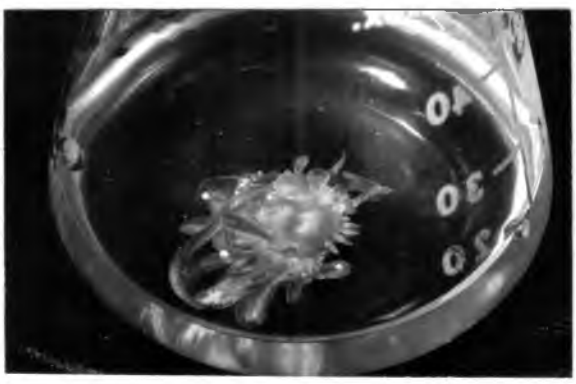

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Table 15. Effect of BA on bud formation from node after 50 days in culture

BA conc. (ppm)	Avg. no. of bud/node
0	0
1	6.0
5	1.3

Table 16. Effect of removal of main shoot on growth of bud obtained by node culture after 30 days in culture

Treatment	Avg. height of shoot (cm)
intact	0.4
removed	0.8

Main shoot was removed after 50 day pre-culture in 1 ppm BA medium.

are shown in Table 17 and Figure 95. Five ppm NAA and 1 ppm 2,4-D induced roots, but 1 ppm NAA was not high enough for root induction. Within 5 months, rooted plants were transferred to pots and cultured as described before (Fig. 96).

Table 17. Effect of NAA and 2,4-D on root formation after 40 days in culture

Treatment	Avg. no. of root/explant
1 ppm NAA	0
5 ppm NAA	5
1 ppm 2,4-D	4

Shoot obtained in 1 ppm BA medium was transferred to the above medium.

Propagation of Spathiphyllum 'Clevelandii'

Figure 88. Spathiphyllum 'Clevelandii' used for culture. 0.1X.

Figure 89. Shoot axis after trimming leaves. 0.5X.

Figure 90. Upper part of the shoot axis after removing leaves. 2X.

Figure 91. Left: node culture in 1 ppm BA showing adventitious bud formation from node. 1.2X, right: node culture in 5 ppm BA showing a few buds formed at node.

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Propagation of Spathiphyllum 'Clevelandii'

Figure 92. Elongation of adventitious buds by removing the main shoot. 2X.

Figure 93. Repeated node culture in 1 ppm BA using excised main shoot showing adventitious bud formation. 1X.

Figure 94. Further elongation of individual shoot in 1 ppm BA. 1X.

Figure 95. Root formation in 5 ppm NAA. 1X.

Figure 96. Potted plantlet (5 months). 0.5X.

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4. Alocasia cucullata (Lour.) Schott

Shoot axis was trimmed as shown in Figure 98 and sterilized. Then, leaves were removed and the shoot tips, including several nodes, were cultured (Fig. 99). The results are shown in Table 18 and Figure 100. Five and 10 ppm BA induced adventitious buds. Rate of node with adventitious buds increased as BA concentrations increased. Since these buds failed to elongate, they were transferred in clumps consisting of several buds with a small portion of nodal tissue to 1, 5 and 10 ppm medium (Fig. 101). In 1 ppm BA medium, all the buds became necrotic and eventually died. In 5 ppm BA, about 30% of the buds began to elongate (Fig. 102). The rest turned yellow and died. In 10 ppm BA medium, no further elongation was noticed although the tissue remained green. Since growth of the shoot in 5 ppm BA became slow, the tissue was transferred to 1 ppm NAA medium when the 2nd leaf appeared. Within 2 months, shoots regained vigor and formed many roots (Fig. 103). Rooted plantlets were transferred to pots and cultured as described before (Fig. 104).

Table 18. Effect of BA on bud formation from node after 40 days in culture

BA conc. (ppm)	Percentage of nodes with buds
1	33
5	44
10	88

Propagation of *Alocasia cucullata* (Lour.) Schott

Figure 97. *Alocasia cucullata* used for culture. 0.2X.

Figure 98. Shoot axis after trimming leaf blades. 0.6X.

Figure 99. Upper part of the shoot axis after removing leaves. 2X.

Figure 100. Adventitious buds induced in 10 ppm BA. 2X.

Figure 101. Developing buds after transfer to 5 ppm BA from 10 ppm BA. 2X.

Figure 102. Elongation of bud in 5 ppm BA. Some buds turned brown and died (arrow). 2X.

Figure 103. Root formation in 1 ppm NAA. 0.7X.

Figure 104. Potted plantlet (5 months). 0.5X.

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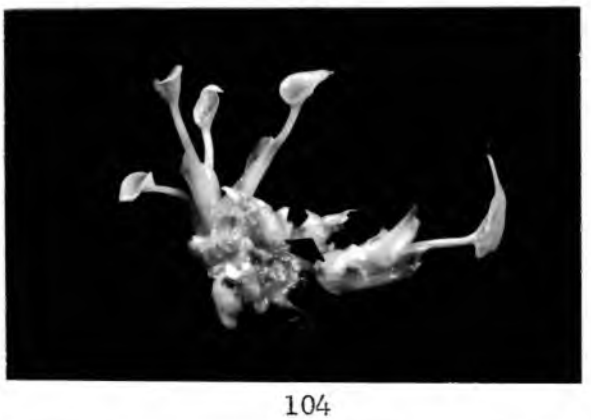

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5. Monstera deliciosa Liebm

Top node was cultured in 1 and 5 ppm BA medium. Tissue did not show any sign of growth after 3 months, although axillary bud still remained green.

B. Other genera

These experiments were conducted to see whether the technique of node culture was applicable to genera other than aroids (Araceae).

1. Zingiber officinale Roscoe

Buds from Zingiber officinale (Zingiberceae) which emerged from rhizome, were excised (Fig. 105) and a few scale leaves were removed. After sterilization, more leaves and basal part of axis were removed to minimize chance of contamination, but 50% of the samples was contaminated. The trimmed bud (about 3 mm in height) was cultured in 0, 1 ppm BA and 1 ppm BA + 1 ppm NAA medium. The results are shown in Table 19 and Figure 106. In 1 ppm BA medium, several buds appeared. Some originated from axillary buds and some adventitiously from node (Fig. 107). Some explants did not produce buds. However, buds were later obtained by removing scale leaves 2 or 3 months after culture (Fig. 108). In 1 ppm BA + 1 ppm NAA medium, only few buds appeared although many roots were formed. Later, buds were individually transferred to 1 ppm BA medium for further growth. Within 2 months, the buds elongated and roots also formed in this medium (Fig. 109). These plantlets formed additional shoots by branching. Finally, they were transferred to pots and cultured as described before (Fig. 110). However, survival percentage was low (20 to 30%).

Propagation of Zingiber officinale Roscoe

Figure 105. Rhizome with sprouting buds. 2X.

Figure 106. Culture of sprouting buds after removing scale leaves. 0.8X. Left: culture in basal medium showing no growth, middle: culture in 1 ppm BA showing bud formation, right: culture in 1 ppm BA + 1 ppm NAA showing formation of many roots.

Figure 107. Morphological observation of the cultured tissue (1 ppm BA) shows that new buds originated from axillary buds and adventitious buds on the node. 4X.

