Anatomical responses to cytokinins of abscised grapevine shoot apices cultured *in vitro**

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Responses of excised shoot apices of *V. vinifera* L. cv. Chenin blanc to different cytokinin treatments were studied at various intervals (days) after the start of *in vitro* culture, using light and scanning electron microscopy. Results clearly indicated that *in vitro* produced shoots were of axillary origin. Shoot clusters were produced by the enhanced release of axillary meristems from apical dominance, due to the application of 6-benzylaminopurine (BAP) singly as well as in combination with zeatin riboside (ZR). Axillary meristems on these axillary shoots were subsequently released from apical dominance, thus giving rise to shoot clusters of high densities. Shoot clusters induced by ZR alone were less dense, probably due to an initial delay in elongation of axillary meristems nearest to the main apical meristem of the shoot. However, elongation of axillary meristems lower down the axes proceeded strongly in the presence of ZR. Application of BAP resulted in more pronounced release of axillary meristems from apical dominance than with ZR.

The anatomical structure of grapevine shoot apices and differentiation pathways of vascular tissues in elongating shoots have been described in detail (Hegedüs, 1957; Thompson & Olmo, 1963; Fournioux, 1972; Pratt, 1974).

Stimulatory effects of cytokinins on *in vitro* shoot production from excised grapevine shoot apices have been reported (Barlass & Skene, 1978; Chée & Pool, 1982; Goussard, 1981, 1982; Harris & Stevenson, 1982). Most authors reporting on rapid *in vitro* propagation of woody plant species stressed the importance that proliferated shoots derived from detached apices should lead to the production of genetically uniform plants (De Fossard, 1976; Abbott, 1978; Cheng, 1978; Anderson, 1980). It is generally accepted (Murashige, 1974; Abbott, 1978; Hussey, 1980) that multicellular organized shoot meristems are genetically highly stable. Although adventitious meristems may enable a substantially faster cloning rate than axillary shoot multiplication, wholly mutant propagules may arise, due to growth from single or very small groups of cells (Murashige, 1977; Anderson, 1980; Hussey, 1980; Wang & Hu, 1980).

In culturing fragmented grapevine shoot apices Barlass & Skene (1978) reported on the enhancement of shoot production in the presence of 6-benzylaminopurine (BAP). Light and scanning electron microscopy (SEM) were used to provide direct evidence that shoots derived *in vitro* from fragmented apices are adventitious in origin (Barlass & Skene, 1980; Barlass, Skene & Clingeleffer, 1981). Non-fragmented shoot apices of *V. vinifera* L. (cv. Chenin blanc) responded with marked shoot proliferation and elongation to single and combined applications of zeatin riboside (ZR) and BAP (Goussard, 1981). Continuous branching of proliferating shoots with BAP alone gave rise to the formation of dense shoot clusters (Goussard, 1982). Chée & Pool (1985) pointed out that within high density clusters the occurrence of adventitious shoots cannot be ruled out.

Objectives of the present study were to investigate anatomical responses to BAP and ZR of excised grapevine shoot apices cultured *in vitro* and whether *in vitro* produced shoots are of axillary or adventitious origin.

MATERIALS AND METHODS

The procedure for the activation of shoot primordia-, excision of shoot apices (0,5 mm - 1 mm) and subsequent culture conditions, except where specified in the text, were according to Goussard (1981) using *V. vinifera* L. (cv. Chenin blanc). The basal medium consisted of the full-strength medium of Murashige & Skoog (1962) supplemented with 30 g/*l* sucrose and 6,8 g/l Difco-Bacto agar.

Anatomical responses of explants to BAP (2 mg/*l*); BAP + ZR (both at 2 mg/*l*) and ZR (10 mg/*l*) were studied at various intervals (days) after the start of culture. Control treatments lacked cytokinins. Sections were also prepared of shoot tips (5 mm) taken from elongating shoots on cane segments of Chenin blanc.

For light microscopy (LM), tissues were fixed in formalin-aceto-alcohol (FAA), infiltrated by means of a tertiary butanol-paraffin wax procedure (Johansen, 1940) and sectioned with a rotating microtome. Sections (8,0 µm) were stained utilising the tannic acid – safranine – fast green technique (De Vos, 1974).

Preparation of material for SEM was according to Hayat (1974). *In vitro* produced shoots were fixed in 25% glutaraldehyde (pH 4) for 24h at 4°C, washed thrice for 30 min periods in a 0,2 M sodium cacodylate buffer (pH 7,2) and dehydrated in a graded acetone-water series. The material was then subjected to critical-point drying with CO_2 and sputter-coated with gold, after which it was studied with an ISI-100A scanning electron microscope.

