our possible realm of using. Now we have within the group at the University of California at Riverside, one of the leading educators and research people in the field of tissue culture, Dr. Toshio Murashige. Toshio has been on the UC campus at Riverside since 1964. He came there from the University of Hawaii. Tosh, you have held tissue culture seminars and lectures for the nurserymen and many of them are practicing tissue culture in their own labs and nurseries; this is all due to your fine work, so come on up and tell us about what is new in tissue culture.[1]

[1] Dr. Toshio Murashige and Ms. Jeanne Jones, U.C. Staff Research Associate, discussed their work in the field of tissue culture.

TISSUE CULTURE OF BROMELIADS[1]

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Abstract. Numerous pineapple [*Ananas comosus* (L.) Merr. variety Smooth Cayenne] plantlets and protocorm-like bodies were produced from shoot tips when a combination of orchid shoot tip technique and callus method for organogenesis was applied sequentially and in correct order. Initially, explants from shoot tip, stem and root tips failed to grow in 42 different media. Meristematic protocorm-like bodies and plantlets were produced from pre-shaken shoot tip cultures in Murashige and Skoog's basal medium plus adenosine, 30 ppm, or adenine, 20 ppm. Ornamental bromeliads were more recalcitrant in culture, but, with slight modifications of cultural media, the same procedures appeared applicable. *Portea petropolipana* and *Guzmania* sp. have shown positive response and a wild pineapple, *Ananas erectifolius*, L.B. Smith has produced several lateral shoots and protocorm-like bodies.

REVIEW OF LITERATURE

The bromeliads, in recent years, have gained popularity as indoor and rock garden ornamentals. They range in appearance from the dull gray *Tillandsia usneoides* (Spanish moss of Florida) to the brilliantly colored flowering aechmeas, billbergias and vriesias. They belong to the pineapple family, Bromeliaceae.

In most bromeliaceous plants, seeds are produced when pollinated but among the best horticultural varieties, only 1 or 2% of the seedlings come true (observations forwarded by Howard Yamamoto, Honolulu bromeliad nurseryman). Then there are other plants like the commercial pinneapple (*Ananas*

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comosus] which do not form seeds. Asexual propagation is by lateral (axillary) shoots or offsets which develop on the stem below the inflorescence axis but these are sometimes limited in number to only a single lateral shoot per plant. The pineapple is unusual in that it produces several planting materials including the *crown*, which surmounts the fruit, and lateral shoots called the *slip*, *hapa* and *sucker*, depending on their position on the stem. Multi-propagation on a commercial scale is made from stump sections, based on early works of Walters (1932), MacLuskie (1939), Skoog (1939 unpublished data) and Siu (1941), and from stem-leaf base techniques subsequently developed by researchers H. Clark, B. Krauss and K. Kerns of the Pineapple Research Institute of Hawaii (unpublished data). Both procedures depend on stimulating the lateral bud, which normally lies dormant at the axil of a leaf, to grow and develop into a shoot. The maximum number of shoots obtained by these methods was 80 per plant (Kerns, unpublished data). When numerous plants of desirable quality are needed, therefore, the utilization of the tissue culture technique appears highly advantageous. Its role in plant multiplication is well known.

Since the principles and aseptic procedures of culturing plant tissues and organs for proliferation of new tissues and organogenesis have been reported earlier to this Society by Murashige (5) and Marston (2), only a brief description of the procedures adapted for culturing bromeliaceous plants will be given.

MATERIALS AND METHODS

Preliminary cultural tests were carried out on September 6, 1970, with 12 rooted crowns of pineapple, *Ananas comosus* (L.) Merr., cv. Smooth Cayenne. Each crown was cut into longitudinal halves, and the shoot apex, the subapical stem tissues, and root tips were cultured aseptically into 26 different media, as shown in Table 1. Solid media of Murashige and Skoog's (6) basal medium (M) and White's (13) basal medium (W) were used with the addition of growth promoting substances to induce callus formation in the tissues. Treatments 1 and 2, as shown, were inoculated with duplicate halves from crown No. 1. Treatments 3 and 4 were from crown #2, etc. Crown No. 12, however, was cut longitudinally twice (quartered) so that 4 shoot tip regions, 4 stem tissues and root tips were available for culturing (Treatments 23-26). The media were poured into the snap-on type of Falcon plastic dishes and one each of the three culturing materials mentioned above was placed on each plate.