Figure 108. Effect of removal of scale leaves from cultured bud on bud formation. 1.4X. Left: intact bud in 1 ppm BA showing no bud formation, right: scale leaves-removed bud in 1 ppm BA showing bud formation.

Figure 109. Five month culture in 1 ppm BA showing root formation. 0.7X.

Figure 110. Potted plantlet (5 months). 0.5X.

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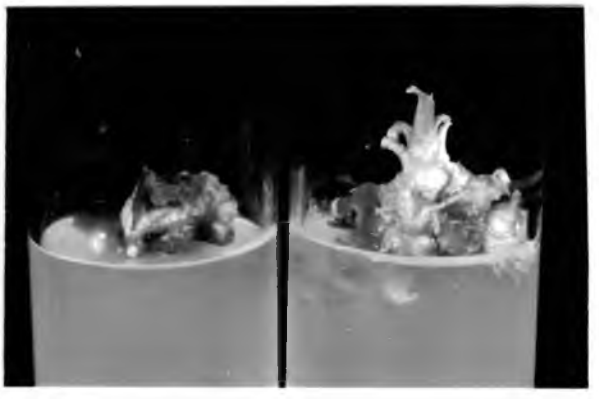

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Table 19. Effect of BA and NAA on shoot and root formation after 2 months in culture

Treatment (ppm)	Avg. no. of shoot	Avg. no. of root
BA 0	0	0
1	5.6 (5.7)[a]	3.0
BA 1 + NAA 1	2.0	6.3

[a]Scale leaves were removed 2 months after culture. Record was taken 1 month after removal.

2. Asparagus myriocladus

Young spear with developing leaves from Asparagus myriocladus (Liliaceae) was used for this experiment. Older spear was discarded because of high percentage of contamination. Several top nodes of the main shoot were excised and cultured in BA, NAA and GA media. The results are shown in Table 20 and Figure 111. One ppm BA produced several adventitious buds. When nodes of axillary shoots were used, 0.1 ppm BA was optimum (data not shown), NAA and GA were without effect. Ten ppm BA produced profuse unorganized buds (Fig. 112) which later turned brown and eventually died, even though they were transferred to 0.1, 1 and 10 ppm BA medium. Anatomical studies (Fig. 113 and 114) show that adventitious buds formed from nodal tissue and then breakage of apical dominance induced other branching shoots. Since buds obtained in 1 ppm BA did not show further growth, their tips (2 mm in height) were excised and transferred to 0, 0.05 and 0.2 ppm BA medium. The results are shown in Table 21 and Figures 115 and 116. Buds in 0.05 ppm BA medium elongated. Those in 0.2 ppm BA induced

Propagation of Asparagus myriocladus Hort

Figure 111. Left: node culture in 0.1 ppm BA showing formation of a few buds from node, right: node culture in 1 ppm BA showing formation of several buds from node. 2X.

Figure 112. Node culture in 10 ppm BA showing profuse unorganized buds from node. 2X.

Figure 113. Anatomical observation of node culture in 1 ppm BA showing adventitious bud formation (longitudinal section). 80X.

Figure 114. Longitudinal section of elongating shoot showing breakage of apical dominance and activated axillary buds. 60X.

Figure 115. Elongation of the induced buds by culturing in lower conc. of BA medium. 0.8X. Left: culture in basal medium showing no growth, middle: culture in 0.05 ppm BA showing normal elongation of shoot, right: culture in 0.2 ppm BA showing abnormal growth with swollen stem.

Figure 116. Further growth in 0.05 ppm BA with expanded leaves. 1X.

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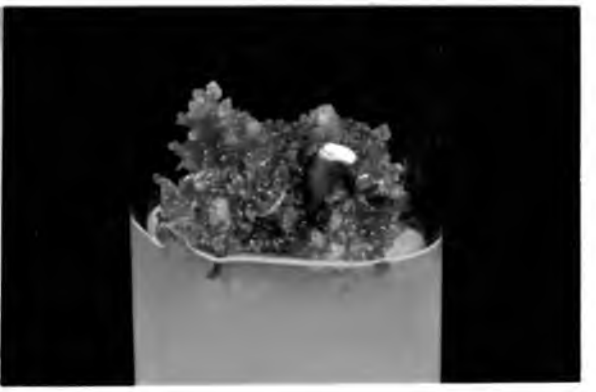

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abnormal stem thickening. No growth was observed in 0 ppm BA medium. Attempts to induce roots were unsuccessful.

Table 20. Effect of BA, NAA and GA on bud formation after 1 month in culture

Treatment (ppm)		Avg. no. of bud/explant
BA	0	0
	0.1	3.3
	1	7.0
NAA	0.1	0
	1	0
GA	0.1	0
	1	0
	10	0

Table 21. Effect of BA on bud elongation

BA conc. (ppm)	Degree of elongation
0	-
0.05	++
0.2	+

- no growth, + slight, ++ fair

Tips of the buds obtained by node culture (1 ppm BA) were excised 2 mm in height and cultured in the above media.

3. Doritaenopsis Dorette

Nodes of Doritaenopsis Dorette were excised from plantlets obtained by aseptic culture of nodes of flower stalk (Fig. 57). They were

cultured in 0 and 1 ppm BA medium. The results are shown in Table 22 and Figure 58. BA induced multiple shoots.

Table 22. Effect of BA on shoot and root formation from node after 3 months in culture

BA conc. (ppm)	Avg. no. of shoot	Avg. no. of root
0	0	0
1	3.0	2.0

4. Neomarica caerulea (Ker-Gawl.) Sprague

Nodes of Neomarica caerulea (Iridaceae) were excised from the base of shoot axis and cultured in 1 ppm BA medium. In this culture, only the main shoot elongated without adventitious bud formation. However, when the node of the main shoot was cultured again in 1 ppm BA, multiple shoots were induced (Fig. 120). After division of shoots, they were cultured in 1 ppm NAA medium to induce roots (Fig. 121).

Coffea arabica (Rubiaceae), Plumeria obtusa (Apocynaceae), Macadamia integrifolia (Proteaceae), Erythrina crista-galli (Leguminosae), and Chrysalidocarpus lutescens (Palmae) were not successful by node culture. All the results on multiple shoot formation by node culture are listed on Table 23.

Propagation of Doritaenopsis Dorette

Figure 117. Plantlet used for node culture. 1X. This plantlet was obtained by aseptic culture of flower stalk.

Figure 118. Culture in 1 ppm BA showing multiple shoot formation from node. 1.2X.

Propagation of Neomarica caerulea (Ker-Gawl.) Sprague

Figure 119. Neomarica caerulea used for culture. 0.25X.

Figure 120. Node culture in 1 ppm BA showing adventitious bud formation. 2X.

Figure 121. Root formation in 1 ppm NAA. 1X.