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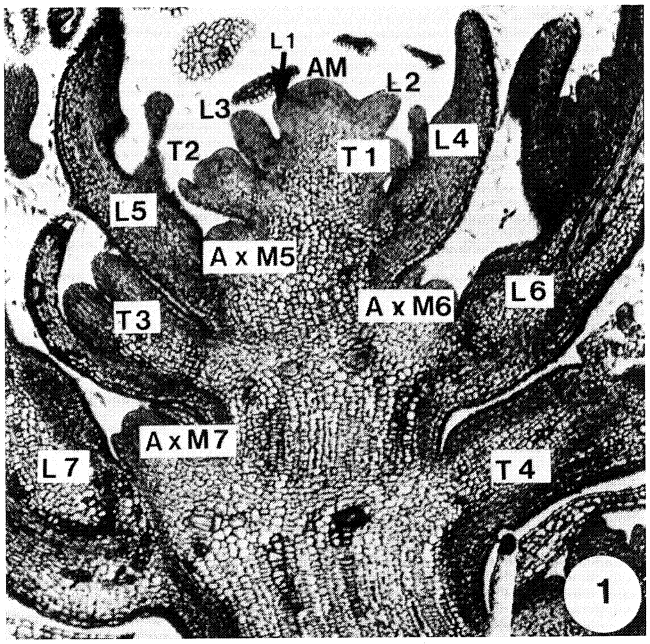

FIG. 1
Apical nodes of elongating shoot from a cane segment of Chenin blanc showing apical meristem (AM), leaf initiation (L1), leaf (L) and tendril (T) primordia and axillary meristems (Ax M). (Numbers indicate basipetal sequences). (LM x 64).

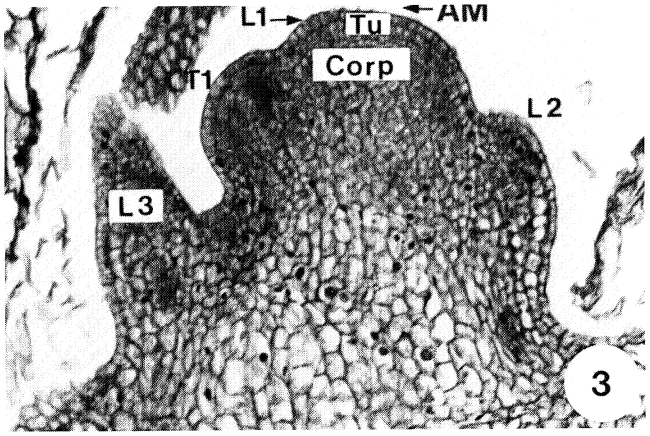

FIG. 3
Shoot apex of Chenin blanc after 40 days of culture on media lacking cytokinins showing apical meristem (AM), 2-layered tunica (Tu), corpus (Corp), leaf initiation (L1) and leaf (L) and tendril (T) primordia. (LM x 210).

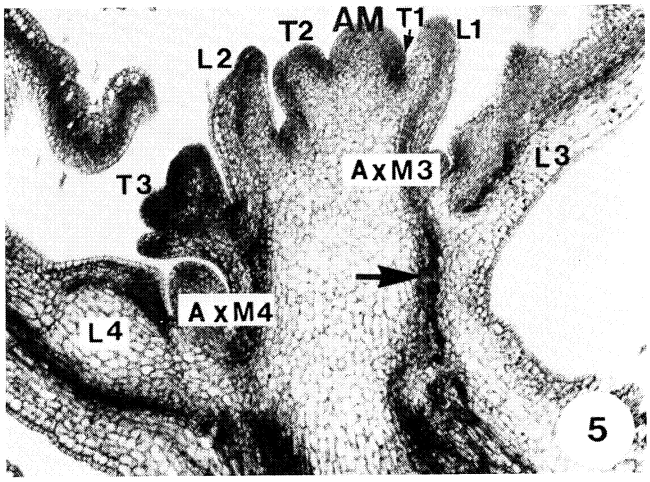

FIG. 5
Elongation of shoot apices, subopposite initiation of leaf (L) and tendril (T) primordia and origination of axillary meristems (Ax M) on shoot derived from apex after 10 days' culture on cytokinin-enriched media. Arrow indicates primary vascularization. (LM x 87).

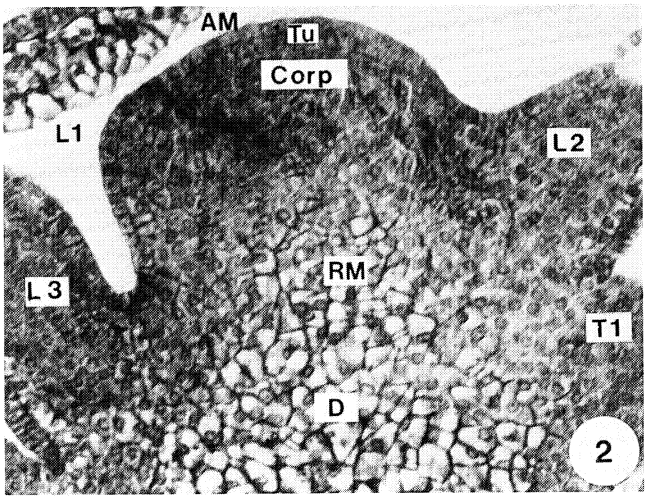

FIG. 2
Shoot apex in Fig. 1 enlarged to show apical meristem (AM), 2-layered tunica (Tu), corpus (Corp), rib meristem (RM), diaphram (D), leaf initiation (L1) and leaf (L) and tendril (T) primordia. (LM x 350).

