Plant Physiol. (1988) 86, 836–840
0032-0889/88/86/0836/05/$01.00/0

Role of Ethylene and Cytokinins in the Initiation of Lateral Shoot Growth in Bromeliads

Received for publication June 5, 1987 and in revised form October 22, 1987

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ABSTRACT

***Aechmea victoriana* var discolor L. B. Foster and *Aechmea dactylina* Bal. are commercially propagated *in vitro* through lateral shoot growth. A modified Murashige and Skoog medium is used which contains both BA and IAA. These growth substances were shown in the present study to synergistically stimulate the production of ethylene by the cultured plants. The stimulation of ethylene production is correlated with the outgrowth of the lateral buds. The rise in ethylene production was concluded to induce lateral shoot growth, because: (a) outgrowth of the shoots was blocked by preventing an increase in ethylene production, (b) 1-aminocyclopropane-1-carboxylic acid (ACC), the natural precursor of ethylene biosynthesis, substituted for IAA in the promotion of ethylene production and lateral bud outgrowth. Although ACC could substitute for IAA, it could not substitute for BA; therefore, cytokinins are concluded to be essential for lateral bud outgrowth *in vitro* in *Aechmea*. These results suggest that cytokinins and ethylene both play roles in natural lateral bud initiation and that the cytokinin function involves two stages of the process.**

The family Bromeliaceae is composed of tropical American genera, many of which are herbaceous epiphytes. Some of them are important ornamentals such as several Aechmea species (5). Several possibilities exist for the multiplication of bromeliads *in vivo* as well as *in vitro* (8). *In vivo* propagation of Aechmea through seed has been a standard practice. The plants obtained are variable and often of poor quality. Poor germination is often another problem which is associated with propagation through seed. Vegetative multiplication through suckers is too slow to be practical (22). Therefore, '*in vitro*' propagation of Aechmea has become more and more widely used. To start the *in vitro* multiplication of Aechmea, plantlets can be obtained from *in vitro* germination or from explanted shoot-tips or axillary buds. These plantlets can be mutiplied vegetatively by induction of lateral shoot growth, on a modified Murashige and Skoog (9) nutrient medium containing 2 mg/L BA and 2 mg/L IAA (8). The importance of IAA and BA was studied in relation to lateral shoot formation and ethylene production.

Tissue Culture. Experiments were performed on the following Aechmea species: *Aechmea victoriana* var discolor L. B. Foster and *Aechmea dactylina* Bal. The liquid basal culture medium used for *in vitro* culture of *Aechmea* included full strength salt concentration of M&S[2] (9), 2% sucrose, 100 mg/L myoinositol, 5 mg/L nicotinic acid, 5 mg/L thiamine-HCl (B1), 0.5 mg/L pyridoxine-HCl (B6), 4 mg/L glycine, and was supplemented with different growth regulators. The germination medium contained only one-third salt concentration of M&S and was solidified with 0.8% Agar (Merck No. 1613). Plants were obtained through *in vitro* germination of seeds surface sterilized with 96% EtOH for 1 min and in a 15% (v/v) aqueous solution of a commercial 7% sodium hypochloride bleach water solution for 1 h. Seeds were placed on a germination medium supplemented with 1 mg/L IAA and 1 mg/L GA_3. Lateral shoot growth was induced on a liquid basal medium, supplemented with 2 mg/L BA and 2 mg/L IAA. Elongation of the plants is obtained on a liquid medium, supplemented with 1 mg/L NAA.

Two types of culture vessels were used: (a) glass honey jars with polypropylene screw caps (MELI-type) and (b) special glass cuvettes adapted for gas measurements under aseptic conditions (15). Culture vessels were kept at 20°C under a 16 h daylight regime of 31 $\mu E/m^2 \cdot s$ of PAR (Philips TLM 65W 33 RS).