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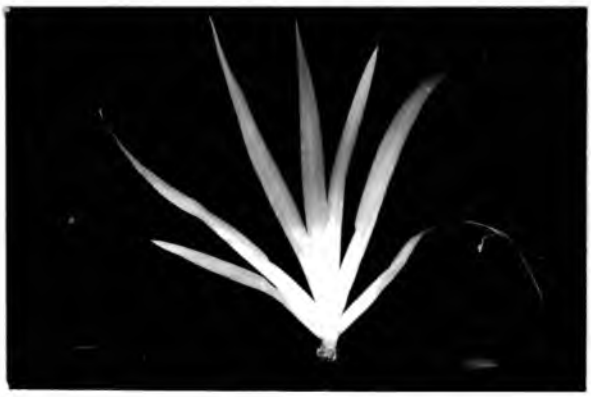

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Table 23. Summary of results on multiple shoot formation by node culture

Species	Family	Sub-class	Result
Scindapsis aureus	Araceae	M	S
Philodendron oxycardium	Araceae	M	S
Philodendron lacerum	Araceae	M	S
Spathiphyllum 'Clevelandii'	Araceae	M	S
Alocasia cucullata	Araceae	M	S
Monstera deliciosa	Araceae	M	F
Zingiber officinale	Zingiberaceae	M	S
Doritaenopsis Dorette	Orchidaceae	M	S
Asparagus myriocladus	Liliaceae	M	S
Neomarica caerulea	Iridaceae	M	S
Chrysalidocarpus lutescens	Palmae	M	F
Macadamia integrifolia	Proteaceae	D	F
Coffea sp. arabica	Rubiaceae	D	F
Plumeria obtusa	Apocynaceae	D	F
Erythrina crista-galli	Leguminosae	D	F

M - monocot, D - dicot, S - successful, F - fail

DISCUSSION

Propagation system through node culture

Currently, there are two methods of propagating plants by tissue culture. One method is through production of callus which is induced to differentiate into plants. This technique has been studied for many years. For example, Skoog and Miller (1957) have succeeded in the induction of shoots and roots from tobacco pith callus. Pillai and Hildebrandt (1972) obtained plantlets from geranium stem callus. Chanhrwedi (1974) obtained complete plants from citrus stem and leaf callus. Hill (1968) obtained plantlets from chrysanthemum.

However, this method has a disadvantage. Callus sometimes shows variation in chromosome number which may be accompanied by undesirable morphological changes in the resulting plants. For example, Shimada and Tabata (1967) reported that 70% of the callus from tobacco pith tissue (2N=48) showed chromosome numbers between 77 and 96 after 1.5 years of culture. Norstog et al. (1969) found that callus which originated from the endosperm of Lolium perenne (3N=21) had numbers ranging from 18 to 50 after 10 years of culture.

Another problem is that shoots cannot be as readily induced as roots. During this study, ginger never formed shoot from callus although many roots appeared. For these reasons, recent studies are shifting to bud production without forming callus.

It is known that some plants produce adventitious buds in nature and treatment with growth regulators promotes their formation. For example, Heide (1965) reported that leaves of Bryophyllum diagremontianum produced more buds with cytokinin treatment. Hackett (1969)

reported that production of bulblets from bulb scales increased by treatment with IAA and kinetin. As these results show, some plants have a capacity to produce adventitious buds in nature and respond well to growth regulators. But, most plants do not respond as the above examples.

Recently, Murashige et al. (1972) succeeded in inducing multiple shoots by culturing asparagus shoot tips. They also reported that the shoot tip method was applicable to gerbera, yucca, begonia and some species in Bromeliaceae (1974). Haramaki (1971) reported that multiple shoots were obtained in gloxinia by shoot tip culture. More recently, Mapes (1974) obtained successful results in taro and pineapple by shoot tip culture. Murashige (1974) reported that buds obtained through shoot tip culture are adventitious in origin or by division of axillary bud. Judging from these results, the shoot tip culture method seems applicable to a wide spectrum of plants.

Recently, Yang and Clore (1973) reported in asparagus culture that propagation through node culture was as successful as through shoot tip culture. They also pointed out that the node culture method has an advantage over the shoot tip method because it does not require tedious manipulation under the stereomicroscope. In my experiments also, the node of Scindapsis aureus produced multiple shoots by single treatment of BA.

Based on the results of culture of Scindapsis aureus, a propagation system for this plant was proposed from the practical point of view (Fig. 122). There are two ways of propagation. For the first way: culture a few nodes from the top in 1 ppm BA medium to promote

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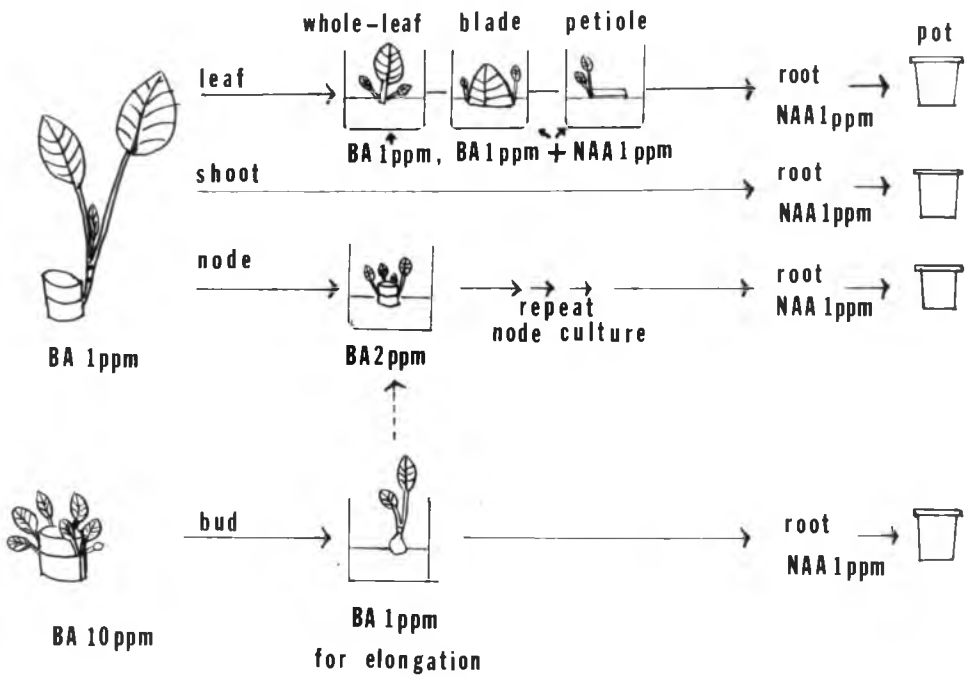

Propagation system of Scindapsis aureus by tissue culture

elongation of axillary bud. Then, from the shoot obtained, excise node and whole leaf (blade + petiole), and culture in 2 ppm BA and 1 ppm BA medium respectively; or, culture leaf blade and petiole in 1 ppm BA + 1 ppm NAA medium (leaf culture will be discussed in detail later). After induction and elongation of bud, transfer to 1 ppm NAA medium to produce roots. Finally, transfer plantlet to pot with vermiculite and cover with plastic bag. Once plantlets are induced, repeat node culture for further multiplication. For the second way: culture top or 2nd node in 10 ppm BA medium to induce adventitious buds. After bud induction, transfer them with a small portion of nodal tissue to 1 ppm BA medium for shoot elongation. Then, transfer individual shoots to 1 ppm NAA medium to induce roots. Finally, transfer plantlets to pot. Once plantlets are induced, repeat node culture in 2 ppm BA medium for further multiplication.

