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Production, Regulation and Mode of Action of Ethylene in Fruit Ripening, Senescence, and Decay

E. Chalutz, Y. Fuchs, J.D. Anderson

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BARD TECHNICAL REPORTS

BARD No. of Proposal I-61-79

Title of Proposed Research Production, Regulation and Mode of Action of Ethylene in Fruit Ripening, Senescence and Decay.

Name(s) of investigator(s):

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Signature

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ספריה המרכזית
למדעי החקלאות
בית דגן

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Abstract

The research work of this BARD project proceeded along two main lines: The first one comprised of studies related to biosynthesis of ethylene. We elucidated some properties and the membrane association of the ethylene forming enzyme, the effect of some drugs - antagonists of calmodulin - on ACC synthase activity and described the role of inorganic phosphate in the biosynthesis of ethylene in higher plants and microorganisms.

The second main research direction centered on the role of ethylene in host-pathogen interactions. We studied the effect of fungal infection on inhibition of ethylene-induced PAL activity in citrus fruit and on the ethylene producing machinary of the host. The contribution of host and pathogen to the ethylene produced was determined in P. digitatum - infected citrus fruit. We also demostrated a rapid induction of ethylene biosynthesis in tobacco leaves when leaf discs were incubated with cell-wall digesting enzymes and demostrated the stimulation of this phenomenon by ethylene, in an autocatalytic manner. The effect of wounding in relation to avocado fruit ripening was also studied. The results obtained have thus deepened our knowledge of these two main research areas.

This BARD project also promoted close, fruitful and productive scientific cooperation between the American and Israeli scientists involved.

Body of the report

The research results of this project can be summarized under two main headings as outlined in the proposal: Biosynthesis and mode of action of ethylene, and the role of ethylene in host-pathogen interactions.

The enclosed papers and manuscripts, which comprise the body of the report, have therefore been arranged accordingly in two main groups.

Evaluation of research achievement with respect to original research proposal

Of the three main research objectives outlined in the porposal, vis. the elucidation of the biosynthesis and mode of action of ethylene, the determination of the role of ethylene in host-pathogen interactions and the development of techniques for suppression or accelerating ethylene production and action to control the ripening process, the research achievements are related mainly to the first two objectives. As to the third objective, we did find a process in which rapid and massive ethylene production occurs but this has not yet been applied for the control of fruit or vegetable ripening.

Description and results of cooperation

Throughout the time span of this project, several visits of the participating scientists at the laboratories of their cooperating investigators took place. These consisted of short visits (1 to 2 weeks) intended for the exchange of information, planning of experiments, development of new research procedures or the summarizing the work and writting reports.In addition, the principal investigator (E. Chalutz) spent an extended period (Sabbatical year) at the laboratory of the cooperating investigator.

Another scientist who cooperated in this project, A.K. Mattoo, had previously spent an extended period (about 2 years) working partly with scientists at the laboratory in Israel and then joined the U.S. laboratory where he has been working, among other projects, on this BARD project, for the past 3 years. Partial salary support for Dr. Mattoo was from this BARD project.

Throughout the duration of the project, special chemicals such as inhibitors and fungal isolates were exchanged between the cooperating laboratories.

On the whole, this project promoted close collaboration between the two research groups with may joint publications and a new BARD proposal submitted last fall.

On January 18, 1982, during the course of this project, M. Lieberman, the cooperating American investigator passed away. His successor as Chief of the Plant Hormone Laboratory BARC, J.D. Anderson, joined in the project and promoted further the research work and the close cooperation.

As a result of the initiative of all the scientists involved in this BARD project (and with the help of others), a special International Symposium on Ethylene, dedicated to the memory of M. Lieberman, was held in Israel in January 1984. This successful event reflected the close cooperation between American and Israeli scientists in the field of ethylene, a research area which has been promoted by BARD.

Thesis

1. Kapulnik, Esther (1981). Production of ethylene by two isolates of the fungus Penicillium digitatum. M.Sc. Thesis. Submitted to the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

2. Achilea, Oded (To be submitted in 1984). The biosynthesis, control and mode of action of ethylene in Penicillium digitatum-infected citrus fruit. Ph.D. thesis, to be submitted to the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

List of publications and abstracts

1. Chalutz, E., A.K. Mattoo and Y. Fuchs (1980). Biosynthesis of ethylene: the effect of phosphate. Plant, Cell and Environ. 3:349-356.

2. Zauberman, G. and Y. Fuchs (1981). Effect of wounding on "Fuerte" avocado ripening. HortScience 16:496-497.

3. Fuchs. Y., A.K. Mattoo. E. Chalutz and Ilana Rot (1981). Biosynthesis of ethylene in higher plants. The metabolic site of inhibition by phosphate. Plant, Cell and Environ. 4:291-295.

4. Mattoo, A.K., O. Achilea, Y. Fuchs and E. Chalutz (1982). Membrane association and some characteristics of the ethylene forming enzyme from etiolated pea seedlings: Biochem & Biophys. Res. Comm. 195: 271-278.

5. Mattoo, A.K., D.O. Adams, G.W. Patterson and M. Lieberman (1982). Inhibition of 1-aminocyclopropane-1-carboxylic acid synthase by phenothiazines. Plant Sci. Letters. 28:173-179.

6. Anderson, J.D., A.K. Mattoo and M. Lieberman (1982). Induction of ethylene biosynthesis in tobacco leaf discs by cell wall digesting enzymes. Bioch. and Biophys. Res. Comm. 107:588-596.

7. Esther Kapulnik, A.K. Mattoo, E. Chalutz and I. Chet (1982). The relationship between the production of ethylene by certain fungi, their enzyme constitution and their ability to accumulate intracellular phosphate. Z. Pflanzenphysiol. 109:347-354.

8. Lisker, N., Lea Cohen, E. Chalutz and Y. Fuchs (1983). Fungal infections supress ethylene-induced phenylalanine ammonia-lyase activity in grapefruits. Physiol. Plant Pathol. 22:331-338.

9. Mattoo, A.K., E. Chalutz, J.D. Anderson and M. Lieberman (1983). Variation in adenylates and adenylate energy charge during phosphate-mediated inhibition of ethylene biosynthesis in Penicillium digitatum. Z. Pflanzenphysiol. 111:301-309.

10. Chalutz, E., A.K. Mattoo, T. Solomos and J.D. Anderson (1984). Enhancement by ethylene of Cellulysin-induced ethylene production by tobacco leaf discs. Plant Physiol. 74: 99-103.

11. Achilea, O., E. Chalutz, Y. Fuchs and I. Rot (1984). Ethylene biosynthesis and related physiological changes in Penicillium digitatum-infected citrus fruit. Physiol. Plant Pathol. (Submitted).

12. Fuchs, Y., E. Chalutz, I. Rot and A.K. Mattoo (1980). Site of phosphate inhibition of ethylene biosynthesis in higher plants. Plant Physiol. 65 Suppl. p. 43, Abstr. No. 272.

13. Mattoo, A.K., Y. Fuchs and E. Chalutz (1981). Regulatory aspects of ethylene biosynthesis in higher plants and microorganisms. Isr. J. Bot. 30:55 (Abstr).

14. Chalutz, E., Y. Fuchs and A.K. Mattoo (1981). Ethylene production by some microorganisms: Role in pathogenicity and regulation by phosphate. Isr. J. Bot. 30:54 (Abstr).

15. Mattoo, A.K. and M. Lieberman (1982). Role of silver ions controlling senescence and conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene. Plant Physiol. 69 Suppl. p. 18, Abstr. No. 94.

16. Anderson, J.D., A.K. Mattoo and M. Lieberman (1982). Cycloheximide-insensitive induction of ethylene biosynthesis in tobacco leaf discs by cell wall digesting enzymes. Proc. 11th International Confer. on Plant Growth Regulators p. 12, Abstr. No. 305.