After 10 weeks (November 16, 1970) when none of the tissues showed signs of new growth or callus formation, the shoot tip tissues were transferred to other media (shown in Table 2)

Table 1. Growth of pineapple explants from crown shoot tip, sub-apical stem tissue, and root tips in Falcon agar plates with two basal media and growth regulators, with (30 ppm) and without adenosine.

Treatment No	Source· Crown No.	Cultural material	Culture Media	Results after 10 weeks
1	1	Shoot tip,	M + CM 10% (V/V) = MCM	No further
2	1	stem and	W + CM 10% (V/V) = MCM	growth in all
3	2	root tip	MCM + NAA 2.5 ppm	treatments.
4	2	on each	WCM + NAA 2.5 ppm	Explant
5	3	treatment	MCM + NAA 5.0	surface
6	3	medium	WCM + NAA 5.0	brown,
7	4		MCM + IAA .5	internal
8	4		WCM + IAA .5	tissues
9	5		MCM + 2,4-D 2.5	white.
10	5		WCM + 2,4-D 2.5	Roots
11	6		MCM + 2,4-D 5.0	white.
12	6		WCM + 2,4-D 5.0	
13	7		Treatment 1 + adenosine	
14	7		Treatment 2 + adenosine	
15	8		Treatment 3 + adenosine	
16	8		Treatment 4 + adenosine	
17	9		Treatment 5 + adenosine	
18	9		Treatment 6 + adenosine	
19	10		Treatment 7 + adenosine	
20	10		Treatment 8 + adenosine	
21	11		Treatment 9 + adenosine	
22	11		Treatment 10 + adenosine	
23	12		Treatment 11 + adenosine	
24	12		Treatment 12 + adenosine	
25	12		MCM + 2,4-D 5 ppm + adenosine	
26	12		MCM + 2,4-D 5 ppm + adenine	

Abbreviations used: Basal media M = Murashige and Skoog's; W = White's.
IAA = indoleacetic acid; NAA = naphthaleneacetic acid;
2, 4-D = 2, 4-dichlorophenoxyacetic acid; ppm = parts per million.
pH of basal media: pH 5.8.

chiefly to induce shoot formation with kinetin or callus formation with coconut milk and 2,4-dichlorophenoxyacetic acid (2,4-D). Both M and W basal media were used and compared. After 2 months lateral shoots appeared in three of the treatments (No.s 29, 34 and 50, indicated with asterisks in Table 2). Two of the treatments (29 and 34) contained kinetin at 10 ppm and one (34) contained, in addition, an auxin, naphthalene-acetic acid (NAA), at 2.5 ppm. Treatment 50 contained coconut milk, 10% by volume, + 2,4-D, 5 ppm + adenine, 20 ppm. The development of the shoots in these media might have been due to chance only or to other factors including the stage of development of bud primordia at transfer, sequential effects from prior treatment (10), etc., but the significant fact was that these buds did emerge and developed into shoots.

Table 2. Subcultures of pineapple shoot tip tissues from Falcon agar plates (Table 1).

Treatment No.	Source	Culture Media	Results
27	Trt 1 (Table 1)	M basal medium = M	No further
28	Trt 2 (Table 1)	W basal medium = W	
* 29	Trt 3 (Table 1)	M + kinetin 10 ppm	growth
30	Trt 4 (Table 1)	W + kinetin 10 ppm	
31	Trt 5 (Table 1)	M + kinetin 20	except in
32	Trt 6 (Table 1)	W + kinetin 20	
33	Trt 7 (Table 1)	M + kin. 10 + NAA 2.5ppm	treatments
* 34	Trt 8 (Table 1)	W + kin. 10 + NAA 2.5ppm	
35	Trt 9 (Table 1)	M + kin. 10 + NAA 5.0	29, 34 and
36	Trt 10 (Table 1)	W + kin. 10 + NAA 5.0	
37	Trt 11 (Table 1)	M + kin. 20 + NAA 2.5	50 with
38	Trt 12 (Table 1)	M + kin 10 + adenosine	
39	Trt 13 (Table 1)	W + kin. 10 + adenosine	lateral
40	Trt 14 (Table 1)	M + kin. + NAA 2.5 + adenosine	
41	Trt 15 (Table 1)	W + kin. + NAA 2.5 + adenosine	buds
42	Trt 17 (Table 1)	M + kin. + NAA 5.0 + adenosine	
43	Trt 19 (Table 1)	MCM + 2,4-D 5 + adenine	
44	Trt 20 (Table 1)	MCM + 2,4-D 5 + adenine	
45	Trt 21 (Table 1)	WCM + 2,4-D 2.5 + adenosine	
46	Trt 22 (Table 1)	MCM + 2,4-D 5.0 + adenosine	
47	Trt 23 (Table 1)	WCM + 2,4-D 5.0 + adenosine	
48	Trt 24 (Table 1)	MCM + 2,4-D 5.0 + adenosine	
49	Trt 25 (Table 1)	MCM + 2,4 D 5.0 + adenosine	
* 50	Trt 26 (Table 1)	MCM + 2,4-D 5.00 + adenine	