The node culture technique can be also applied to other plants in Araceae. The conditions for bud induction, bud elongation, and root induction for five aroids are listed in Table 24. From this table, it is possible to establish a general procedure for propagation of aroids, although some modifications are necessary depending on the species (Fig. 123).

Moreover, the results on node culture of orchid, asparagus, ginger and neomarica suggest possibility of wide application of this method for propagation of monocots.

From the results of chromosome counting, the chances of polyploidy appear much less than that of callus.

Table 24. Hormonal requirements for propagation of aroids by node culture

Species	Induction of bud	Elongation of bud	Induction of root
Scindapsis aureus	BA 10 ppm	BA 1 ppm	NAA 1 ppm
Philodendron oxycardium	BA 10 ppm	BA 1 ppm	NAA 1 ppm
Philodendron lacerum	BA 5-10 ppm	BA 1 ppm	NAA 0.1 ppm
Spathiphyllum 'Clevelandii'	BA 1 ppm	BA 1 ppm	NAA 5 ppm
Alocasia cucullata	BA 5-10 ppm	BA 5 ppm	NAA 1 ppm

Step. 1. Induction of bud: culture top few nodes in relatively high concentration BA medium (1-10 ppm). If adventitious buds do not form, repeat this process using axillary shoot obtained in the above medium.

Step. 2. Elongation of bud: transfer the induced buds with some portion of mother tissue to relatively low concentration BA medium (1-5 ppm).

Step. 3. Induction of root: transfer shoots to auxin medium (0.1-5 ppm NAA or 0.1-1 ppm 2,4-D).

Step. 4. Transfer plantlets to pot and cover with plastic bag until they are established (7-10 days).

(Leaf culture was not included in the general procedure because it was not successful in plants other than Scindapsis aureus.)

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Step 1
induction of adventitious bud

BA high conc. (1—10 ppm)

Step 2
elongation of bud

BA low conc. (1—5 ppm)

Step 3
induction of root

NAA 0.1—5 ppm, 2-4D 0.1—1ppm

Step 4
pot culture

vermiculite medium

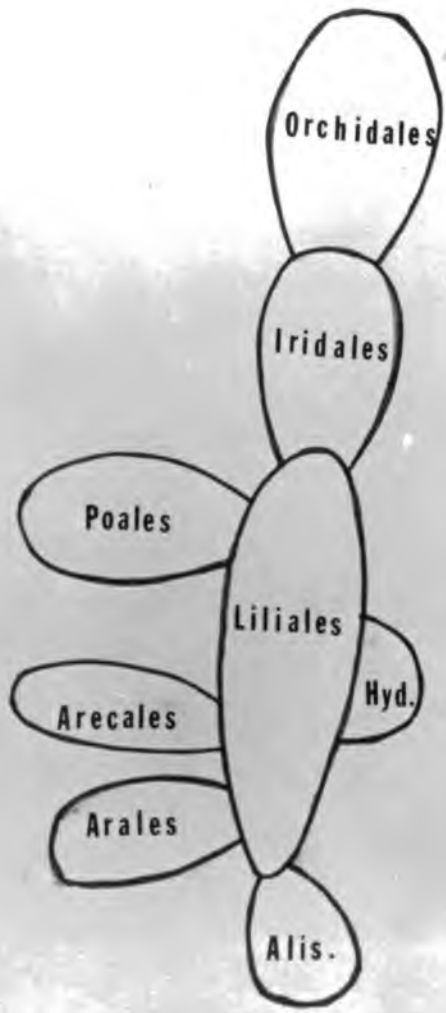
General procedure for propagation of aroids

It is interesting to look at multiple shoot formation from phylogenetic point of view. Figure 124 shows a chart of phylogeny of monocots (Bessey, 1915). Asparagus myriocladus and Doritaenopsis Dorette which belong to Liliales and Orchidales respectively showed multiple shoot formation. Therefore, it can be expected that some plants in Iridales which is between the two previous orders could produce multiple shoots. The result of Neomarica caelurea (Iridales) supports the above idea.

Although the node culture method is widely applicable, dicots and woody monocots (e.g. palm) did not respond.

Hormonal studies on bud formation from node and internode (Scindapsis aureus)

Data show that cytokinin and auxin are important factors for bud formation (Heide, 1965; Skoog and Miller, 1957). In internode culture of Scindapsis, both auxin (NAA) and cytokinin (BA) were required. On the other hand, in node culture, only BA was required (Fig. 125). Leaf culture will be discussed later. Although exogenous auxin is not necessary in node culture, endogenous auxin seems to be involved in bud formation. The middle of Figure 125 shows the following hypothesis. Node tissue can produce enough endogenous auxin for bud formation. Therefore, application of NAA is not necessary. On the other hand, the internode produces too little or no auxin for bud formation. Therefore, application of NAA is necessary. In fact, TIBA (antagonist of auxin) applied through the medium (Fig. 125) suppressed bud formation from node (Ex. I. A. 1. f.). This result suggests



Phylogeny of monocot (Bessey, 1915)

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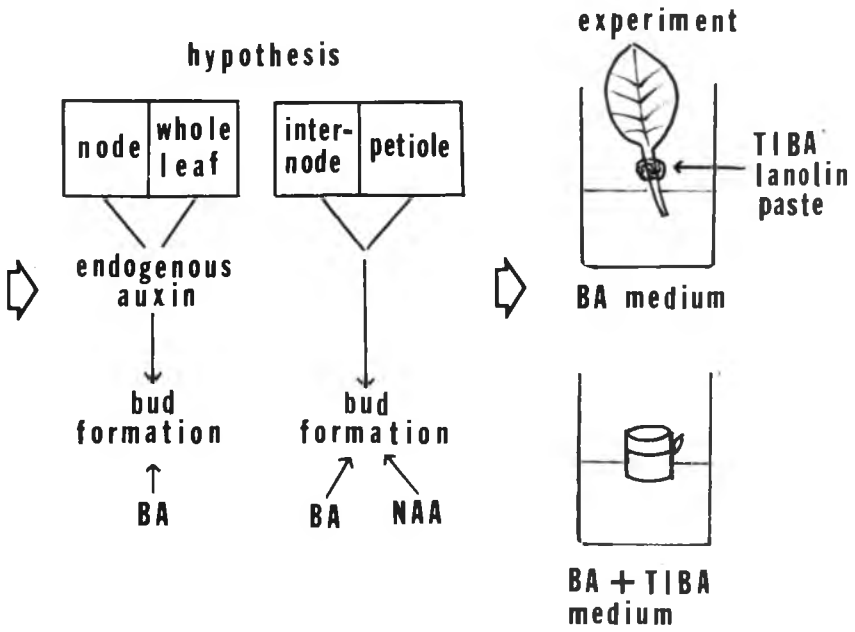
requirement for bud formation

