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Relationship Between the Activity of the Ethylene-Forming Enzyme and the Level of Intracellular 2,4-Dichlorophenoxyacetic Acid in Pear Cell Cultures *In Vitro*

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Abstract. Ethylene production by auxin-dependent pear cells cultured *in vitro* falls rapidly when they are deprived of 2,4-D. This phenomenon is associated with a decrease in ACC production. Readdition of 2,4-D causes a resumption of ACC production and ethylene synthesis. Ethylene-forming enzyme (EFE) activity, although never limiting, decreases sharply during 2,4-D depletion and rises again upon addition of 2,4-D. This increase in the EFE activity is not a rapid response to 2,4-D, since it requires several hours. Changes in EFE activity follow the same pattern as changes in 2,4-D concentration; the decrease in EFE activity is also concomitant with a decrease in the ability of 2,4-dinitrophenol to inhibit ethylene production. The possible role of auxins in the modulation of EFE activity is discussed.

The pathway of ethylene biosynthesis appears mainly to be controlled by two key enzymes: ACC synthetase, a cytoplasmic enzyme which catalyzes the conversion of SAM to ACC (Yu et al. 1979, Acaster and Kende 1983), and ethylene-forming-enzyme (EFE), a membrane bound complex which has not yet been isolated but appears to be at least partly associated with the tonoplast (Guy and Kende 1984). Ethylene production is reported to be dependent, in most plant tissues, on the activity of ACC synthetase, EFE activity being usually nonlimiting (Yang and Hoffman 1984). In some cases, however, EFE is either not present in detectable amounts as in immature fruits (unpublished data), or it decreases so much that its activity limits ethylene production as in senescing fruit or flower tissues (Hoffman and Yang 1980, Bufler et al. 1980). It is well known that auxins play a major role in the induction of ACC synthetase, at least in juvenile tissues (Yoshii and Imaseki 1982). Furthermore, it

was recently hypothesized that auxins could also be involved in the conversion of ACC to ethylene through their effects on membrane polarization (John 1983). If this is true, auxin deprivation of plant cells would be expected to decrease EFE activity, and, conversely, auxin application would be expected to quickly activate it. A study was therefore undertaken to test this hypothesis by using a culture of auxin-dependent pear cells grown in bioreactors and maintained in quiescent conditions as described in previous papers (Pech and Romani 1979, Balagué et al. 1982). We followed ethylene production and ACC content of the cells under auxin depletion and upon readdition of 2,4-D. We also examined the relationship between the changes in EFE activity and in the level of intracellular 2,4-D.

Materials and Methods

Plant Material

A suspension culture established in 1981 from young Passe-Crassane pear fruit (*Pyrus communis* L.) was used in these experiments. The conditions for growth during successive transfers were the same as previously described (Pech and Romani 1979, Codron et al. 1979). They include the use of a standard medium supplemented with 45 μM 2,4-dichlorophenoxyacetic acid (2,4-D) as the only exogenous hormone.

Culture in a Closed-Continuous Bioreactor

Growing cells were first subcultured for 9 days without medium renewal in the standard medium minus 2,4-D. They were then transferred into the bioreactor fed with an "aging medium" containing one-fourth concentration of the mineral and organic nutrients of the standard medium, plus 0.37 M mannitol and 0.03 M sucrose. The system and conditions of culture have been described in previous papers (Pech and Romani 1979, Balagué et al. 1982). Each experiment was carried out with two bioreactors, inoculated with the same cells. The first was free of 2,4-D throughout; the second was supplemented to 2.3 μM 2,4-D after 7 days. In some experiments 0.1 M 1-aminocyclopropane-1-carboxylic acid (ACC) was added both to the medium present in the bioreactor before transferring cells and to the in-flow medium.

Determination of ACC and ACC Conjugate

Freeze-dried cells (500 mg) were ground in a mortar in the presence of acid-washed sand and 15 ml of 70% ethanol. After boiling for 15 min, the extract was filtered through glass wool and the filtrate was concentrated under vacuum at 35°C to the desired volume. Quantification of ACC conjugate was carried out by hydrolyzing it to ACC in 6 N HCl at 110°C for 25 min. The concentration of ACC in the extract was determined by converting ACC to ethylene ac-

cording to the method of Lizada and Yang (1979). Ethylene was determined by gas chromatography. Each determination was run in duplicate.

Measurement of Ethylene Production and EFE Activity

Vials (25 ml) containing 3 ml of cell suspensions were incubated at 25°C in the dark with constant shaking, in the absence (actual ethylene production) or in the presence (EFE activity) of saturating concentration of ACC (1 mM). The vials were capped with a septum for 6 h, and a 1-ml gas sample of head space was withdrawn with a hypodermic syringe for ethylene determination by gas chromatography. It had been previously verified that ethylene production in the presence or absence of exogenous ACC was linear during the 6-h incubation period and that oxygen was not limiting.

Extraction and Determination of Intracellular 2,4-D

The method described by Mousdale (1981) was used with some minor adaptations. One gram of fresh cells was ground in a mortar with liquid nitrogen and acid-washed sand. The cell powder was extracted with 5 ml of methanol for 16 h at −15°C and then centrifuged at 10,000*g* for 5 min. The supernatant was collected and buffered with 15 ml of 0.5 M K_2HPO_4 (pH 8.5). The HPLC system used was manufactured by Waters and was equipped with a model 600A pump, a UGK injector, and a 440 absorbance detector at 280 nm. The solvent system was 30% methanol in 0.01 M tetramethylammonium phosphate (pH 6.5) at a flow rate of 1 ml/min.

Results

Effect of the Readdition of 2,4-D on Ethylene Production of Quiescent Cells

2,4-D Depletion of quiescent cells in the close-continuous bioreactor dramatically reduced ethylene production, from 100 $pl.h^{-1}/10^5$ cells at day 1 to 20 pl at day 3 and around 10 pl thereafter (Fig. 1). Concomitantly the ACC content of the cells decreased from 50 pmole/10^5 cells to 10 pmole. The readdition of 2,4-D (2.3 μM) at day 7 caused a rapid increase of ethylene production and ACC content of the cells to a level which was 15 times higher than the control at day 18. A slight decrease was observed by the end of the culture (Fig. 1).

EFE Activity as a Function of the Intracellular Level of Free 2,4-D

The EFE activity of 2,4-D-deprived cells decreased rapidly from 0.72 $nl.h^{-1}/10^5$ cells at day 1 to 0.22 nl at day 5. The same pattern was observed for the

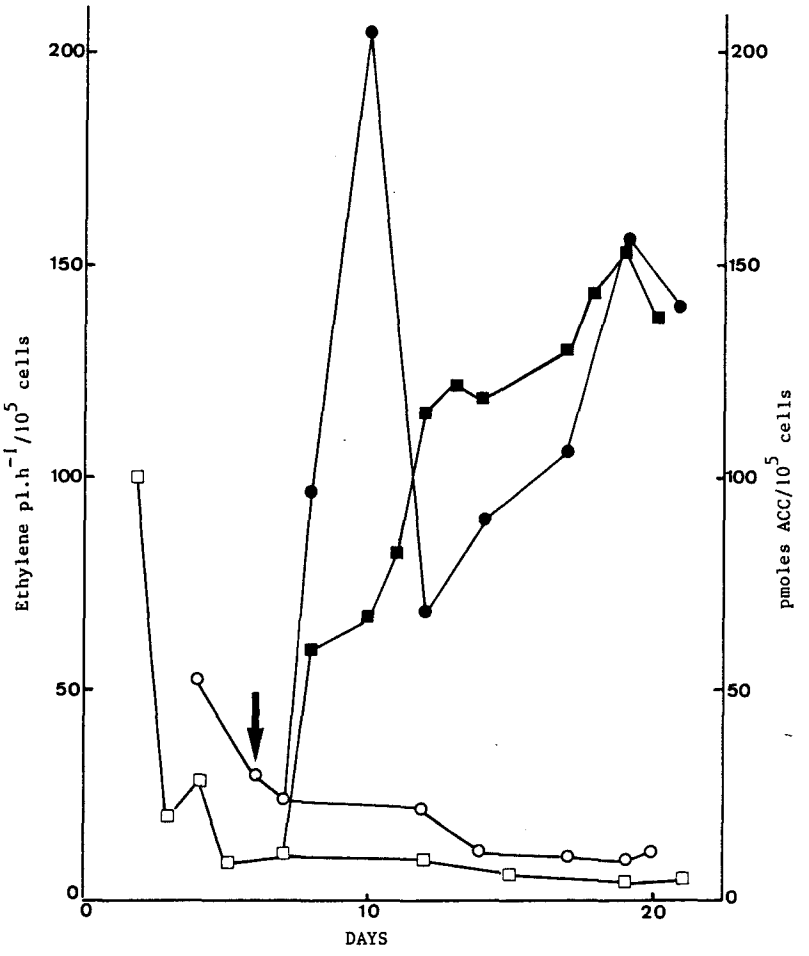

Fig. 1. Ethylene production and ACC accumulation by quiescent pear cells cultured in the absence of 2,4-D (open symbols) and in response to readdition of 2,4-D (closed symbols). Ethylene (□—□, ■—■); ACC (○—○, ●—●); addition of 2.3 μM 2,4-D (↓).

intracellular level of 2,4-D which fell from 155 nmole/10^5 cells the first day to 12 nmole at day 5 (Fig. 2).

The readdition of exogenous 2,4-D in the culture medium at day 7 caused a 3-fold increase in EFE activity 24 h later (0.5 nl.h^{-1}/10^5 cells). The decrease in the intracellular level of 2,4-D observed during the first days in the presence of 2.3 μM exogenous 2,4-D is paralleled by a decrease in EFE activity (Fig. 2). However, its level always remained much higher than in control cells. The kinetics of the EFE increase during the first hours after addition of 2,4-D was estimated in a separate experiment. Results reported in Table 1 show that no increase could be detected during the first 6 h and that the maximum activity was reached after 16 h. The increase in EFE activity is therefore a long-term response to the readdition of 2,4-D.

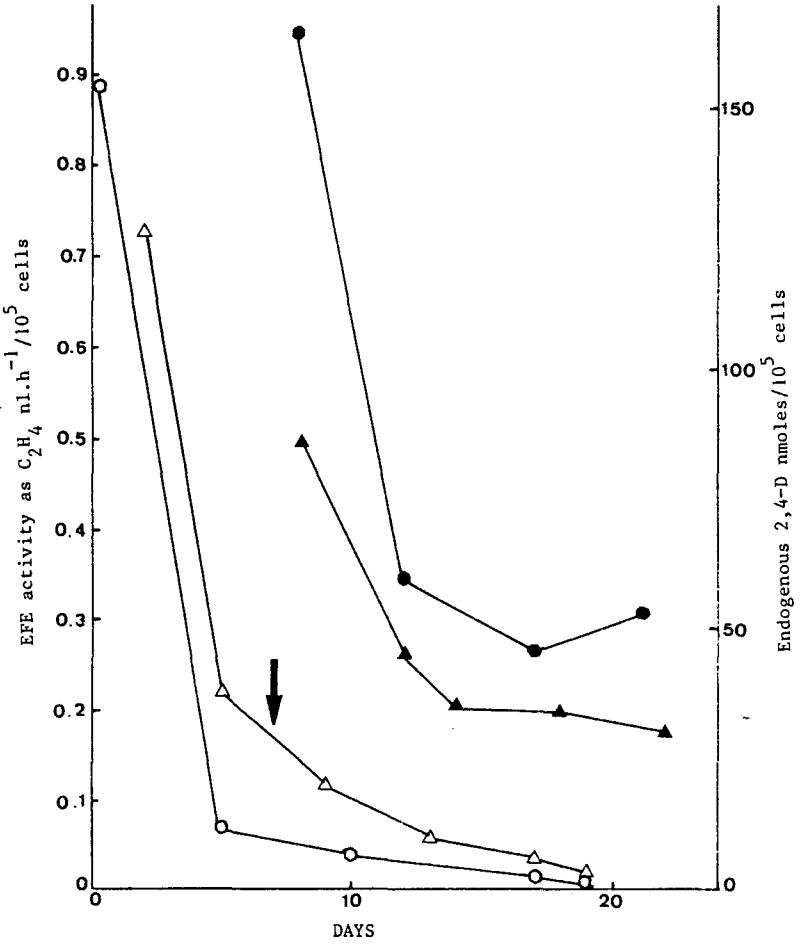

Fig. 2. Intracellular level of free 2,4-D and EFE activity in quiescent pear cells cultured in the absence of 2,4-D (open symbols) and in response to readdition of 2,4-D (closed symbols). 2,4-D (○—○, ●—●); EFE activity (△—△, ▲—▲); addition of 2.3 μM 2,4-D (↓).

Ethylene Production by 2,4-D-deprived Cells in the Presence of Exogenously Supplied ACC in the Culture Medium

When auxin-deprived cells were continuously fed with exogenous ACC (0.1 mM), ethylene production was as high as 1 nl.h^{-1}/10^5 cells during the first days of culture, fell rapidly to 0.17 nl at day 10, and reached a steady level of approximately 0.1 nl thereafter (Fig. 3). The level of ACC inside the cells increased to a peak at 15 days as ethylene production fell, then fell again. Ethylene production in these conditions was 10–20 times higher than in 2,4-D-deprived cell suspensions cultured in the absence of exogenous ACC (see Fig. 1). ACC conjugate concentration was about 1.4 nmole/10^5 cells at day 7. It fell rapidly thereafter to reach a steady level of about 0.6 nmole/10^5 cells. ACC conjugate content of cells not supplied with exogenous ACC was always around 0.04 nmole/10^5 cells.

Table 1. EFE activity of quiescent pear cells upon readdition of 2,4-D (2.3 μM). Cells were previously cultured for 7 days in the bioreactor with 2,4-D-deprived medium.

	Time (h)				
EFE activity	0	6	16	22	40
Ethylene nl · $h^{-1}/10^5$ cells	0.15	0.14	0.36	0.30	0.30

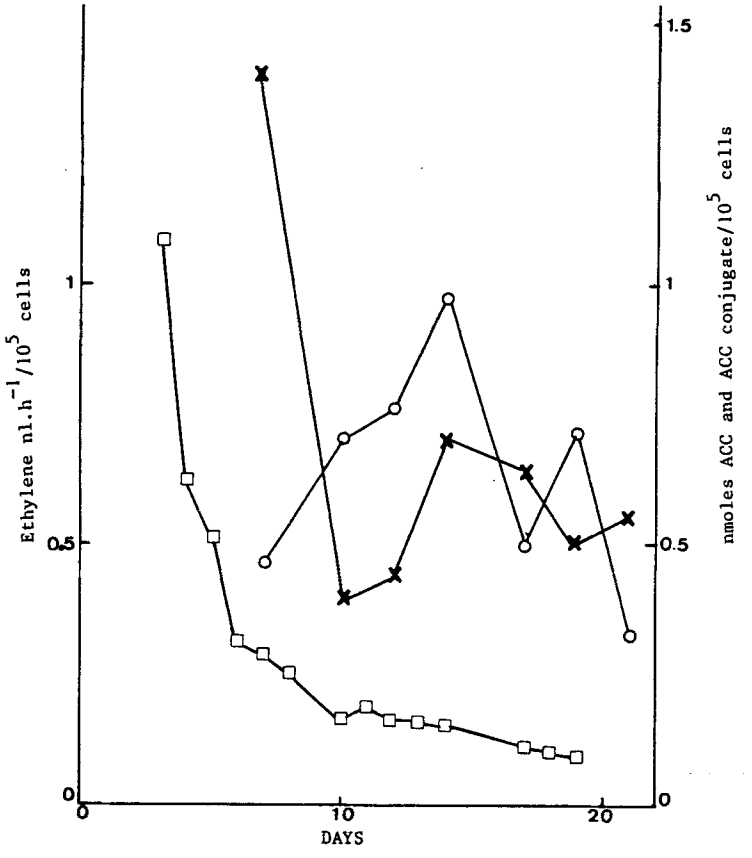

Fig. 3. Ethylene production, ACC, and ACC conjugate accumulation by quiescent pear cells in the absence of 2,4-D but with 0.1 mM exogenous ACC. Ethylene (□—□); ACC (○—○); ACC conjugate (×—×).

Effects of 2,4-Dinitrophenol (DNP) on EFE Activity

The effects of DNP (100 μM) on EFE activity were investigated at various times during culture in the absence of 2,4-D. Table 2 shows that DNP inhibited ethylene production by 69%, 56%, and 35% after 2, 5, and 9 days, respectively. This decrease of the efficiency of DNP in inhibiting ethylene production was correlated with the reduction in intracellular 2,4-D.

Table 2. EFE activity of quiescent cells after 6 h of incubation with ACC (0.5 mM) or ACC (0.5 mM) + DNP (0.1 mM).

	Ethylene nl · $h^{-1}/10^5$ cells		
Day	ACC	ACC + DNP (% inhibitions)	Endogenous 2,4-D nmole/10^5 cells
2	4.2	1.3 (69)	100
5	1.25	0.55 (56)	13
9	0.66	0.43 (35)	7

Discussion

Ethylene production by cell suspension cultures has been found to be associated with the stress following the transfer into a new medium or with the exponential phase of cell division (Mackenzie and Street 1970, Lieberman et al. 1979, Adams et al. 1981). In the study reported here, cell suspensions were cultured in a medium that was continuously renewed and which suppressed cell division (Balagué et al. 1982). The level of a specific metabolite or hormone is easily controlled, and the system is therefore particularly suitable for studying the possible regulatory role of auxins on the activity of the EFE complex. The method used for modulating the level of auxins-(2,4-D) consists in a depletion, by suppressing the exogenous supply, and resupplementation. After a long period (12 days) of auxin depletion, ethylene production was very low—around 10 pl.$h^{-1}/10^5$ cells. Growing cells of the same strain cultured in the presence of 4.5 μM of 2,4-D produced around 1 250 pl.$h^{-1}/10^5$ cells just before the stationary phase (data not shown). Readdition of IAA (10^{-4} M) or 2,4-D (10^{-5} M) after 10 days of auxin depletion respectively led to an ethylene production of about 3,500 pl.$h^{-1}/10^5$ cells and 1,000 pl.$h^{-1}/10^5$ cells (Puschmann and Romani 1983). After a longer period (16 days), we found that the readdition of 2,4-D (2.3 μM) gave a maximum ethylene production of 500 pl.$h^{-1}/10^5$ cells.

Since these changes were paralleled by changes in the level of intracellular ACC, and since the activity of the EFE was always higher than the potential ethylene production, even in the presence of auxin, it can be concluded that, as in many other tissues (Yu et al. 1979, Jones and Kende 1979, Yoshii and Imaseki 1981, 1982), the activity of ACC synthetase is the rate-limiting step and is modulated by auxins. However, the activity of the EFE fell 10-fold during the first 10 days of culture, even in the continuous presence of ACC (0.1 mM) in the culture medium. This decrease closely parallels the depletion of the intracellular 2,4-D; moreover, readdition of 2,4-D stimulates EFE activity about 3-fold. Pushmann and Romani (1983) also showed an increased rate of the conversion of ACC to ethylene upon addition of IAA even though the magnitude of the increase was much lower, probably because of a less complete auxin depletion.

The loss of EFE activity during auxin deprivation may be associated with some molecular events occurring in the membranes. It is generally accepted that EFE is a membrane-bound complex and that any changes in membrane conformation or integrity greatly affect its activity (Lieberman 1979). It has also been suggested that membrane polarization could be involved in the conversion of ACC to ethylene (John 1983). However, polarity effects of auxins are rapid (Bates and Goldsmith 1983), whereas in the present experiments the response to the readdition of 2,4-D was rather slow. Auxins could therefore probably act more through changes in the synthesis of proteins or enzymes directly involved in the structure of the EFE complex. Such effects of auxin on protein synthesis have been found to require several hours (Zurfluh and Guilfoyle 1980, Meyer et al. 1984).

In agreement with the results of Yu et al. (1980), 0.1 mM DNP inhibited ethylene production by about 70% during the first days of the culture. When auxin deprivation becomes more complete, the percent inhbition of ethylene production by DNP becomes less important (56% at day 5 and 35% at day 9). A similar decrease in the efficiency of DNP was also observed for amino acid uptake (Balagué, unpublished). This could be related to the gradual loss of membrane integrity and/or of the energy supply necessary for ACC conversion to ethylene. Indeed we have observed previously that respiration greatly decreases during auxin starvation, thus limiting the energy supply to the cell (Balagué et al. 1982). A loss of membrane integrity (clearance of inner mitochondrial membrane, vesiculation of endoplasmic reticulum, sinuosity of plasmalemma) was evident from electron microscopy observations (Balagué et al. 1983). It therefore becomes obvious that the loss of energy supply and the loss of membrane integrity are interrelated or interdependent.

A decrease in EFE activity associated with a loss of membrane integrity also occurs in senescing or ripening organs (Mayak et al. 1981, Platt-Aloia and Thomson 1981), and Ca^{++} and Mg^{++} have been shown to be able to sustain ethylene production (Lieberman and Wang 1982, Legge et al. 1982, Evensen 1984). But the effective concentrations used are very high (50–100 mM), well out of the range of physiological levels of these ions in cells. Auxins appear to be rather a candidate for the maintenance and modulation of membrane integrity and EFE activity.

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