*Emergence of lateral bud in culture.
See Table 1 for the explanation of abbreviations.

When these aseptically grown shoots became available for study, a switch to an approach used in orchid culture and termed "mericloning" (3, 4, 14) seemed appropriate. One of the shoots, however, was first transferred to M + coconut milk, 10% by volume, agar medium (MCM). It continued to grow to a size suitable for transplanting into soil and greenhouse conditions. No lateral shoots or calluses were formed and thus there was no increase in plant number. The two remaining shoots were treated like orchid shoot tips, employing the method described by Sagawa, et al. (7). They were cultured in modified Knudson's liquid medium which was supplemented with coconut milk, 20% by volume, and adenine, 20 ppm (designated KCM20). The medium was prepared in 50 ml. Erlenmeyer flasks with 15 ml. of the medium in each flask. The flasks were shaken on a gyrorotary shaker under continous light for several weeks. One of the shoots was lost from bacterial contamination; the remaining shoot tip culture from Treatment 50 responded remarkably well.

RESULTS

A typical nodular growth associated with orchid shoot tip cultures appeared on the cut end of the shoot in 8 weeks while on the shaker. The shoot tip was then transferred to an agar tube of M + adenosine, 30 ppm. A medium with coconut milk was avoided since it was good for plant growth but not for callus formation in pineapple tissues. Adenosine was found to be beneficial in growing several of the cultures under study. Fortunately, this medium helped to trigger off the production of meristematic globular bodies (Fig. 1) which were reminiscent of protocorms in orchid cultures. Each globular body, here termed a protocorm-like body, contained a shoot apex which subsequently developed into a shoot. Numerous plantlets developed in the older part of the culture while the meristematic protocorm-like bodies were formed at the advancing margins. Pineapple plantlets in various stages of development are shown in Figures 2 and 3.

In recent studies, adenine, 20 ppm, was substituted for adenosine and produced similar results (Fig. 4).

Some of the protocorm-like meristematic cultures were transferred to various liquid media in T-shaped tubes and rotated at 1 rpm, as carried out by Dr. F. C. Steward and associates at Cornell University (11). Abundant plantlets were obtained in a liquid medium of Schenk and Hildebrandt (9) and in modified basal medium of Murashige and Skoog. Effects of pH, sucrose concentrations and growth regulators are under study.

When it appeared feasible to produce numerous pineapple plantlets in culture, the ornamental bromeliads were also tried but with limited success. The recalcitrant ornamental bromeliad tissues seem to require additional stimulants for growth. The cultures under investigation are: *Viesia* spp., *Neoregelia* hybrid, *Aechmea racine* and *Guzmania* sp. from H. Yamamoto and *Ananas erectifolius* (a wild pineapple), *Portea petropolipana* and *Bromelia* sp. from Pineapple Research Institute of Hawaii. *Portea and Guzmania* cultures look promising with positive response in modified Knudson's medium with 20% coconut milk and adenine. *Ananas erectifolius* is further along with several lateral buds (Fig. 5) and protocorm-like bodies in culture.

SUMMARY

Based on the results with pineapple cultures, a feasible procedure for culturing and multiplying bromeliaceous plants is a combination of the orchid shoot tip technique and the callus method, applied sequentially and in correct sequence. After prior shaking in a liquid medium, the shoot tip cultures are grown on a medium which stimulates the induction and growth of protocorm-like bodies and subsequent development into plantlets. Cloning of