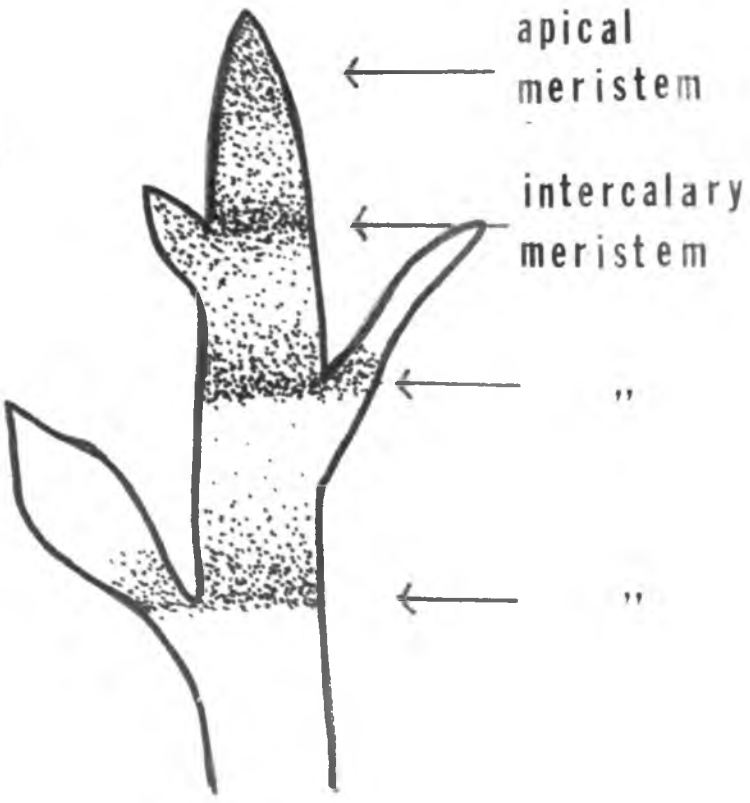
tissue	NAA	BA
node	−	+
whole leaf	−	+
inter-node	+	+
petiole	+	+



participation of endogenous auxin in bud formation. In Ex. I. A. 1. h., also, a correlation between auxin content in the node and ability of bud formation was found. Therefore, it seems that both auxin and cytokinin are crucial factors for bud formation from node.

It is known that auxin is produced in meristematic tissues such as shoot tip and developing fruit. In node culture there are two meristematic regions: one is axillary bud, the other is intercalary meristem. In Ex. I. A. 1. g., removal of axillary bud from node did not affect the number of adventitious buds. This indicates that the axillary bud is not necessary and existence of intercalary meristem is enough for bud formation. Probably, auxin which is provided in intercalary meristem participants in bud formation.

A correlation between ability of bud formation and distribution of intercalary meristem of a grass plant is shown in Figure 126. In the 2nd node culture (Ex. I. A. 1. a.), adventitious buds appeared just on the nodal zone, where intercalary meristem is located. In the top node culture, adventitious buds appeared from the basal part of the stem section, which is internode in position. However, in close proximity to shoot tip, the intercalary meristem is distributed even in internode as shown in Figure 126. In the internode culture between 2nd and 3rd node (Ex. I. A. 1. e.), adventitious bud did not form by single treatment with BA. This experiment can be explained as follows: intercalary meristem is so sparse in this region (Fig. 126) that auxin production is poor. Therefore, buds were not induced. When auxin (NAA) besides BA was supplied to the medium, adventitious buds emerged again. However, the internode below the 3rd node did



Location of meristems of a grass plant

(Eames and MacDaniels 1947)

not produce buds even with a combination treatment of BA and NAA (data not shown). Therefore, in this region, factors other than auxin seems involved in bud formation.

In Ex. I. A. 1. d., nodes of plantlets pre-cultured in 10 ppm BA produced more buds than those pre-cultured in 1 ppm BA. It is known that cytokinin keeps tissue from aging (Richmond and Lang, 1957). Probably, high concentration of BA kept nodes meristematic, so that more buds formed. BA concentration which was optimum for bud induction was too high for elongation in Scindapsis aureus, Philodendron oxycardium, Philodendron lacerum, Alocasia cucullata and Asparagus myriocladus. These results show that different concentrations are required for different stages in growth.

Anatomical, histochemical and microautoradiographic studies on bud formation from node (Scindapsis aureus)

Anatomical study by safranin-fast green staining of paraffin sections shows that cell elongation of a sub-epidermal layer of internode occurred within 15 days after culture in 10 ppm BA medium. On the other hand, subepidermal cells in the bud forming zone, which is located on the nodal zone, are smaller in size than surrounding cells on the 15th day. This result suggests that cells on the bud forming zone did not elongate and entered into cell division. More striking is the change in size of the nucleus. Several epidermal cells and sub-epidermal cells in the bud forming zone showed larger nuclei than those of surrounding cells. It is recognized that nuclear size increases prior to morphogenesis (Ajello, 1941). Therefore, several epidermal cells and cells of some sub-epidermal

layers seem to be activated and involved in bud formation. However, the exact number of cells could not be determined because some cells had already started dividing. On the 30th day, the shoot tip had already appeared.

As for DNA, a higher content per unit area was found in the bud forming zone. This is due to a larger number of small cells in this zone. A similar case is known in tobacco callus (Thorpe, 1972), where bud forming callus showed a higher content of DNA per unit area prior to bud formation. On the other hand, non-bud forming callus showed uniform DNA distribution through the sections. However, intensity of stain of nuclei was the same between bud forming end non-bud forming callus. In Scindapsis node culture also, this was noticed, although some cells in the bud forming zone showed more intense staining of the nuclei. These cells probably have just completed DNA duplication and are ready for mitosis. Stem epidermal cells prior to culture showed higher intensity of stain in some nuclei. These cells seem to remain dormant without mitosis after DNA duplication. The significance of DNA duplication of epidermis was not clear because no appreciable morphological changes followed. Staining of DNA by methyl green showed result similar to Feulgen reaction.

It is known that RNA synthesis precedes visible signs of morphogenesis. Kahl (1973) reported that parenchyma cells of sliced potato tuber synthesized RNA prior to periderm formation. In Scindapsis node culture also, active RNA synthesis (microautoradiographic study) was found in the bud forming zone in advance of cell division or accompanying cell division. However, some RNA synthesis was observed

in the nuclei of the tissue prior to culture. Probably, resting cells can produce a certain amount of RNA and store in the nuclei without discharging to cytoplasm. On the 15th day, more RNA synthesis was observed in the bud forming zone. Although the distinction between nucleus and cytoplasm was not so sharp because of compression by paraffin sectioning (4μ), RNA seems to be distributed in cytoplasm, too. Probably, RNA was discharged to cytoplasm for protein synthesis in the cells in the bud forming zone.

In fact, ninhydrin reaction shows existence of cytoplasmic proteins in the bud forming zone. However, denser stain of protein was found in nuclear region. Probably, this is due to the fact that nucleoprotein is more condensed than cytoplasmic protein. A similar observation was also reported in tobacco callus by Thorpe and Murashige (1970). Shono (1965) demonstrated that an inhibitor of protein synthesis suppressed shoot formation in carrot callus. Fosket and Miksche (1966) reported that protein synthesis preceded the first visible signs of wound xylem differentiation. Probably, proteins are indispensable as components of membranes, ribosomes, enzymes, other structures of new cells.