Morphological Parameters. Two macromorphological parameters were monitored during the experiments: (a) the swelling at the base of the plants was described by means of four arbitrary stages quantifying the increase in the stem diameter (0, no swelling: +, 0–50%; + +, 50–100%, + + +, 100–200%); (b) the number of lateral shoots per culture vessel (each vessel containing 5 plants). Both parameters are separated by a slash. In the tables a dash means no observations were made.

Ethylene Determinations. The ethylene production rates were measured in a flow through system as described by De Greef *et al.* (3) and De Greef and De Proft (4). This measuring technique was adapted for measurements under aseptic conditions (15). For all ethylene data points the standard errors were less than 2.5% of the measured values.

Ethylene Inhibitors. Suppression of the ethylene production was obtained by using: 5 mg/L AVG (10); 500 mg/L AIB (12, 13); 13 mg/L Co^{2+} (7), and for inhibition of ethylene action 66 mg/L STS (2, 17).

RESULTS

After *in vitro* germination, Aechmea plants were grown on a elongation medium containing only 1 mg/L 1-NAA during a first subculture of 10 weeks. These plants were used for experiments performed during subsequent subcultures. During the second subculture the pattern of the ethylene evolution differed with the type of culture medium used (Fig. 1). The rate of ethylene

[1] Recipient of an IWONL grant.

[2] Abbreviations: M&S, Murashige and Skoog medium; ACC, 1-aminocyclopropane-1-carboxylic acid; AIB, 1-aminoisobutyric acid; AVG, aminoethoxyvinylglycine; NAA, 1-naphthylacetic acid; STS, silver thiosulfate.

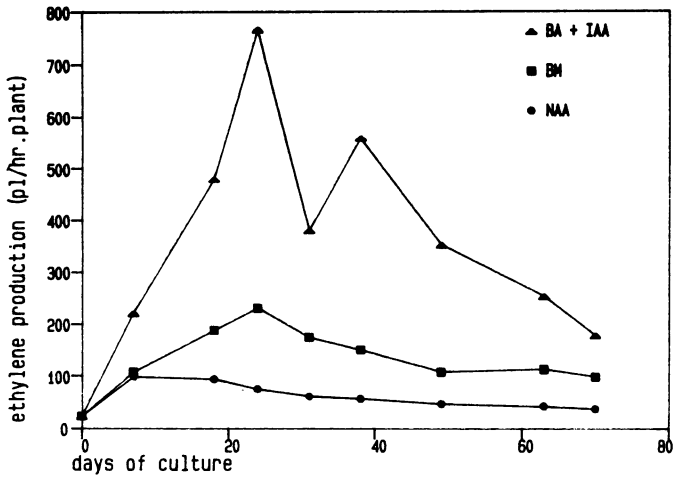

FIG. 1. Evolution of ethylene during a subculture of Aechmea plants on a basal medium containing either no growth substances (BM), 2 mg/L BA, and 2 mg/L IAA or 1 mg/L NAA.

production rose to a modest peak and then declined on the basal medium which contained no growth substances.

The multiplication medium which contained 2 mg/L BA and 2 mg/L IAA stimulated the ethylene production rate. Ethylene production peaked during the fourth and sixth week of the subculture. The first of these peaks coincides with the basal swelling of the plant and the second with the outgrowth of the lateral shoots (Table I). The elongation medium containing 1 mg/L NAA produced only a modest inhibition of the ethylene production compared to the control.

No outgrowth of lateral shoots was observed in either the elongation medium with NAA or in the basal medium without growth substances. Shoot formation only occurred where there was a strong promotion of ethylene synthesis. We examined the necessity of the combination of BA and IAA in the medium, in relation to the stimulated ethylene production and lateral shoot growth (Fig. 2).

Neither the medium containing only BA nor the medium containing only IAA stimulated the ethylene production of the plants. The results clearly demonstrate the synergistic effect of BA and IAA on the production of ethylene (Fig. 2). Neither BA nor IAA alone induced any outgrowth of lateral buds (Table I). Cytokinins are known to break or release apical dominance (11), but in the case of the Aechmea plants they are not the only controlling factor for outgrowth, because BA alone did not cause outgrowth.