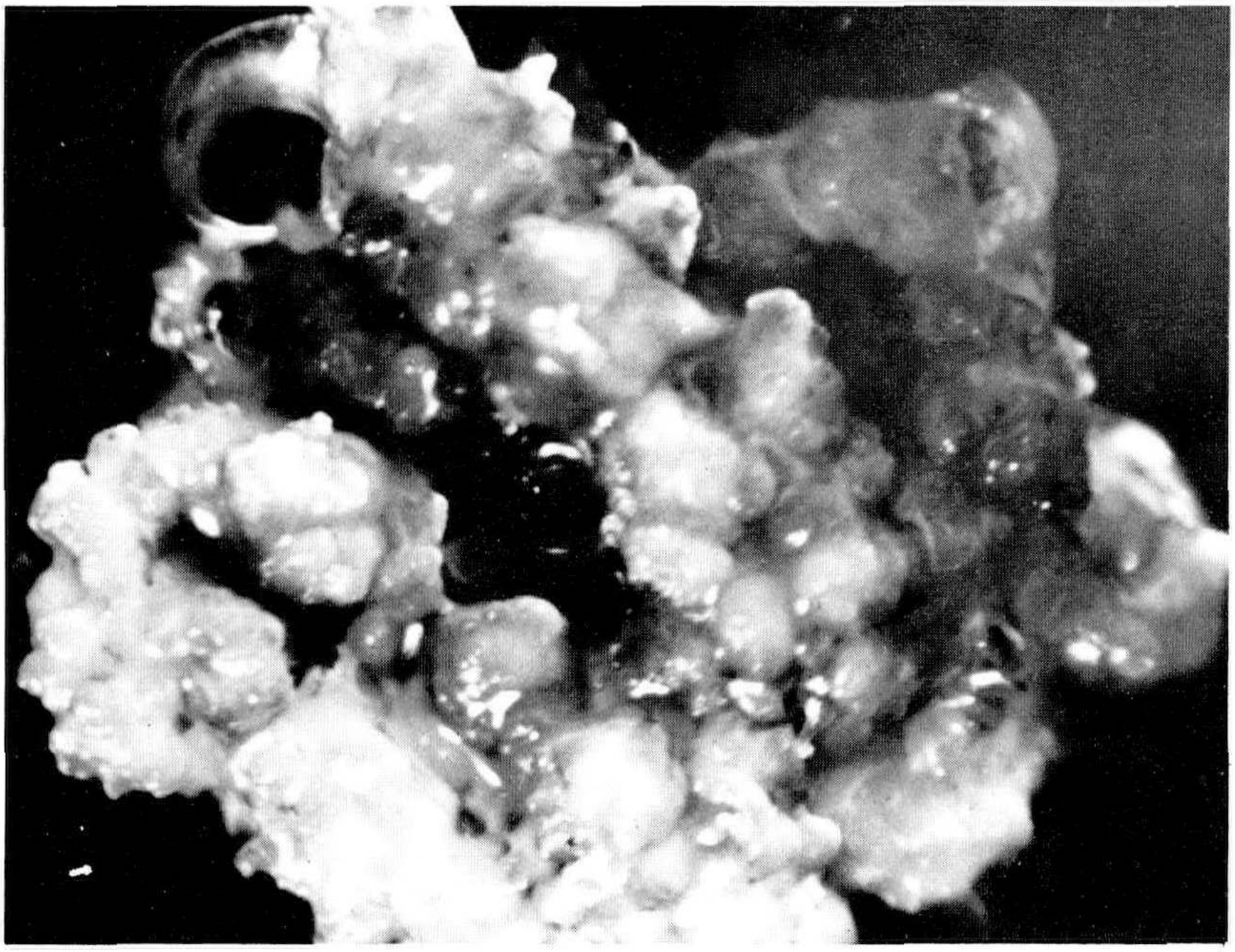

Figure 1. Growth of callus-like tissues and protocorm-like bodies in pineapple on Murashige and Skoog's basal medium plus adenosine, 30 ppm.

Figure 2. Growth of protocorm-like bodies and subsequent development of shoots and pineapple plantlets in Murashige and Skoog's solid basal medium plus adenosine, 30 ppm, after 2 months.

Figure 3. The pineapple culture in Fig. 2 at higher magnification to show the protocorm-like bodies and early stages of shoot development.

Figure 4. Profuse growth of pineapple plantlets and protocorm-like bodies in Murashige and Skoog's solid basal medium plus adenine, 20 ppm, after 3 months.