Hydrocarbon metabolism is often involved in morphogenesis in relation to energy supply. In fact, Thorpe (1968) found that starch accumulation is heaviest in cells of loci which ultimately produced bud, and the callus which was treated with GA prevented starch accumulation and also organ formation. Since GA promoted de novo synthesis of α-amylase of barley endosperm (Varner and Chandra, 1964), induced enzyme probably causes digestion of starch, resulting in failure of

bud formation. They suggested that physiological significance of starch accumulation may reflect high energy requirement for organogenetic process. In node culture of Scindapsis, however, no starch accumulation was found all over the tissues by I-KI reaction. Also, no accumulation of polysaccharides other than starch was observed in the cytoplasm by PAS reaction. However, the nuclei in the bud forming zone were stained for polysaccharides. This may be due to the existence of pentose sugar in DNA and RNA. Therefore, accumulation of polysaccharides in cytoplasm does not seem to be required in the case of Scindapsis. But, chemically fixed tissue does not give information on accumulation of water soluble compounds because they leach out during hydration. In fact, Fehling reaction by fresh hand section was positive in the bud forming zone on the 15th day, indicating accumulation of reducing sugars (water soluble). Probably, sucrose from the medium is converted to reducing sugars such as glucose and fructose. Accumulation of reducing sugars has been often reported in relation to growth and development. For example, Ishida and Takano (1971) reported that shoot tip of Aster savatieri accumulated reducing sugars after vernalization. They suggested that accumulated sugars will be consumed for the following shoot elongation. In node of Scindapsis also, the accumulated sugars will be consumed as energy sources for bud formation. On the 30th day, apical meristem was highly stained, indicating more energy requirement for bud development.

In relation with energy metabolism, it is expected that respiration increases in process of bud formation. Although gas

change of oxygen and carbon dioxide was not measured, it is possible to estimate the degree of respiration from enzyme activity. Succinate dehydrogenase showed high activity in bud forming zone on the 15th day, suggesting enhancement of oxidative respiration. Avers (1958) found that root cells which developed into hairs showed higher activity of this enzyme. This result suggests difference of enzyme activity in advance of differentiation. However, significance of slight stain of stem epidermis remained unclear because no morphological changes were observed through the culture period.

Change of cytochrome oxidase activity is also observed in process of differentiation. Baba (1953) in experiments on wound meristem formation found an increase in cytochrome oxidase activity in resting parenchyma cells in advance of cell division. In Scindapsis node culture, however, no appreciable activity was found in bud forming zone on the 15th day. Even apical meristem on the 30th day, which is supposed to be high in activity, was negative. The same negative reaction was observed in root primordia and shoot tip in preliminary experiment. These regions coincided with those showing high activity of succinate dehydrogenase. Vanden Born (1963) also found a negative result on cytochrome oxidase activity in shoot tip of white spruce in which succinate dehydrogenase activity was high. Horwig (1960) mentioned this problem and explained that dehydrogenase may be responsible for reduction of indophenol blue (oxidized form of Nadi reagent) to colorless compounds. Probably, in node culture also, indophenol blue colored by activity of cytochrome oxidase was reverted to colorless reduced from (original

Nadi reagent) by succinate dehydrogenase with hydrogen. In such a masking condition, it seems to be difficult to obtain real information on cytochrome oxidation.

Glucose-6-phosphate dehydrogenase is another important respiration enzyme catalyzing the first reaction which shifts EMP pathway to pentose phosphate pathway and the reaction mode is formulated as G-6-P + TPN + $H_2O \rightleftharpoons$ 6-phosphogluconic acid + TPNH + H^+. Significance of pentose phosphate pathway is in synthesis of pentose rather than respiration. On the 15th day, bud forming zone showed high activity. As described before, DNA and RNA synthesis was active in this zone. Therefore, it is probable that synthesis of pentose, which is a component of nucleic acids, is also promoted.

As for peroxidase, Van Fleet (1959) reported that axillary loci for the origin of buds gave reactions for peroxidase prior to actual cell division in bud formation. He also suggested that peroxidase may catalyze the reduction of hydrogen accepters essential to cell division because an increase of peroxidase was often found in transition from rest to cell division. In Scindapsis node culture also, high activity was found in bud forming zone on the 15th day, although some cells had already started dividing. Of physiological significance of this enzyme, there is a possibility that a new respiration pathway via peroxidase is functioning in the activated cells. In the tissue prior to culture, high activity of stem epidermis and vascular tissue were observed. However, the physiological significance remains unclear although peroxidase might be involved in lignin formation in vascular tissue as reported by Ishikawa and Takaichi (1957).

In conclusion, the following statement could be made: BA and endogenous auxin activate several epidermal cells and cells of sub-epidermal layers. DNA duplication and mitosis start. RNA is synthesized in nuclei and discharged to cytoplasm for the following protein synthesis. For evergy supply, reducing sugars are accumulated and respiration enzymes (succinate dehydrogenase and G-6-P dehydrogenase) are activated. Probably, pentose also would increase and be used for synthesis of nucleic acids. These events will contribute to formation of new enzymes, membranes, wall components and other structures of new cells of bud.

Most of the Scindapsis plantlets obtained by node culture showed a marbled type of variegation pattern similar to mother plant. This result seems reasonable because multiple cells are involved in bud formation, judging from the results of anatomical and histochemical studies. But, some plants (5-10%) showed albino or entire green plant. Probably, in this case, all the cells in bud forming zone must have been all albino or green in origin.

Studies on leaf culture

In Ex. I. B. 1. a. and c., whole leaf (blade + petiole) and blade segment formed buds with single BA treatment, but petiole section required NAA as well as BA. From these results, as in node culture, it is possible to speculate that auxin is produced in the blade, flows down along the petiole, accumulates at the cut end, and interacts with BA in the medium, resulting in bud formation (Fig. 125). In fact, in whole leaf culture (Ex. I. B. 1. b.), bud did not form by stopping auxin flow along petiole with TIBA, indicating involvement

of endogenous auxin in bud formation. Petiole segment, by itself, maybe, cannot produce auxin or produces too little for bud formation (Fig. 125). Therefore, supply of NAA is necessary as well as BA (Ex. I. B. 1. a.).

In leaf segment culture, buds formed only at the proximal cut end of midvein by BA treatment, but combination treatment induced buds across the length of the cut surface. These results can be explained by hypothesizing a basipetal flow of auxin (Fig. 127). Probably, auxin is produced in the marginal meristem in the blade and flows basipetally along veins. Then, it is accumulated at the proximal cut end of midvein, resulting in bud formation by interaction with BA from medium. Such auxin accumulation may be supported by a positive reaction of Ehlrich reagent at the proximal cut end of midvein (Ex. I. B. 1. d.). If medium contains both NAA and BA, any cut surface of leaf segment is activated and able to produce buds. From a practical point of view, combination treatment of BA and NAA seems better because more buds are formed.

Buds obtained by leaf culture was slow in growth. This problem was solved by transferring them to the liquid medium. Probably, nutrients are more easily absorbed by whole tissue in liquid than on solid medium.

Anatomical study on leaf blade culture suggests that several cell layers above cut surface are activated and form tumor-like tissue. Then, within 30 days, the tissue forms apical meristem on the peripheral zone.

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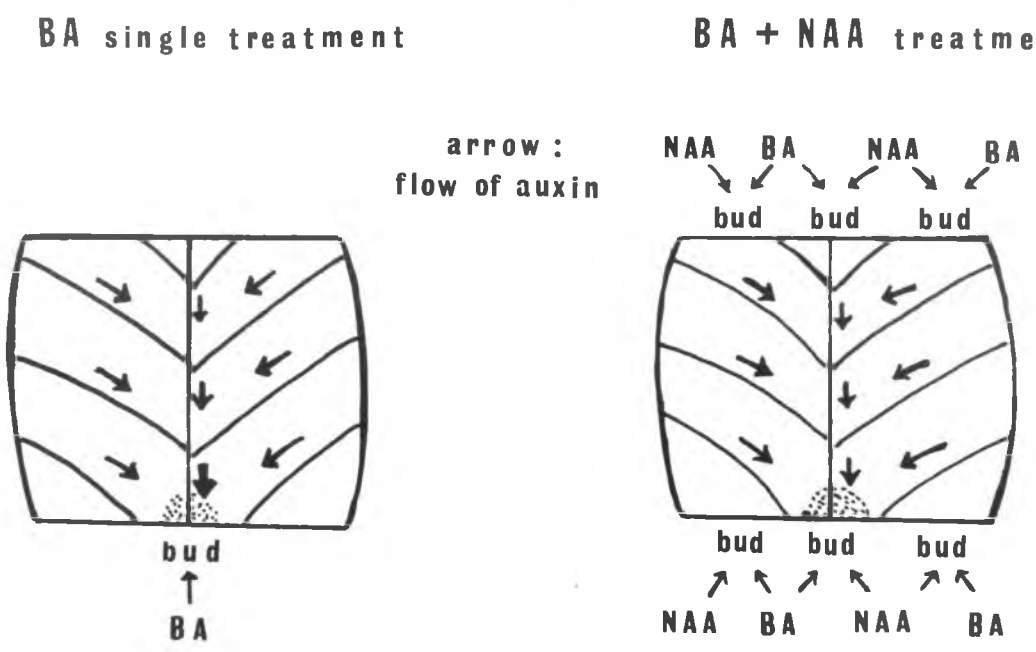

Possible mechanism of bud formation

SUMMARY

Experiments were conducted to establish more efficient propagation systems for aroids and other genera through tissue culture. Physiological, morphological, anatomical, histochemical and microautoradiographic studies were used to elucidate the process of adventitious bud formation.

I. Scindapsis aureus

A. Node culture

1. Morphological and physiological studies

a. Adventitious buds were induced on top first and second nodes cultured in Murashige-Skoog medium which was supplemented with 10 ppm BA; no adventitious buds were produced on the third and fourth nodes.

b. Buds induced by 10 ppm BA elongated when transferred to 1 ppm BA medium and formed roots when transferred to 1 ppm NAA medium. Rooted plantlets were transferred to pots and covered with plastic bags to avoid desiccation. With 4 months, plants were established by the above method.

c. Node, which was excised from the plantlets induced in 10 ppm BA, produced more buds than that of plantlets induced in 1 ppm BA.

d. Internode between second and third nodes formed adventitious buds by combined treatment of 10 ppm BA and 0.1 ppm NAA.

e. Involvement of endogenous auxin in bud formation was suggested by antiauxin, TIBA, treatment in node culture.

f. Removal of axillary bud did not affect the numbers of adventitious buds on the node.

g. A correlation between site of intercalary meristem and occurrence of bud formation was found in node culture.

h. Polyploidy was not found in the plantlets obtained through node culture.

2. Anatomical study of node culture suggests that several epidermal cells and cells of subepidermal layers are involved in forming a bud.

3. Histochemical studies of node cultures showed an increase in DNA per unit area, an increase in proteins, particularly nucleoprotein (histone), an increase in reducing sugars, increases in respiration enzyme activities (succinate dehydrogenase and glucose-6-phosphate dehydrogenase) and an increase in peroxidase activity on the nodal zone prior to bud formation.

4. Microautoradiographic study of node culture showed an increase in RNA on the nodal zone prior to bud formation.

B. Leaf culture

1. Morphological and physiological studies

a. Whole leaf withpetiole and leaf blade segment produced adventitious buds by single 1 ppm BA treatment. Petiole produced buds only by combined treatment with BA and NAA.

b. Involvement of endogenous auxin in bud formation was suggested by antiauxin, TIBA, treatment around petiole in whole leaf culture.

c. In leaf segment culture in BA medium, buds formed at the proximal cut end of midvein. This was explained by hypothesizing a basipetal movement and accumulation of endogenous auxin which may be produced in the marginal meristem.

d. Accumulation of IAA on bud forming side in leaf segment culture was suggested by test of Ehlrich reagent.

e. Buds induced in leaf blade culture elongated faster in liquid medium than solid medium.

2. Anatomical studies of leaf blade and petiole cultures showed that several cell layers above cut surface were activated and formed tumor-like tissue. Buds emerged from the peripheral zone of this tumor.

II. Other genera

A. Other aroids. The applicability of node culture was tested with plants related to Scindapsis in Araceae.

1. In Philodendron oxycardium, nodes cultured in 10 ppm BA medium formed adventitious buds. They elongated and formed roots by transferring to 1 ppm BA and then to 1 ppm NAA medium. Plantlets were successfully cultured in the pots.

2. In Philodendron lacerum, top node cultured in 5 and 10 ppm BA medium did not form adventitious buds. But, a node, which was excised from axillary shoot obtained in the above medium, formed adventitious buds. They elongated in 1 ppm BA medium and then rooted when transferred to 0.1 ppm NAA medium. Plantlets were successfully cultured in pots.

3. In Spathiphyllum 'Clevelandii', nodes of upper stem cultured in 1 ppm BA formed adventitious buds. They elongated by removing a main shoot. Individual shoot was further cultured in the same medium and then transferred to 5 ppm NAA or 1 ppm 2,4-D medium. Rooted plantlets were successfully cultured in pots.

4. In Alocasia cucullata, nodes of upper stem cultured in 5 or 10 ppm BA medium formed adventitious buds. They elongated when transferred to 5 ppm BA medium, but some shoots became necrotic and died. Surviving shoots were transferred to 1 ppm NAA medium when the second leaves emerged. Rooted plants were successfully cultured in pots.

5. In Monstera deliciosa, top node culture in 5 and 10 ppm BA did not produce any adventitious buds. General procedure for propagation of aroids: based on all the results of aroids, a general propagation system of this family was suggested. First step: culture top or second node in relatively high concentration BA medium for bud induction. If buds do not form, repeat this step using axillary shoot obtained in the above medium. Second step: transfer the buds to relatively low concentration BA medium for elongation. Third step: transfer the shoots to NAA or 2,4-D medium for root formation. Fourth step: transfer the plantlets to pots and culture with plastic cover. The same method can be applied to some in monocots.

B. Other genera. The applicability of node culture was tested in genera other than aroids.

1. In Zingiber officinale (Zingiberaceae), nodes obtained from sprouting buds on the rhizome were cultured in 1 ppm BA medium.

Some formed buds. Some did not. However, by removing scale leaves, all the nodes produced buds. Later, they were individually transferred to 1 ppm BA medium for further growth. Rooted plants were transferred to pots, but survival percentage was not high (20-30%).

2. In Asparagus myriocladus (Liliaceae), nodes cultured in 1 ppm BA medium formed adventitious buds. When their tips were excised and cultured in 0.05 ppm BA medium, they elongated, but roots did not form in any medium examined.

3. In Doritaenopsis Dorette (Orchidaceae), node, which was excised from plantlets in vial culture of flower stalk, was cultured in 1 ppm BA medium. Adventitious buds and roots formed. They were successfully cultured in pots.

4. In Neomarica caerulea (Iridaceae), which is in Iridales and is phylogenetically located between Orchidales and Liliales, multiple shoots were produced by node culture in 1 ppm BA medium. After root formation in 1 ppm NAA medium, plantlets were successfully cultured in pots.

5. In Chrysalidocarpus lutescens (Palmae), Coffea arabica (Rubiaceae), Macadamia integrifolia (Proteaceae), Plumeria obtusa (Apocynaceae) and Erythrina crista-galli (leguminosae) node cultures were not successful.

LITERATURE CITED

Ajello, L. 1941. Cytology and cellular interrelations of cystolith formation in Ficus elastica. Amer. Jour. Bot. 28: 589-594.

Avers, CH. 1958. Histochemical localization of enzyme activity in root epidermis of Phleum pratense. Amer. Jour. Bot. 45:609-613.

Bessey, C. E. 1915. Phylogenetic taxonomy of flowering plants. Ann. Mo. Bot. Gard. 2:1-55.

Baba, S. 1953. A histochemical study of wound periderm formation. Mem. Coll. Sci. Univ. Kyoto Ser. B 20:195-201.

Chaturvedi, H. C. and G. C. Mitra. 1974. Clonal propagation of citrus from somatic callus cultures. HortScience 9(2):118-120.

Eames, A. J. and L. H. MacDaniels. 1947. An introduction to plant anatomy. 2nd. MacGraw-Hill Book Company, Inc. New York and London. 427 pp.

Fosket, D. L. and J. Miksche. 1966. Protein synthesis as a requirement for wound xylem differentiation. Physiol. Plant. 19:982-991.

Hackett, W. P. 1969. Aseptic multiplication of lily bulblets from bulb scales. Proceedings of the International Plant Propagators Society Annual Meeting. pp. 105-108.

Haramaki, C. 1971. Tissue culture of gloxinia. Int. Plant. Prop. Soc. 21:442-448.

Harwig, J. 1967. The use of histochemical regents for cytochrome oxidase in plant tissues. Bot. Rev. 33:116-129.

Hasegawa, P. M., T. Murashige and F. H. Takatori. 1973. Propagation of asparagus through shoot apex culture. II. Light and temperature requirements, transplantability of plants, and cytohistological characteristics. Jour. Amer. Hort. Sci. 98(2): 143-148.

Hawk, P. B., B. L. Oser and W. H. Summerson. 1951. Practical Physiological Chemistry. The Blakiston Company. Toronto, Philadelphia. 1323 pp.

Hay, J. R. 1956. The effect of 2,4-dichlorophenoxyacetic acid and 2,3,5-triiodobenzoic acid on the transport of indoleacetic acid. Plant Physiol. 31:118-120.

Heide, O. M. 1965. Effects of 6-benzylaminopurin and 1 naphthalene acetic acid on epiphyllous bud formation in Bryophyllum. Planta 67:281-296.

Herrmann, R. G. and W. O. Abel. 1972. Microautoradiography and electron probe analysis. Ed. U. Lüttge. Springer-Verlag, New York, Heidelberg, Berlin. 242 pp.

Hill, G. P. 1968. Shoot formation in tissue cultures of Chrysanthemum 'Bronze Price'. Physiol. Plant. 21:386-389.

Ishida, A. and T. Takano. 1971. Studies on the forcing of Aster savatieri MAKINO. XIII. Histochemical changes in the shoot apex in cold and non-cold treated plants. Jap. Soc. Hort. Sci. 40(2):179-182.

Ishikawa, H. and K. Takaichi. 1957. Lignin and lignification. 6. The formation of lignin in young plants. Jour. Japan Forest Soc. 39(70-73).

Jensen, W. A. 1962. Botanical Histochemistry. W. H. Freeman and Company. San Francisco and London. 408 pp.

Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill. New York. 523 pp.

Kahl, G. 1973. Genetic and metabolic regulation in differentiating plant storage tissue cells. Bot. Rev. 39:273-299.

Mapes, M. O. 1973. Tissue culture of bromeliads. Int. Plant Prop. Soc. Combined Proc. 23:47-55.

Mapes, M. O. and W. J. Cable. 1974. Mericloning of taro (Colocasia esculenta). Proc. 3rd Int. Symp. of Tropical Root and Tuber Crops.

Murashige, T. 1974. Plant propagating through tissue cultures. Ann. Rev. Plant Physiol. 25:135-166.

Murashige, T., M. N. Shabde, P. M. Hasegawa, F. H. Takatori and J. B. Jones. 1972. Propagation of asparagus through apex culture. I. Nutrient medium for formation of plantlets. Jour. Amer. Hort. Sci. 97(2):158-161.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nachlas, M. M., D. G. Walker and A. M. Seligman. 1958. The histochemical localization of triphosphopyridine nucleotide diaphorase. Jour. Biophysic. and Biochem. Cytol. 4(4):467-473.

Norstog, K., W. E. Wall and G. P. Howland. 1969. Cytological characteristics of ten-year-old rye-grass endosperm tissue cultures. Bot. Gaz. 130:83-86.

Pillai, S. K. and A. C. Hildebrandt. 1972. Induced differentiation of geranium plants from undifferentiated callus in vitro. Amer. Jour. Bot. 56(1):52-58.

Poovaiah, B. W., H. P. Rasmussen and M. J. Bukovac. 1973. Histochemical localization of enzymes in the abscission zones of maturing sour and sweet cherry fruit. Jour. Amer. Soc. Hort. Sci. 98(1):16-18.

Richmond, A. E. and A. Lang. 1957. Effect of kinetin on protein content and survival of detached Xanthium leaves. Science 125:650-651.

Shimada, T. and M. Tabata. 1967. Chromosome numbers in cultured pith tissue of tobacco. Japan Jour. Gen. 42(3):195-201.

Shōno, K. 1965. Physiological and biochemical changes of carrot root callus during successive cultures. Plant Cell Physiol. 6:371-392.

Skoog, F. and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11:118-130.

Thorpe, T. A. and T. Murashige. 1968. Starch accumulation in shoot-forming tobacco callus cultures. Science 160:421-422.

Thorpe, T. A. and T. Murashige. 1970. Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. Jour. Bot. 48:277-285.

Vanden Born, W. H. 1963. Histochemical studies of enzyme distribution in shoot tips of white spruce. Can. Jour. Bot. 41:1509-1527.

Van Fleet, D. S. 1959. Analysis of the histochemical localization of peroxidase related to the differentiation of plant tissues. Can. Jour. Bot. 37:449-458.

Varner, J. E. and G. Ram Chandra. 1964. Hormonal control of enzyme synthesis in barley endosperm. Proc. Nat. Acad. Sci. U.S.A. 52:100-106.

Yang, Hsu-Jen and W. J. Clore. 1973. Rapid vegetative propagation of asparagus through lateral bud culture. HortScience 8(2):141-143.