In order to establish the exact involvement of ethylene, we asked the following two questions: (a) does stimulation of the ethylene production at given stages of the subculture correspond with an induction of lateral shoot growth? (b) Does inhibition of a stimulated ethylene production (on a medium containing BA and IAA) prevent lateral shoot growth? For the first question Aechmea plants were grown on a M&S medium containing only 2 mg/L BA, as a control medium. Under these conditions apical dominance is expressed (11). Ethylene production remains low (Fig. 2), and outgrowth of the lateral buds is not observed (Table I). At two different stages of this subculture, d 0 and d 14, 2 mg/L IAA was added to the nutrient medium under aseptic conditions. With both additions of IAA, ethylene production was promoted but with the delayed addition a shift of 14 d was observed in the ethylene production pattern (Fig. 3), as well as in the outgrowth of the lateral buds (Table II). Adding IAA at d 0 resulted in the appearance of lateral shoots from the 4th week on. Adding IAA at d 14 resulted in an appearance of lateral shoots from the 6th week on. These results demonstrate another correlation between high ethylene production rates and the outgrowth of lateral buds.

For the second question Aechmea plants were grown on a normal multiplication medium containing 2 mg/L BA and 2 mg/L IAA to which inhibitors of the ethylene biosynthesis pathway or ethylene action are added during a first subculture.

AIB and Co^{2+} reduced ethylene production to a very low rate (Fig. 4). AVG suppressed ethylene production to an even lower rate of 10 pl/h · plant (results not shown). STS did not block the ethylene production (Fig. 4). However, the STS-concentration used, decreased the number of lateral buds which formed lateral shoots. The number diminished from 30 down to 8 per culture vessel with STS. AIB, Co^{2+}, and AVG prevented lateral shoot growth (Table III). These results indicate that ethylene production is necessary for the outgrowth of the lateral buds of Aechmea plants. In routine propagation culture ethylene production occurs in response to the presence of both 2 mg/L BA and 2 mg/L IAA in the medium.

To evaluate the exact involvement of IAA and ethylene during the induction of the outgrowth of lateral shoots, Aechmea plants were grown on a M&S medium to which 2 mg/L BA was added. IAA was replaced by 50 mg/L ACC, which was added to the medium on d 0 or d 22 of the subculture. As in other systems ACC was rapidly converted into ethylene (Fig. 5). When added at d 0 the ethylene production increased up to 12 nl/h plant at d 20; when added at d 22 a very sharp increase of the ethylene production was observed, reaching a maximum of 14 nl/h plant around 8 d later. This pattern of the ethylene evolution with its two subsequent peaks was typical and repeatable. The pattern is also analogous to the pattern of the ethylene evolution by Aechmea plants on commercial multiplication medium (Figs. 1 and 2). The postponement of the ethylene stimulation until d 22 also seems to induce a postponement in the outgrowth of the lateral shoots (d 35 instead of 21) (Table IV).

In order to evaluate the necessity of BA for induction of the outgrowth of lateral shoots, 50 mg/L ACC was added to a basal medium, along the either 2 mg/L BA or 1 mg/L 1-NAA. During the subculture on the medium without BA the rate of ethylene release fluctuated around 1 nl/h plant (Fig. 6). This in sharp contrast to the higher ethylene production on the medium containing BA (Fig. 6). However, it should be noted that ethylene production is still 5 to 10 times higher than ethylene production of Aechmea plants grown on a multiplication medium with BA

Table I. *Outgrowth of Lateral Buds on a Basal Medium*

Medium	Culture Time (d)									
	7	14	21	28	35	42	49	56	63	70
Control	—[a]	0	—	—	0	0	—	0	—	0
IAA, BA	—	0	—	++	++/10	++/20	—	++/22	—	++/28
BA, 2 mg/ml	—	0	—	0	0	0	0	0	—	0
IAA, 2mg/ml	—	0	—	—	0	0	—	0	0	0
NAA, 1 mg/ml	—	0	—	0	0	0	0	0	—	0

[a] No observations were made.

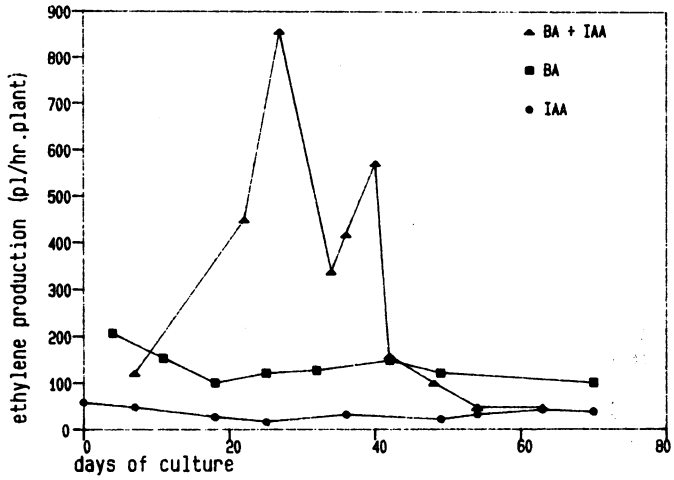

FIG. 2. Evolution of ethylene during subculture of Aechmea plants on a basal medium containing either 2 mg/L BA, 2 mg/L IAA, 2 mg/L BA, and 2 mg/L IAA.

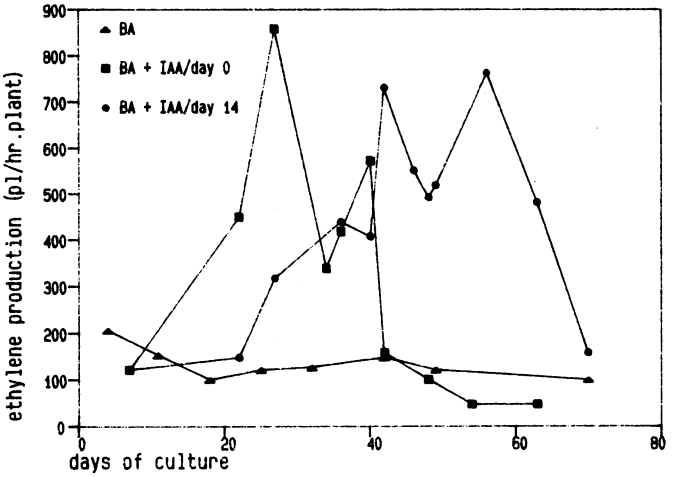

FIG. 3. Evolution of ethylene during subculture of Aechmea plants on a basal medium containing 2 mg/L BA, to which 2 mg/L IAA was added at two different stages of the subculture.

and IAA (Fig. 1). In this experiment (Figs. 1 and 2) it was demonstrated that ethylene production of 800 pl/h plant during multiplication on a M&S medium containing 2 mg/L BA and 2 mg/L IAA, was sufficient to induce outgrowth of lateral shoots. As can be seen, although ethylene production level is adequate (Fig. 6) the absence of BA seems to prevent the outgrowth of lateral shoots (Table V).

In order to more accurately define the rate of ethylene production which is sufficient to induce lateral shoot growth, smaller concentrations ACC were added (1 mg/L and 20 mg/L instead of 50 mg/L). Ethylene production dropped to reach maxima of, respectively, 1000 pl/h · plant and 3500 pl/h · plant (Fig. 7).

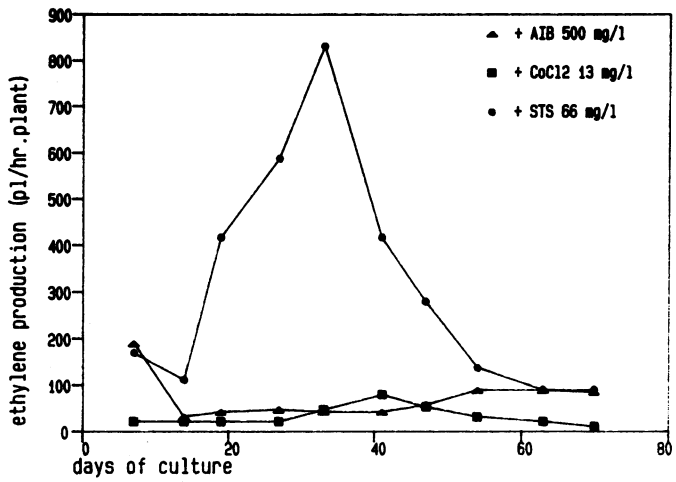

FIG. 4. Evolution of ethylene during a subculture of Aechmea plants on a basal medium containing 2 mg/L BA and 2 mg/L IAA and to which different ethylene inhibitors were added.

In all cases outgrowth of lateral shoots was induced (Table VI). However, high amounts of ACC and therefore high ethylene production rates seemed to hasten outgrowth of the lateral shoots. For example, BA and IAA were used, shoots were observed from d 28 to 35 onward as was the case when 1 mg/L ACC was used. Twenty mg/L ACC advanced this date by 7 d while 50 mg/L ACC advanced it 14 d (Table VI).

DISCUSSION

Stimulation of ethylene production was only obtained when both BA and IAA were present in the medium. The stimulation of ethylene production up to a rate of 800 pl/h plant is the result of the synergistic effect of BA and IAA on ethylene biosynthesis. It is known that cytokinins and auxins have a synergistic effect on the *de novo*-synthesis of ACC-synthase (6, 21). ACC-synthase controls the conversion of s-adenosyl-methionine (SAM) to ACC, the precursor of ethylene. The outgrowth of lateral buds was correlated with this stimulation of ethylene production. A delay in the onset of the high ethylene production rate resulted in a corresponding delay in the appearance of the first lateral shoots. Inhibition of this rise in ethylene production prevented outgrowth of lateral shoots. STS only partially prevented the outgrowth of lateral buds probably due to suboptimal concentration. Preferential accumulation of STS in certain parts of the plant (16) may also account for the observations made. An increase in the ethylene production rate above a threshold is necessary for the outgrowth of lateral buds in Aechmea. Analogous observations were made on lateral bud outgrowth in *Phaseolus vulgaris* (19, 20), *Pisum sativum* (1), and *Lilium speciosum* (14). A possible underlying control or involvement by cytokinins and auxins was not excluded in previous work. The fact that ACC will successfully substitute for IAA confirms the importance of high ethylene production rates for the control of the outgrowth of lateral shoots and seems to exclude a direct involvement of

Table II. *Outgrowth of Lateral Buds on a Basal Medium Containing 2 mg/l BA*

Medium	Culture Time (d)									
	7	14	21	28	35	42	49	56	63	70
Control	—[a]	0	—	0	0	0	—	0	—	0
IAA d 0	—	0	—	++	++/10	++/20	—	++/22	—	++/28
IAA d 14	—	—	0	0	++	—	++/3	—	++/6	++/10

[a] No observations were made.

Table III. *Outgrowth of Lateral Buds on a Basal Medium + 2 mg/L BA and 2 mg/L IAA, After Adding of Ethylene-Inhibitors*

Medium	Culture Time (d)									
	7	14	21	28	35	42	49	56	63	70
STS 66 mg/L	0	—[a]	—	++	+++/4	+++/6	—	+++/8	+++/8	—
AIB 500 mg/L	0	0	—	—	0	0	—	0	0	0
Co^{+2} 13 mg/L	0	—	—	0	0	0	—	0	—	0
AVG 5 mg/ml	0	0	0	—	0	—	0	0	—	0

[a] No observations were made.

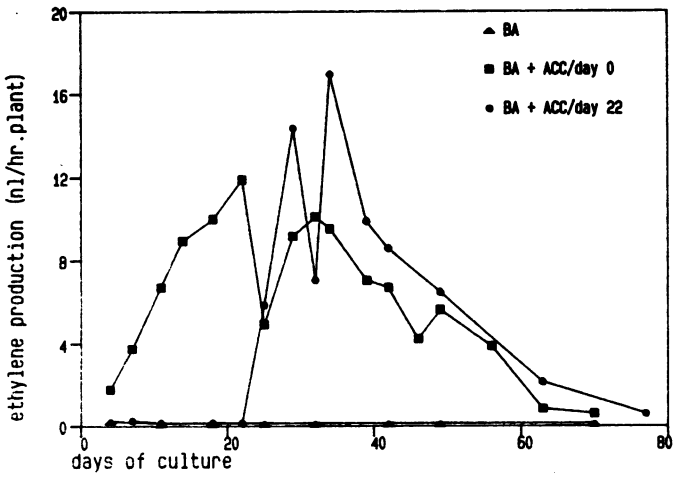

FIG. 5. Evolution of ethylene during a subculture of Aechmea plants on a basal medium containing 2 mg/L BA, to which 50 mg/L ACC was added at two different stages of the subculture.

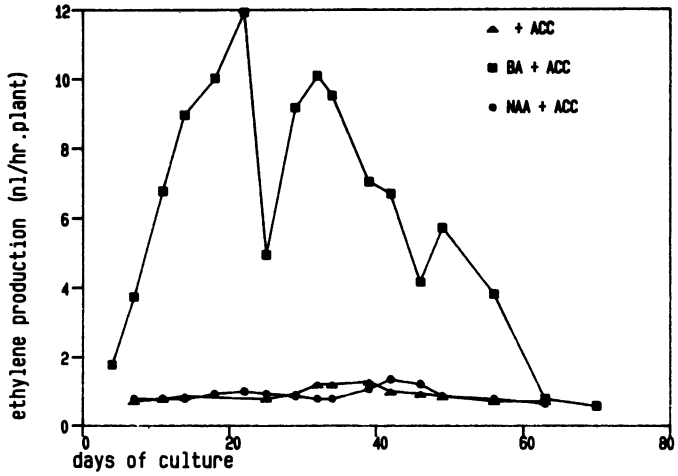

FIG. 6. Evolution of ethylene during a subculture of Aechmea plants on three different media containing either no growth substances (BM), 2 mg/L BA or 1 mg/L NAA, and to which 50 mg/L ACC was added.

IAA in the outgrowth of the shoots, apart from the stimulation of the ethylene production.

The occurrence of two peaks in the pattern of the ethylene evolution was consistent during all our experiments on a liquid basal medium containing BA and IAA. Due to the broadness of the intervals between measurements, the exact timing of the ethylene production maxima, as well as the exact amounts of the ethylene production ratios, peaks can differ slightly among the graphic representations. This may explain the single peak pattern observed in Figure 4. The two peaks in ethylene production correspond to the swelling of the basal tissues and to the outgrowth of lateral buds. In the first case bud differentiation and development are apparently occurring, and in the latter the actual bud outgrowth had begun.

In initial experiments with ACC the ethylene rate increased up to 12 nl/h · plant. Much of this ethylene seems to be surplus because lateral shoot outgrowth can be induced with 1 mg/L ACC rather than the 50 mg/L level used initially. One mg/L ACC stimulates the production up to a maximum of 1 nl/h · plant, which is in the same range as the observed 0.800 nl/h · plant with 2 mg/L IAA.

The addition of 50 ppm ACC to M&S media containing no BA demonstrated the importance of cytokinins for lateral bud differentiation and outgrowth. Even though ethylene production rates reached 1 nl/h · plant with ACC but without any BA or NAA no outgrowth of lateral shoots was observed within the normal 6 week experimental period. The addition of ACC to a medium containing BA resulted in higher ethylene production

Table IV. *Outgrowth of Lateral Shoots on a Basal Medium Containing 2 mg/L BA and to which 50 mg/L ACC Was Added at d 0 and 22*

Medium	Culture Time (d)								
	14	21	28	35	42	49	56	63	70
Control	0	—[a]	0	0	0	—	0	—	0
ACC (d 0)	+++	+++/2	+++/8	+++/15	+++/17	+++/21	—	+++/30	—
ACC (d 22)	0	+	+++	+++/10	+++/15	+++/19	—	+++/25	—

[a] No observations were made.

Table V. *Outgrowth of the Lateral Shoots on Basal Media Containing Different Growth Substances and 50 mg/L ACC*

Medium	Culture Time (d)					
	14	21	28	35	42	63
Control	0	0	0	0	0	0
+2 mg/L BA	+++	+++/5	+++/8	+++/15	+++/21	+++/30
+1 mg/L NAA	0	0	0	0	0	++

Table VI. *Outgrowth of the Lateral Shoots on Basal Media Containing 2 mg/L BA and to Which Different Concentrations of ACC were Added*

Medium	Culture Time (d)								
	7	14	21	28	35	42	49	56	63
+ACC									
1 mg/L	0	0	++	+++	+++/2	+++/4	+++/8	+++/15	+++/17
5 mg/L	0	0	+++	+++	+++/5	+++/7	+++/10	+++/16	+++/19
15 mg/L	0	++	+++	+++/4	+++/7	+++/11	+++/14	+++/23	+++/25
20 mg/L	—[a]	++	+++	+++/5	+++/7	+++/12	+++16	+++/25	+++/29

[a] No observations were made.

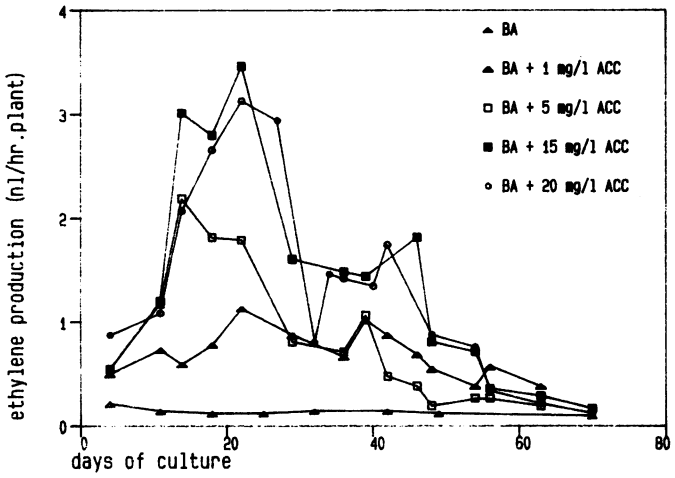

FIG. 7. Evolution of ethylene during subculture of Aechmea plants on a basal medium containing 2 mg/L BA, to which different concentrations ACC were added.

rates and sharper increases, when added at d 22 rather than at d 0. This indicates that the involvement of BA in the lateral bud outgrowth must not be limited to its stimulatory effect on ethylene production. Apparently BA is also active during early stages of lateral bud differentiation as indicated by the observed swelling at the basal part of the plants. This swelling preceded the outgrowth of lateral shoots and was always correlated with the presence of BA. In contrast, outgrowth of lateral shoots was always correlated with high ethylene production rates. Different stages during initiation of adventitious buds by embryos of *Picea abies* have been described by Von Arnold and Eriksson (18). Further work should provide more insight into the exact succession of the stages of lateral shoot formation in Aechmea and their control by plant growth substances.

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