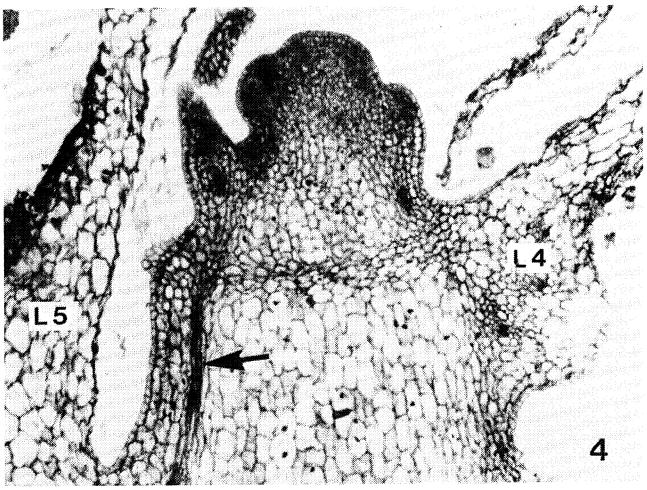

FIG. 4
Apical part of shoot in Fig. 3. Arrow indicates primary vascularization acropetally to No. 5 leaf base. (LM x 130).

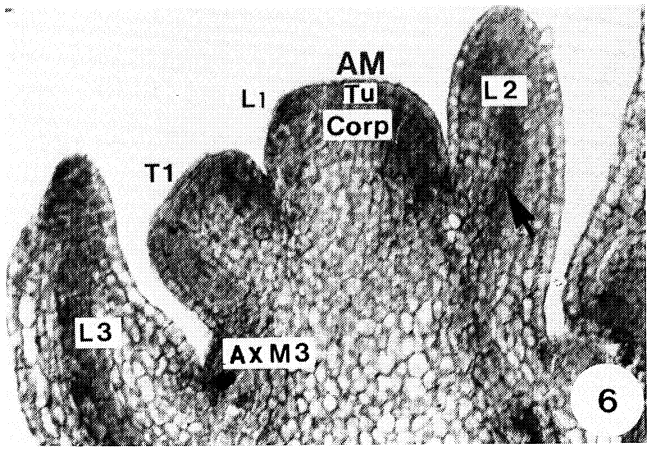

FIG. 6
Shoot apex as in Fig. 5 showing the apical meristem (AM), 2-layered tunica (Tu), corpus (Corp), leaf initiation (L1), leaf (L) and tendril (T) primordia and origination of axillary meristem (Ax M) in the axil of leaf 3. Arrow indicates procambial traces (leaf 2). (LM x 210).

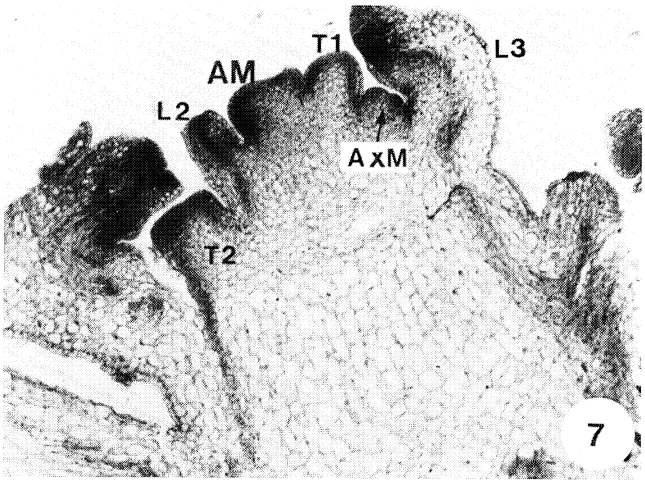

FIG. 7
Apical part of shoot after 20 days' culture on media containing 2 mg/l BAP. Light micrograph (LM) showing increase in diameter of stem part and apical meristem (AM) less prominently elevated above the youngest tendril (T) and axillary meristem (Ax M). (LM x 87).

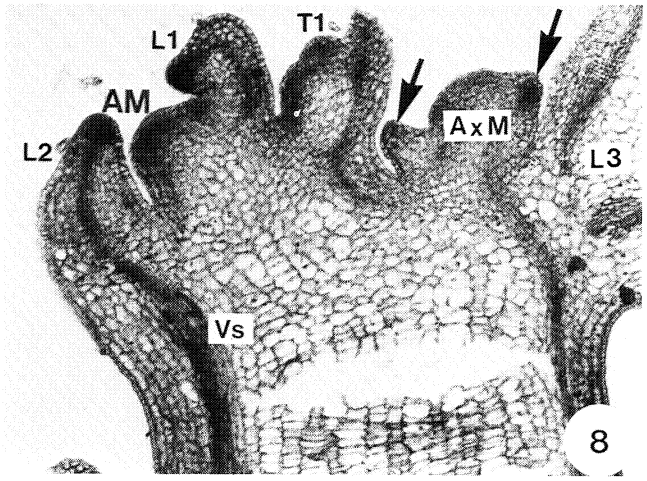

FIG. 8
Apical part of shoot after 25 days' culture with 2 mg/l BAP. Light micrograph (LM) showing leaf (L) and tendril (T) primordia and the overcoming of apical dominance as expressed by the strong development of the axillary meristem (Ax M) nearest to the apical meristem (AM). Arrows indicate leaf primordia on the axillary meristem (Ax M). Vascularization (Vs) proceeded strongly (base leaf 2). (LM x 105).

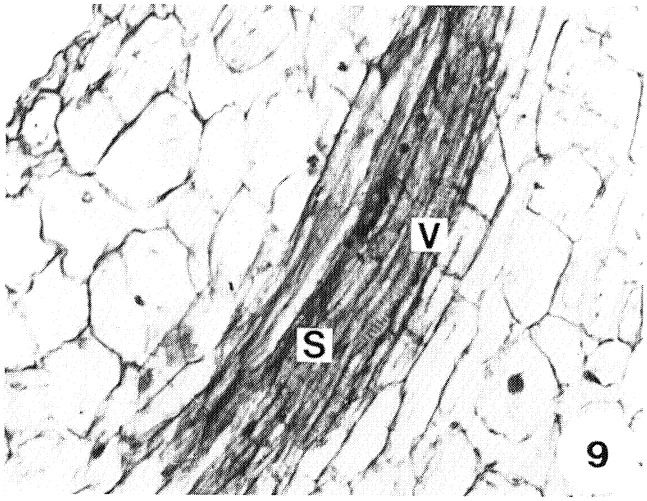

FIG. 9
Differentiated sieve elements (S) and spirally thickened vessels (V) at the base of leaf 2 in Fig. 8 (LM x 250).

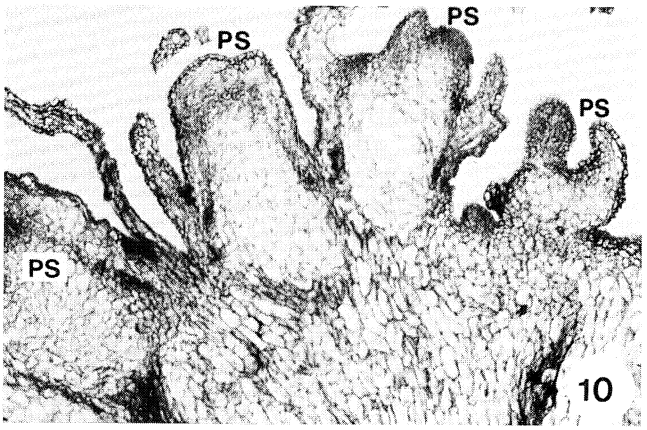

FIG. 10
Part of shoot clump derived from shoot apex after 35 days' culture on media containing 2 mg/l BAP, showing protruding shoots (PS). (LM x 87).

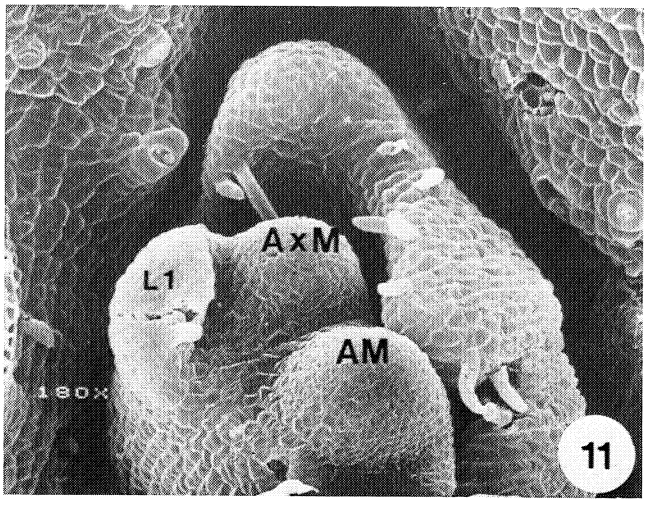

FIG. 11
Proliferated shoot in clump as in Fig. 10 showing axillary meristem (Ax M) at the same level with the apical meristem (AM). (SEM x 180).

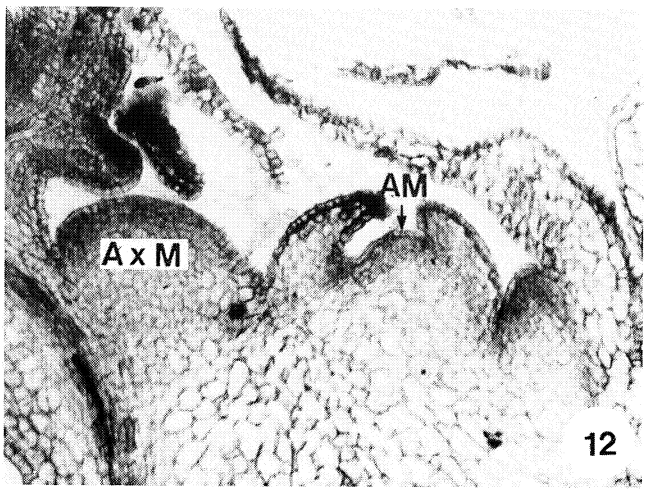

FIG. 12
Proliferated shoot in clump showing shoot apical meristem (AM) in a slight depression below developing axillary meristem (Ax M). (LM x 140).

RESULTS

Apical part of elongating shoot:

The anatomical structure of the apical nodes of an elongating shoot of Chenin blanc with initiation of leaf and tendril primordia and origin of axillary meristems is shown in Fig. 1. The apical meristem of the shoot (AMS) consists of two tunica layers over a poorly defined corpus (Fig. 2). A leaf primordium (L1) is initiated on the flank of the AMS by periclinal divisions in the second tunica layer.

Growth response in the absence of cytokinins:

Culture media lacking cytokinins resulted in limited elongation of excised shoot apices. Serial sections obtained at 40 days after the start of culture showed leaf and tendril initiation on the flanks of the AMS (Fig. 3). However, subsequent development of the youngest leaves and tendrils was reduced. Primary vascularization was observed acropetally to the No. 5 leaf bases (Fig. 4). Over the culture period of 45 days explants lacked initiation of axillary meristems.

Growth response in the presence of cytokinins:

After 10 days' culture, shoot apices responded with marked elongation and concomitant leaf and tendril initiation to the range of cytokinins applied (Fig. 5). The typical discontinuous arrangement of tendrils of *Vitis* (exception *V. labrusca*) persisted. Sieve elements and spirally thickened vessels differentiated at the base of leaf 3. The anatomical structure of the apical meristems resembled that of elongating shoots on established vines (Fig. 6). Procambial traces are clearly visible (leaf 2). The youngest axillary meristem originated in the axil of leaf 3.

Benzylaminopurine alone and in combination with zeatin riboside

In response to BAP and BAP + ZR, the apical part of the primary shoot increased in diameter, with the apical meristem less prominently elevated above the youngest tendril and axillary meristems after 20 days of culture (Fig. 7). After 25 days apical dominance was overcome, expressed by the strong development of the axillary meristem nearest to the AMS (Fig. 8). Pronounced vascularization occurred. Sieve elements and spirally thickened vessels differentiated at the base of leaf 2 (Fig. 9). Shoot clumps with several protruding shoots resulted after 35 days (Fig. 10). Light and scanning electron microscopy of clumps revealed that some shoot apical meristems appeared practically at the same level as axillary meristems (Fig. 11), or in a slight depression below it (Fig. 12). This phenomenon was especially discernible in response to application of BAP at 2 mg/*l*.

Once released from apical dominance, the apical meristem of the axillary shoot duplicated the pattern found in the main AMS and proceeded with initiation of leaves and axillary meristems. Subsequent elongation of axillary shoots in response to BAP alone was limited (Fig. 13). With a combination of BAP and ZR elongation of axillary shoots was slightly more pronounced, with origination and subsequent development of axillary meristems lower down the axes (Fig. 14).

Although apical branching of proliferated shoots in response to BAP and BAP + ZR was observed at earlier stages during culture, it was more pronounced after 40 days (Fig. 15). The apical regions of many shoots took on a forked appearance (Fig. 16) with organized apical meristems. They elongated with subsequent initiation and development of axillary meristems in leaf axils. However, in response to BAP alone, elongation of forked shoots was limited and coupled with a strong release of the youngest axillary meristems from apical dominance (Fig. 17). With a combination of BAP and ZR elongation was more pronounced with axillary meristems originating at slightly lower levels down the axes (Fig. 18). Continuous axillary branching of proliferated shoots coupled with a strong release of axillary meristems from apical dominance gave rise to the formation of dense shoot clusters with numerous proliferating shoots (Fig. 19).

Zeatin riboside

Excised shoot apices responded markedly to the application of ZR at high concentrations (10 mg/*l*). Pronounced elongation at 20 days after the start of culture with concomitant leaf initiation and origination of axillary meristems is shown in Fig. 20. Elongating shoots lacked tendril initiation. After 25 days the AMS still appeared well elevated above the youngest leaf primordium and axillary meristem (Fig. 21). Axillary meristems nearest the AMS lacked active development and elongation. Primary vascularization proceeded strongly (base of leaf 3). Marked differentiation of sieve elements and spirally thickened vessels occurred (Fig. 22). After 30 days of culture, axillary meristems lower down the axis of the primary shoot responded with pronounced development and elongation (Fig. 23). Proliferated shoots lacked any branching and resulted in shoot clumps of low density, even at 40 days after the start of culture. However, they elongated rapidly and exhibited long internodes. Stretching of xylem elements occurred as expressed by the uncoiling of spirals (Fig. 24).

DISCUSSION

The structure of the apical part of an elongating shoot of Chenin blanc with leaf and tendril initiation, axillary meristem origination and primary vascularization agrees with descriptions of Hegedüs (1957), Thompson & Olmo (1963), Fournioux (1972) and Pratt (1974).

Results of the present investigation stressed the dependency of detached grapevine shoot apices on the presence of an external source of cytokinin in order to elongate, with a concomitant production of leaves, each of which bears in its axil an anatomical replica of the main apex. Upon release from apical dominance, these axillary meristems were capable of producing their own axillary meristems. The potential of cytokinins to stimulate development and elongation of axillary meristems, thus inducing *in vitro* shoot multiplication, is in agreement with reports for other woody species (De Fossard, 1976; Abbott, 1978; Cheng, 1978; Anderson, 1980; Hussey, 1980; Wang & Hu, 1980).

Shoots derived from fragmented grapevine shoot apices were adventitious in origin (Barlass & Skene, 1980; Barlass *et al.*, 1981). In the present investigation no evidence was found that proliferated shoots originated adventitiously. Shoot clusters were produced by

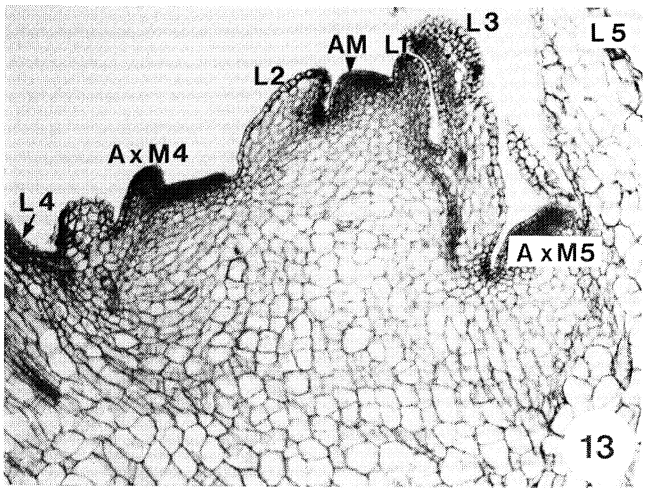

FIG. 13
Axillary shoot once released from apical dominance with BAP (2 mg/*l*) showing leaf (L) primordium and origination of axillary meristems (Ax M). (LM x 95).

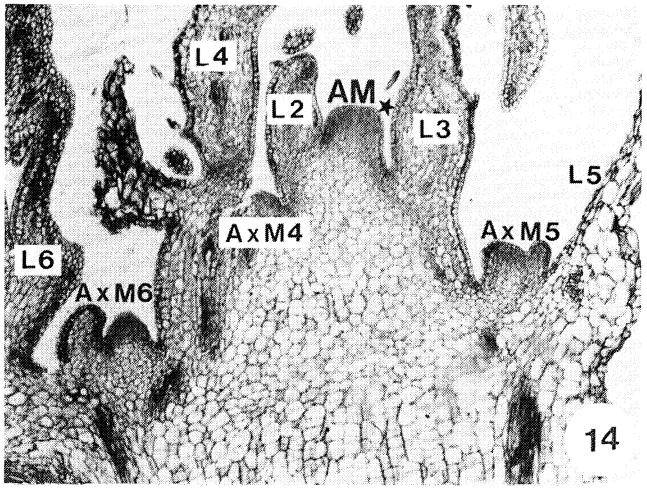

FIG. 14
Axillary shoot once released from apical dominance with 2 mg/*l* ZR plus 2 mg/*l* BAP, showing the apical meristem (AM) and leaf (L) primordia. Asterix indicates leaf initiation on the flank of the apical meristem (AM). Elongation is more pronounced with origination and subsequent development of axillary meristems (Ax M) at lower levels down the axis. (LM x 80).

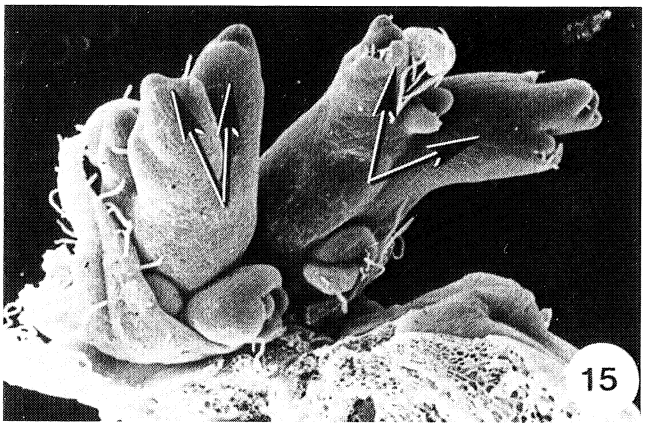

FIG. 15
Branching of proliferated shoots (arrowed) in response to BAP and BAP + ZR after 40 days' culture. (SEM x 20).

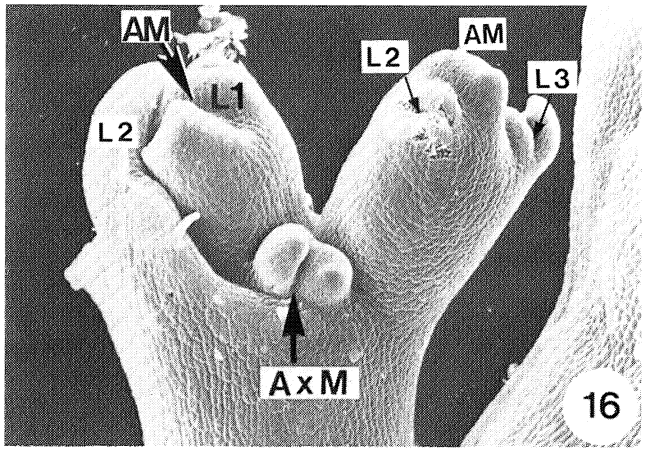

FIG. 16
Forking of apical regions of shoots as in Fig. 15. The apical meristem (AM) of the shoot on the left is concealed by leaf (L) primordia. Axillary meristem (Ax M) with leaf primordium occurred in the axil of leaf 2 (shoot on left). The apical meristem (AM) of the shoot on the right is well organized and proceeded with initiation of leaf (L) primordia (SEM x 70).

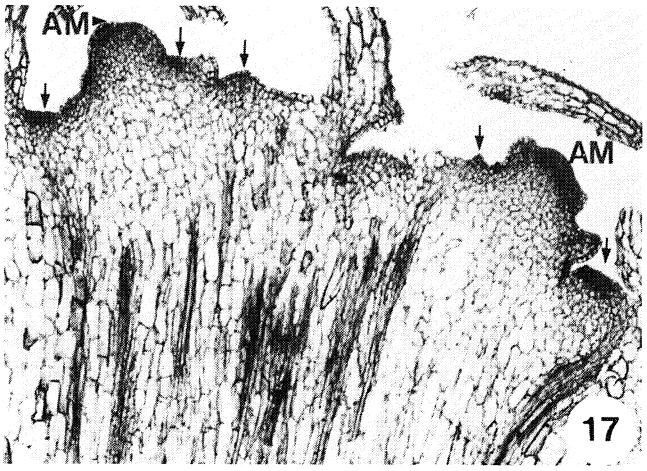

FIG. 17
Limited elongation of shoots after forking at 40 days after the start of culture in response to BAP (2 mg/*l*). Well organized apical meristems (AM) are coupled with a strong release of axillary meristems (arrowed) from apical dominance. (LM x 87).

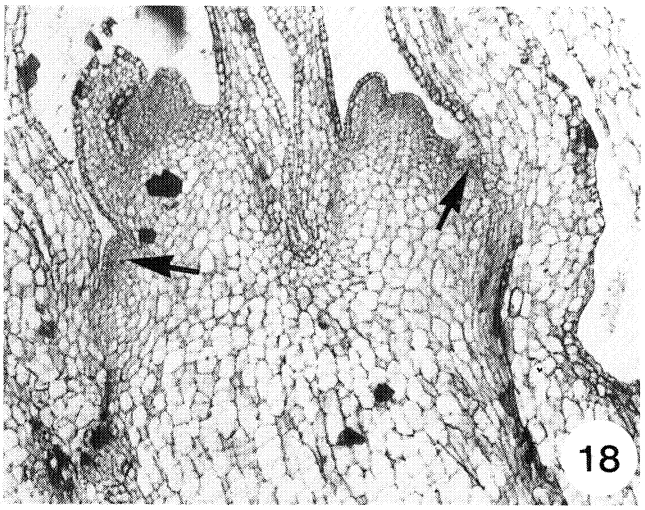

FIG. 18
More pronounced elongation of shoots after forking with combinations of BAP and ZR (both at 2 mg/*l*) after 40 days' culture. Axillary meristems (arrowed) originated at lower levels down the axes. (LM x 95).

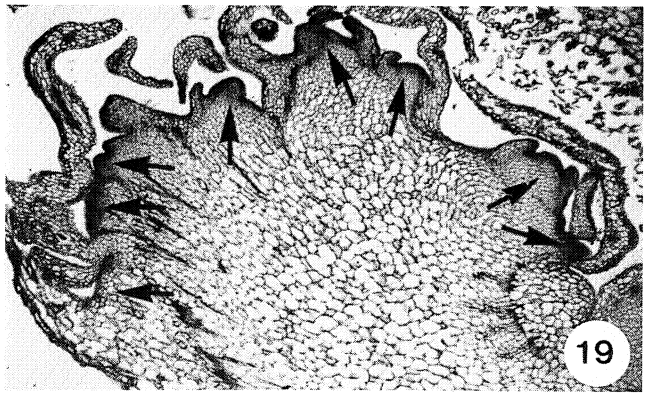

FIG. 19
Part of dense shoot clump after 40 days' culture on media enriched with BAP and BAP + ZR. Arrows indicate proliferating shoots. (LM x 35).

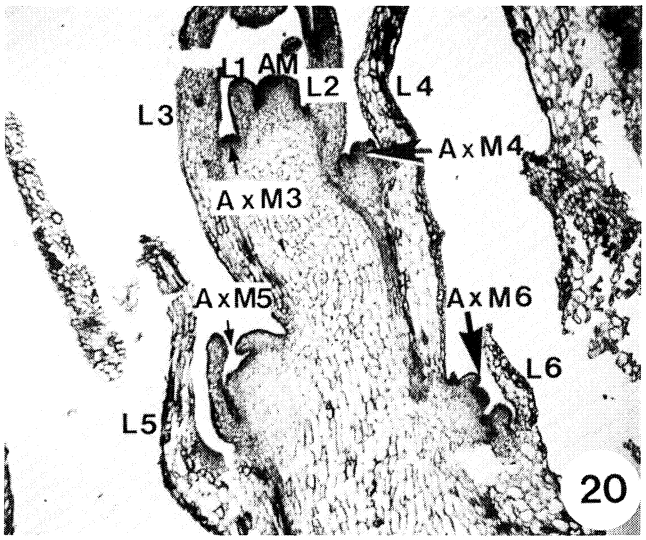

FIG. 20
Marked elongation of shoots derived from apices after 20 days' culture on media enriched with 10 mg/l ZR. Origination of axillary meristems (Ax M) in leaf (L) axils occurs at lower levels down the axes. Note absence of tendrils. (LM x 40).

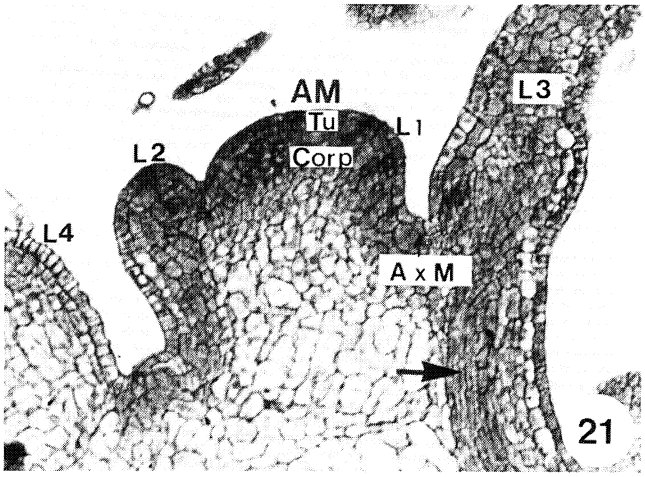

FIG. 21
Shoot apex after 25 days' culture on media enriched with 10 mg/l ZR, showing apical meristem (AM), 2-layered tunica (Tu), corpus (Corp), leaf initiation (L1), leaf (L) primordia and primary vascularization (arrowed). The youngest axillary meristem (Ax M) differentiated in axil of leaf 3. (LM x 140).

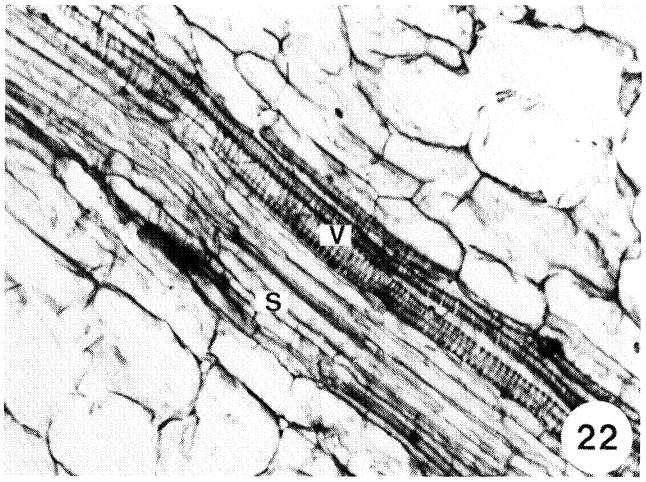

FIG. 22
Differentiated sieve elements (S) and spirally thickened vessels (V) at base of leaf 3 in Fig. 21. (LM x 220).

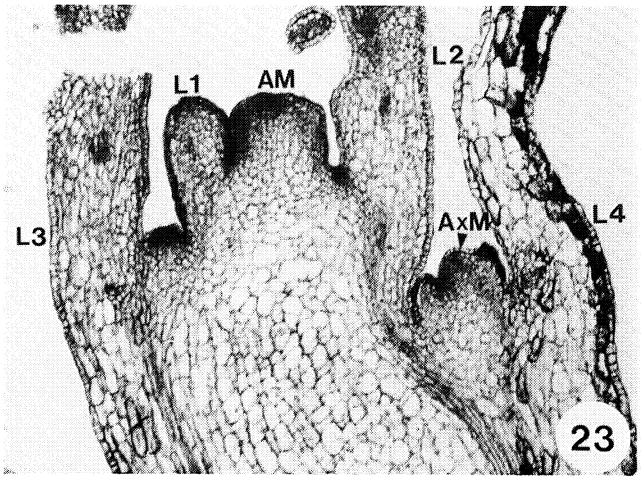

FIG. 23
Pronounced development and elongation of axillary meristem (Ax M) in axil of leaf 4 after 30 days' culture with 10 mg/l ZR. (LM x 87).

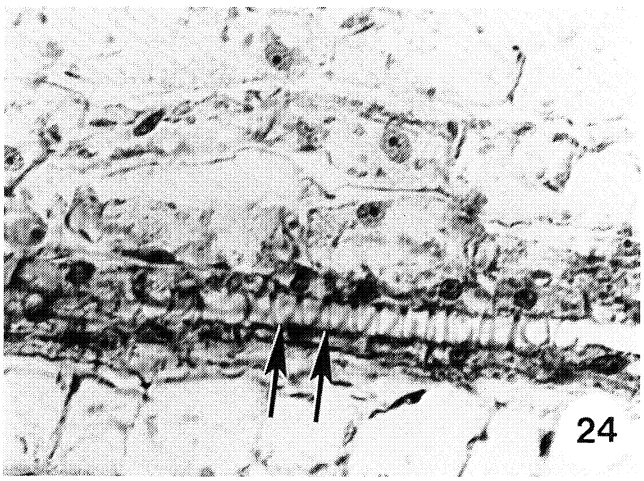

FIG. 24
Stretching of xylem elements as expressed by the uncoiling of spirals (arrowed) in response to 10 mg/l ZR. (LM x 520).

the enhanced release of axillary meristems from apical dominance, due to the application of BAP alone, as well as in combination with ZR. Axillary meristems on these axillary shoots were subsequently released from apical dominance, thus giving rise to dense clusters with the main AMS occasionally encircled by developing axillary meristems. Branching of the apical parts of shoots protruding from these clusters could be traced to the strong release from apical dominance of axillary meristems nearest the main AMS. Due to strong development and elongation of the axillary meristems, forklike branches resulted. Subsequent branching contributed to the formation of clusters of variable densities.

Shoots produced with 10 mg/*l* ZR lacked any branching and elongated rapidly. Growth of axillary meristems nearest to the main AMS was initially limited. However, lower down the axes the axillary meristems elongated rapidly. Shoot clusters of low densities may be attributed to this response.

Shoots derived from abscised shoot apices cultured *in vitro* in the presence of cytokinins proceeded with vascularization according to the sequence described for growing shoot tips of established vines (Hegedüs, 1957; Fournioux, 1972; Pratt, 1974). In the present investigation a tendency for sieve elements and vessels to differentiate at levels nearer to the main AMS was observed, especially in shoot clumps of high densities. It is concluded that proliferated shoots derived from non-fragmented shoot apices is of axillary origin. Applications of BAP at 2 mg/*l*, alone or in combination with 2 mg/*l* ZR, resulted in more pronounced depression of apical dominance than with 10 mg/*l* ZR.

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