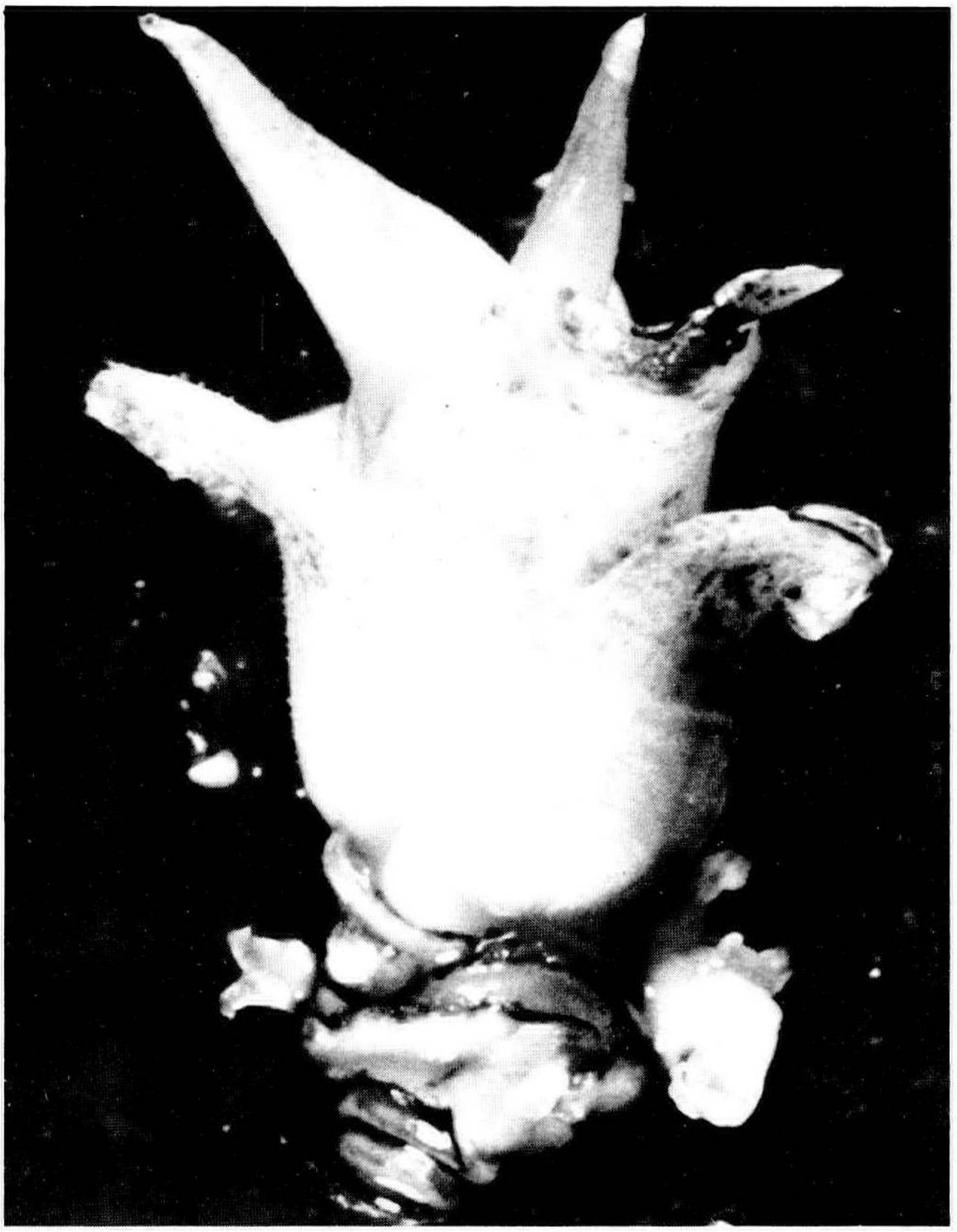

Figure 5. Shoot tip culture of *Ananas erectifolius* showing two lateral buds and a callus-like growth formed laterally across the stem. One of the shoots on the back was removed prior to photographing. Age 3 months in culture.

desirable, high quality plants in great numbers should be possible.

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VICE-PRESIDENT OKI: Thank you, Marion, and thank you, Dick, for a good job of moderating. Going on now to the second part of this morning's program we are going to change moderators. Edsal Wood, would you please take over now?

MODERATOR WOOD: Our first speaker in the second half of this morning's session graduated from the University of British Columbia about 16 years ago in plant science and then started a nursery business with his father. They are general ornamental growers, with many species and varieties, but their specialty is in propagation of plant liners. Les Clay: