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In vitro organogenesis from leaf explants of *Annona squamosa* Linn*

S. NAIR, P.K. GUPTA, M.V. SHIRGURKAR and A.F. MASCARENHAS**

Biochemistry Division National Chemical Laboratory Poona 411 008, India.

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Abstract. Multiple shoot formation was induced from excised leaf explants of *Annona squamosa* Linn. (custard apple) seedlings on a Murashige and Skoog basal medium containing benzylaminopurine and kinetin. Various auxins in combination with the above medium produced callusing of the explants. In an investigation of environmental factors affecting shoot induction it was seen that the maximum number of shoots were obtained using the leaf base with petiole at a temperature of 27 °C and a light intensity of 1000 lux. Roots were initiated erratically when individual shoots were treated with an auxin and then transferred to an auxin free medium. The process of the development of adventitious buds in leaf culture was analysed histologically..

Introduction

Custard apple (*Annona squamosa* Linn.) is a common fruit tree native to South America and the West Indies and now cultivated in several parts of India. The pulp of the fruit is edible [8]. Seed germination in nature is low. being about 30–40%.

Several reports using shoot tips, axillary buds or nodal segments are now available on the propagation of fruit trees by tissue culture [5, 6, 14, 15, 17]. Organogenesis or embryogenesis has also been induced directly from leaf explants of many herbaceous species [2, 3]. Reports on regeneration of shoots from leaves of woody species are still rare [4, 13].

This paper describes (1) a study of various hormonal and physical factors affecting in vitro shoot regeneration from leaf explants of *Annona squamosa* and (2) histological studies to determine the origin of shoot buds.

Materials and methods

Culture media

The various culture media used are abbreviated as follows:

MS: Murashige and Skoog [11] from which edamin (a lactalbumin hydrolysate, Sheffield Chemical), Kinetin (KN) and indole-3-acetic acid (IAA) were omitted.

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**To whom all correspondence should be addressed.

MS-1: MS containing 0.2 mgl^{-1} KN and 0.5 mgl^{-1} BAP.
MS-2: MS containing 0.5 mgl^{-1} KN and 2 mgl^{-1} BAP.
A-1: Anderson medium [1] 1/2 strength containing 0.25% activated charcoal (E. Merck) and 0.8% agar.
A-2: Anderson (1/2 strength) liquid medium without activated charcoal.

The cytokinins BAP, KN, Zeatin (Z), N^6-(Δ^2-isopentenyl) adenine (2iP) and the auxins (Table 4) were added individually or in combination at different concentrations indicated in the text. Coconut milk (CM) was the liquid endosperm obtained from tender green coconuts. Casein hydrolysate (CH) was obtained from Difco. The pH of all media was adjusted to 5.8. For studies on shoot initiation semisolid media containing 0.8% agar and distributed in 20 ml aliquots in 25 mm x 150 mm Corning brand test tubes were used. Liquid medium (20 ml per test tube, with a filter paper platform) was used for the induction of rooting. All cultures were incubated at 27 ± 2 °C with a 16 h photoperiod (1000 lux) supplied by cool daylight fluorescent tubes. Chemicals used were of analytical grade from British Drug House (BDH), Merck, Sigma or Difco.

Five replicates were used for shoot initiation studies and ten replicates for determining the conditions for rooting. All experiments were repeated at least twice and found reproducible. Data were recorded at the end of a 4-week culture period and are presented as the average number of shoot buds per culture tube ± standard deviation (S.D.). The results of rooting experiments are presented as percetage of cultures showing a rooting response.

Sterile leaves used for the studies were obtained from shoots derived from seedling explants grown in vitro on MS-1 medium. Leaf explants of approximately 7 mm^2 surface area (Figure 1A) and consisting only of the laminar region including midrib (except where mentioned otherwise) were used as inocula in all experiments.

Histological studies

For histological studies cultured leaf explants were fixed at intervals of 48 h in a formalin – acetic – alcohol mixture (FAA) followed by dehydration in an alcohol-xylol series before embedding in paraffin wax (MP 59–60 °C) [7]. Transverse sections cut at 8–10 microns were stained with 1% light green in clove oil mounted in DPX-4 189-(2-chloro-N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl amino carbonyl) benzene sulfonamide) (BDH) and observed microscopically.

Results

Effect of cytokinins and growth regulators on shoot bud formation from leaf explants

Leaf explants of *Annona* seedlings showed direct shoot regeneration accompanied by varying amounts of callus formation when cultured on MS medium

Table 1. Effect of different growth regulators on shoot bud formation from leaf explants

Sr. No.	Basal medium (MS)	Shoot bud formation (% response)	Average no. of shoot buds per leaf explant ± S.D.
1.	KN (0.5)	60	5.2 ± 0.40
2.	BAP (0.5)	80	6.0 ± 0.63
3.	2iP (0.5)	40	3.8 ± 0.40
4.	Z (0.005)	30	3.4 ± 0.48
5.	KN (0.50) + Z (0.005)	50	3.4 ± 0.48
6.	KN (0.5) + 2iP (0.5)	60	5.6 ± 0.48
7.	KN (0.5) + BAP (0.5)	100	9.8 ± 0.74
8.	KN (0.5) + BAP (0.5) + Z (0.005)	70	7.6 ± 0.80
9.	KN (0.5) + NAA (0.2)	50	3.8 ± 0.40[a]
10.	BAP (0.5) + NAA (0.2)	60	5.4 ± 0.80[a]
11.	KN (0.5) + BAP (0.5) + NAA (0.2)	70	8.2 ± 0.40[a]
12.	KN (0.5) + CM (10)	40	3.0 ± 0.63
13.	BAP (0.5) + CM (10)	60	4.0 ± 0.63
14.	KN (0.5) + CH (0.05)	50	4.6 ± 0.80
15.	BAP (0.5) + CH (0.05)	65	5.6 ± 0.48

The results are expressed as the average number of shoot buds ± S.D., from 5 culture tubes. Concentrations are given in parenthesis in mgl^{-1} for the growth hormones and as percentages for CM and CH.
[a]Callus formation at the cut ends of the explant

to which different growth regulators were added either singly or in different combinations at a single concentration (Table 1). Best response was observed when KN and BAP were added in combination each at 0.5 mgl^{-1} (Table 1, no. 7). However, when NAA was supplied to this medium there was a depression in the response, with the formation of callus at the cut ends of the explant. This tendency to develop callus was observed in all media containing NAA (Table 1, nos. 9, 10 and 11). When cytokinins were tested one at a time, most shoots were obtained with BAP followed by KN, 2iP and Z. However, Z was added at 100 times lower concentration in this experiment. Neither CH nor CM enhanced the number of shoot buds at the concentration tested (Table 1, nos. 12 to 15). These results indicated that KN and BAP were the most effective cytokinins for shoot bud formation and that their

Table 2. Effect of KN concentration on shoot bud formation from leaf explants [Basal medium: MS + BAP (0.5) to which KN was added at different concentrations.]

Sr. No.	KN concentration (mgl^{-1})	Average no. of shoot buds per leaf explant ± S.D.
1.	Nil	6.2 ± 0.40
2.	0.1	7.4 ± 0.48
3.	0.5	9.4 ± 0.80
4.	1.0	8.8 ± 0.74
5.	2.0	7.8 ± 0.74
6.	5.0	4.8 ± 0.40

The results are expressed as the average number of shoot buds ± S.D., from 5 culture tubes.

Table 3. Effect of BAP concentration on shoot bud formation from leaf explants [Basal medium: MS + KN (0.5) to which BAP was added at different concentrations.]

Sr. No.	BAP concentration (mgl^{-1})	Average no. of shoot buds per leaf explant ± S.D.
1.	Nil	5.4 ± 0.48
2.	0.1	6.2 ± 0.75
3.	0.5	9.2 ± 0.97
4.	1.0	11.2 ± 0.74
5.	2.0	13.2 ± 0.40
6.	5.0	7.2 ± 0.74

The results are expressed as the aveage number of shoot buds ± S.D., from 5 culture tubes.

response was more pronounced if they were added in combination (Table 1, No. 7). In order to determine the optimum concentrations of these two cytokinins in combination, two sets of experiments were carried out. In the first set (Table 2) the BAP level was kept constant at 0.5 mgl^{-1} in MS medium and the KN concentration was varied. The optimum KN concentration was 0.5 mgl^{-1} (Table 2, No. 3) when the BAP level was 0.5 mgl^{-1}. In a second set of experiments the KN level was maintained at 0.5 mgl^{-1} and the BAP concentration varied (Table 3). The optimum BAP level was found to be 2 mgl^{-1} (Table 3, No. 5) when the KN concentration was 0.5 mgl^{-1}. Based on these results MS medium supplemented with 0.5 mgl^{-1} KN and 2 mgl^{-1} BAP (MS-2) was used for further studies on shoot regeneration.

Table 4. Effect of different auxins on shoot bud formation from leaf explants. Basal medium: MS-2 to which the different auxins were added.]

Sr. No.	Auxins (mgl^{-1})	Average no. of shoot buds per leaf explant ± S.D.
1.	Nil	13.6 ± 0.80
2.	IAA (0.1)	10.4 ± 0.48
	IAA (0.5)	8.2 ± 0.74
	IAA (1.0)	6.0 ± 0.89
3.	NAA (0.1)	10.0 ± 0.63
	NAA (0.5)	6.4 ± 0.80
	NAA (1.0)	5.0 ± 0.63*
4.	IBA (0.1)	11.0 ± 1.40
	IBA (0.5)	7.4 ± 0.80
	IBA (1.0)	6.2 ± 0.74
5.	2,4-D (0.1)	8.2 ± 0.40
	2,4-D (0.5)	5.4 ± 0.48*
	2,4-D (1.0)	4.2 ± 0.40*

The results are expressed as the average number of shoot buds ± S.D., from 5 culture tubes.
*Callus formation at cut ends of the explant.

Table 5. Effect of different leaf regions on shoot bud formation from leaf explants. [Basal medium: MS-2]

Sr. No.	Leaf region	Average no. of shoot buds per leaf explant ± S.D.
1.	Leaf base with petiole	15.6 ± 0.80
2.	Leaf base without petiole	13.4 ± 0.48
3.	Lamina with midrib	12.8 ± 0.74
4.	Lamina without midrib	5.2 ± 0.40
5.	Petiolar region without lamina	3.8 ± 0.40
6.	Apical region with midrib	No shoot bud formation

The results are expressed as the average number of shoot buds ± S.D., from 5 culture tubes.

Effect of different auxins on shoot bud formation from leaf explants

The effect of IAA, NAA, IBA and 2,4-D on shoot bud development was tested at concentrations 0.1, 0.5 and 1.0 mgl^{-1} added to MS-2 medium (Table 4). Callus formation was observed with 2,4-D at 0.5 and 1.0 mgl^{-1} and with NAA at 1.0 mgl^{-1} with simultaneous reduction in the number of shoot buds. In general, the number of shoot buds decreased with all concentrations of every auxin tested.

Effect of different leaf regions on shoot bud formation from leaf explants

The different regions from which explants of the seedling leaf were taken include: (1) leaf base with petiole (4 mm leaf base with 2–3 mm petiole); (2) leaf base without petiole (7 mm^2); (3) Lamina with midrib (7 mm^2); (4) Lamina without midrib (7 mm^2); (5) Petiolar region without lamina (7 mm length); and (6) Apical region with midrib (6–7 mm). Maximum shoot bud formation was observed from explants taken from the basal portion of the leaf containing the petiole (Table 5, No. 1) followed by the region of the leaf containing the leaf base without the petiole and by the lamina with midrib respectively. In the absence of the midrib or when the petiole alone was cultured, the number of shoot buds was low. In explants where the midrib region was included a number of shoot buds formed first along the midrib with a few scattered buds on the laminar region of the leaf. The apical region of the leaf merely showed a curling response but developed no buds.

Table 6. Effect of light intensity on shoot bud formation from leaf explants [Basal medium: MS-2]

Sr. No.	Light intensity (lux)	Average number of shoot buds per leaf explant ± S.D.
1.	500	7.0 ± 0.80
2,	1000	13.6 ± 0.80
3.	1500	10.4 ± 0.48

The results are expressed as the average number of shoot buds ± S.D., from 5 culture tubes. Temperature was kept constant at 27 ± 2 °C.

Table 7. Effect of temperature on shoot bud formation from leaf explants [Basal Medium: MS-2]

Sr. No.	Temperature °C	Average no. of shoot buds per leaf explant ± S.D.
1.	5	–
2.	15	2.2 ± 0.40
3.	25	12.0 ± 1.41
4.	27	15.8 ± 1.16
5.	30	9.4 ± 0.80
6.	35	7.4 ± 0.48

The results are expressed as the average number of shoot buds ± S.D., from 5 culture tubes.
– No shoot bud formation. The light intensity was kept constant (1000 lux) for 16 h.

Physical factors

1. Effect of light intensity Cultures were incubated at 27 ± 2 °C but were provided with different intensities of light for 16 h (Table 6). The amount of light supplied had a distinct effect on the number of developing shoot buds. The optimum light intensity was found to be 1000 lux (Table 6, No. 2). The number of shoot buds decreased with lower or higher light illumination.

2. Effect of temperature A wide range of temperatures from 5 to 35 °C using a series of growth chambers (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) was tested. All cultures for these experiments were supplied with a constant light intensity (1000 lux) for 16 h (Table 7). The optimum temperature was found with cultures incubated at 27 ± 2 °C (Table 7, No. 4) followed by 25 and 30 °C. Shoot bud development was totally or partially suppressed at 5 and 15 °C and at 35 °C there was a 50% inhibition in the number of shoot buds.

Rooting of leaf-derived shoots

In order to induce rooting from leaf-derived shoots the effect of 4 different filter-sterilized auxin solutions IAA, IBA, IPA and NAA contained in half strength MS medium was tested individually. The auxins were used at concentrations of 0.1, 0.5, 1, 2, 5, 10, 15, 20 and 25 mgl^{-1} for varying periods of time (24, 48, 72 and 96 h). Shoots (15–20 mm long) grown on MS-2 medium (Figure 1B) were excised after 25–30 days and 2 mm of the cut end immersed in half strength MS containing the auxins. Cultures were then incubated in dark at 25 °C for different time periods as mentioned above. After auxin treatment explants were transferred to A-1 medium and incubated at 27 °C in light (16 h photoperiod). This medium was tested after earlier negative results using half strength MS with and without activated charcoal. With NAA, IBA, IAA and IPA at various concentrations for 24 and 48 h the shoots remained green but there was no rooting (Table 8). At concentrations above 20 mgl^{-1} for periods above 48 h shoots appeared unhealthy, leaves turned

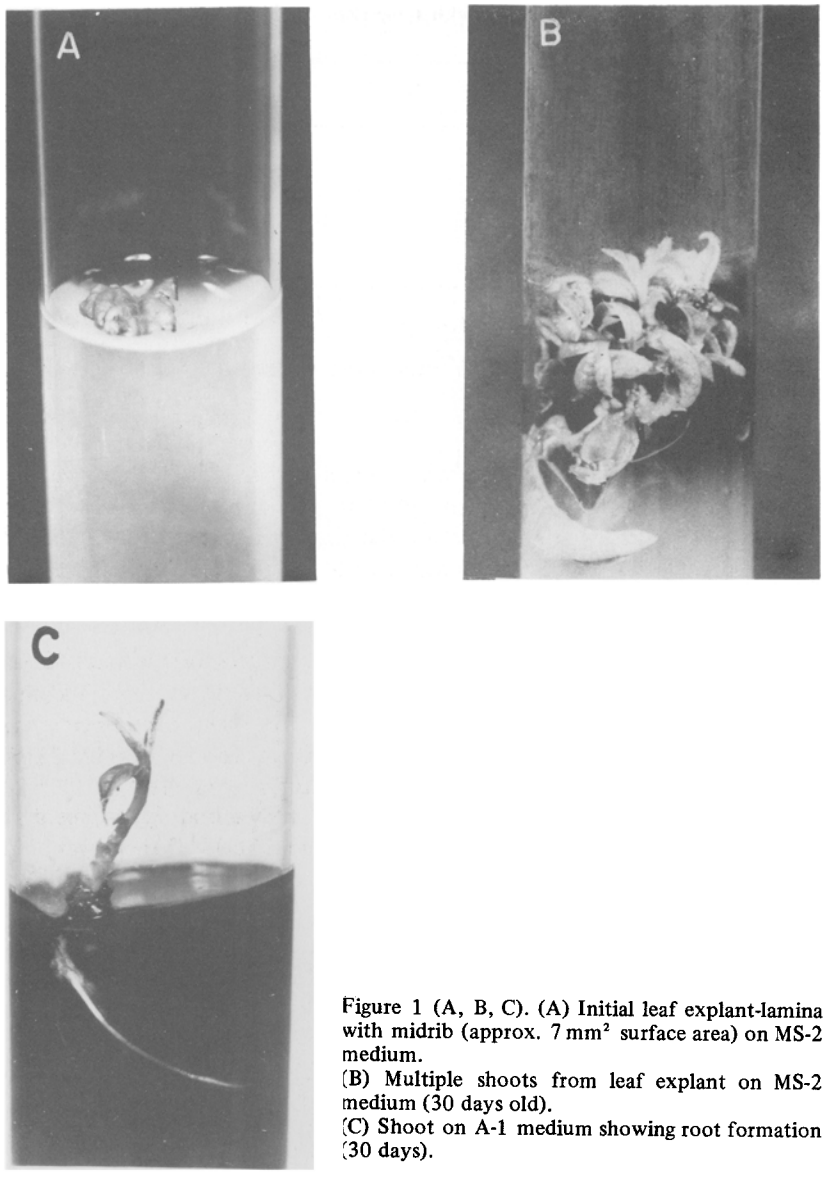

Figure 1 (A, B, C). (A) Initial leaf explant-lamina with midrib (approx. 7 mm^2 surface area) on MS-2 medium.
(B) Multiple shoots from leaf explant on MS-2 medium (30 days old).
(C) Shoot on A-1 medium showing root formation (30 days).

yellow and eventually dropped off. However, 10% of the cultures treated with 20 mgl^{-1} IBA for 72 h (Table 8, No. 4) developed roots on A-1 medium within 25–30 days (Figure 1C). All other treatments were ineffective and shoots died within 45–50 days on A-1 medium. Further growth of plantlets was obtained by subsequent transfer to A-2 medium. Rooted plantlets grown in this medium were transferred to pots containing a sterile soil : sand : compost (farm yard manure) mixture (3 : 3 : 1). Potted plants were incubated at 27 °C in light (16 h photoperiod), covered with glass beakers to maintain

Table 8. Effect of different auxin treatments on root formation

S. No.	Auxin concentration (mgl^{-1})	Rooting response (%) Treatment (h)		
		24 & 48	72	96
1.	NAA (0.1–20.0)	–	–	–
2.	NAA (25.0)	–	–	*
3.	IBA (0.1–15.0)	–	–	–
4.	IBA (20.0)	–	10	*
5.	IBA (25.0)	–	*	*
6.	IAA (0.1–20.0)	–	–	–
7.	IAA (25.0)	–	*	*
8.	IPA (0.1–20.0)	–	–	–
9.	IPA (25.0)	–	–	*

Shoot explants (15–20 mm long) were treated at 25 °C in dark by dipping 2 mm of the cut end of the shoots for varying periods in MS (1/2 strength) liquid medium to which the different auxins were added. After auxin treatment the explants were transferred to A-1 medium and incubated at 27 °C in light (16 h photoperiod, 1000 lux). Results given in this table are the response of the shoots on A-1 medium at the end of 25–30 days, after the respective auxin treatments.

– No root initiation, shoots remain green * Shoots unhealthy

Percentage rooting is from 10 culture tubes and was repeated three times.

humidity and watered daily. Only 10% of these potted plantlets survived (Figure 1D).

Further studies to improve the conditions for rooting and for transfer of plantlets to soil are in progress.

Histological studies

Leaf explants cultured on MS-2 medium were used for studies of the gross anatomical changes occuring during bud development. A transverse section of a cultured leaf explant at day 'zero' consisted of epidermis, mesophyll and

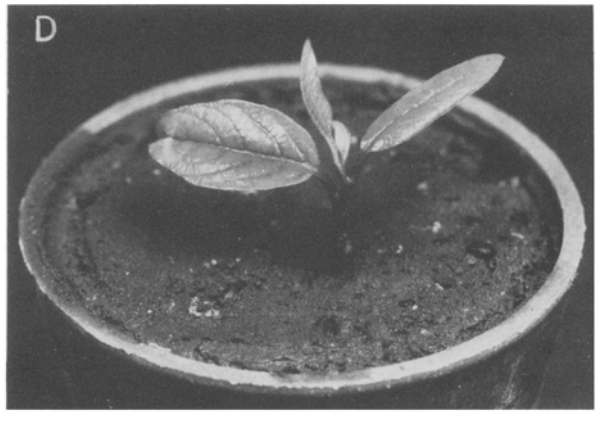

Figure 1 (D). Plantlet in pot – 15 days after transfer

vascular bundles (Figure 1E). Each of these tissue layers consisted of differentiated cells and possessed characteristic cellular organisation. Through systematic observation of microscopic sections of leaf explants it was found that significant cellular changes occurred as early as 4 days in culture. During this time proliferation of new cells in the epidermal and hypodermal (mesophyll) layers was observed. These cells continued to divide, forming a group of small cells with dense cytoplasm and prominent nuclei. Within 6 days in culture this cell mass became pronounced and could readily be identified as a meristemoid (Figure 1F), the precursor of an adventitious bud.

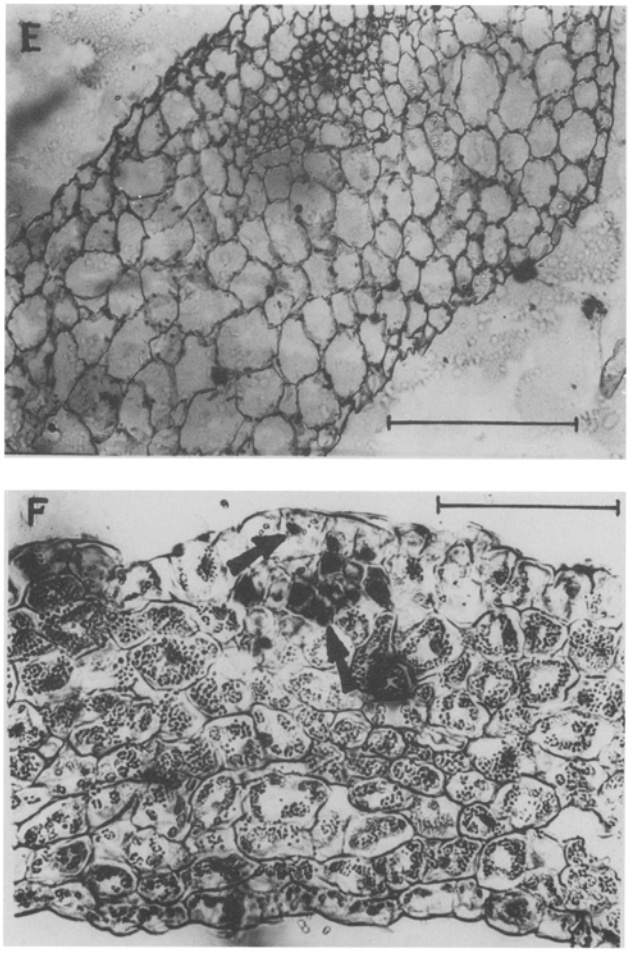

Figure 1 (E, F). (E) Leaf explant showing tissue layers of epidermis, mesophyll and a vascular bundle at day 'zero' culture. Bar represents 10 μm.
(F) Meristemoid stage showing meristematic centre after 6 days in culture. Arrows show active cells of epidermis and mesophyll. Bar represents 10 μm.

Continued proliferation of small meristemoid cells resulted in the rapid expansion of the surface area and the total volume. A consequence of this expansion of cell mass was the protrusion of a bud primordium above the leaf surface which became visible after 20 days in culture (Figure 1G). After 25 days the bud primordia progressed into the early stage of adventitious bud development (Figure 1H). The distribution of adventitious buds on the surface of leaf explants revealed the existence of numerous loci capable of responding to in vitro stimuli for adventitious bud production.

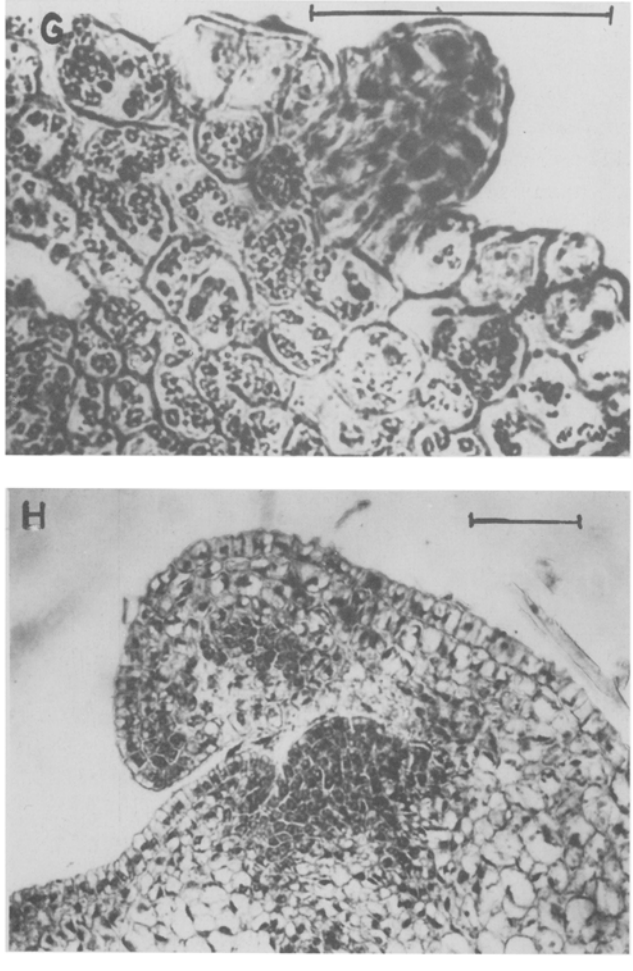

Figure 1 (G, H) (G) Bud primordium stage arising from the meristematic centre after 20 days in culture. Bar represents 10 μm.
(H) Early stage of adventitious bud development showing apical meristem flanked by a pair of leaf primordia after 25 days. Bar represents 10 μm.

Discussion

The present studies clearly demonstrate that BAP and KN act synergistically to produce healthy and vigorous shoots from leaves of *Annona squamosa* in culture. This result is unlike earlier reports in which an auxin and a cytokinin were used for regeneration of shoots in vitro [9, 10].

The portion of leaf tissue used for culture is also a very important factor in shoot formation. The base of the leaf with the petiole yielded the highest number of shoots. This observation suggests that the petiolar and midrib regions contain some factor(s) which favour(s) shoot proliferation and which is absent in the laminar region. A similar report was made for *Populus* hybrid leaf culture [16].

Studies on rooting require improvements but have shown that two treatments are necessary. The first one includes an auxin treatment in a half strength salt medium followed by transfer of the shoot to a different basal salt medium containing low amounts of nitrate, and potassium iodide but high levels of iron. This medium also contained activated charcoal but lacked auxins [12]. However, further studies are in progress to obtain higher percentages of rooting and survival in field.

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