17. Mattoo, A.K., J.E. Baker and M. Lieberman (1983). Copper induced ethylene production by Spirodela oligorrhiza is a consequence of singlet oxygen mediated membrane damage. Plant Physiol. Suppl. 72, p. 39. Abstr. No. 215.

18. Achilea, O., E. Chalutz, Y. Fuchs and Ilana Rot (1983). Ethylene biosynthesis and related physiological changes in Penicillium digitatum-infected citrus fruit. Plant Physiol. Suppl. 72, p. 163, Abstr. No.925.

19. Chalutz, E., A.K. Mattoo, T. Solomos and J.D. Anderson (1983). Enhancement by ethylene of Cellulysin-induced ethylene production by tobacco leaf discs. Plant Physiol. Suppl. 72 p.158, Abstr. No.902.

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21. Anderson, J.D., E. Chalutz and A.K. Mattoo (1984). Purification and properties of ethylene inducing factor from the cell wall digesting enzyme mixture, cellulysin. International Conference on Biochemical, Physiological and Applied Aspects of Ethylene, Jerusalem, Israel, January 1984 (Abstract).

22. Mattoo, A.K. and J.D. Anderson (1984). Characterization of wound-induced 1-aminocyclopropane-1-carboxylic acid synthase activity. International Conference on Biochemical, Physiological and Applied Aspects of Ethylene. Jerusalem, Israel, January 1984. (Abstract).

23. Chalutz, E., A.K. Mattoo and J.D. Anderson (1984). Enhancement by ethylene of cellulysin-induced ethylene production by tobacco leaf discs. International Conference on Biochemical, Physiological and Applied Aspects. Jerusalem, Israel, January 1984 (Abstract).

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Biosynthesis of ethylene: the effect of phosphate*

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Abstract. Biosynthesis of ethylene in tomato and avocado fruit slices, carrot root, pea seedling and tomato shoot segments, *Penicillium expansum* and *Escherichia coli* was found to be inhibited by inorganic phosphate. Compared with microbial systems, relatively high concentrations of phosphate in the incubating medium were necessary to bring about a significant inhibition of ethylene production in higher plants. The degree of inhibition in higher plants correlated with the increased internal cellular concentration of phosphate and not with that of the incubating medium. Phosphate concentrations inhibitory for ethylene biosynthesis did not affect the respiration of tomato fruit slices. The phosphate effect was reversible, confined to only the biological systems and was not due to a change in the ionic strength. The differential inhibitory effects of aminoethoxyvinylglycine on ethylene biosynthesis in tomato fruit slices of various stages of ripening, were markedly influenced by high phosphate concentrations. The data indicate a biological significance to the phosphate control of ethylene biosynthesis.

Introduction

Ethylene, a plant hormone, is synthesized by higher plants and many micro-organisms (for a recent review see Lieberman, 1979). Methionine is now well established as the precursor of ethylene in higher plants (Baur & Yang, 1972; Lieberman *et al.*, 1966). However, biosynthesis of ethylene by microorganisms, which has been studied mostly in *Penicillium digitatum* Sacc., the green mould of citrus fruit, may involve different pathways in which glutamate or methionine can be utilized as precursors, depending on the culture conditions under which the fungus is grown (Chalutz, Lieberman & Sisler, 1977, Chan & Yang, 1973; Mattoo *et al.*, 1979b).

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Although pathways of its biosynthesis have been extensively studied and reviewed (Lieberman, 1979; Yang, 1974), the regulation of ethylene production by micro-organisms and higher plants still remains obscure. Chalutz *et al.* (1978) demonstrated that inorganic phosphate is a potent inhibitor of ethylene production by shake cultures of *P. digitatum*. The phosphate control, which involved repression of the ethylene producing system in the fungus (Mattoo *et al.*, 1979b), was accompanied by a rise in the intracellular level of ATP (Chalutz *et al.*, 1978), inhibition of alkaline phosphatase and decreased incorporation of the labelled precursor glutamate into ethylene (Mattoo *et al.*, 1979b). In view of these observations and the findings concerning changes in phosphatases (Mattoo *et al.*, 1975; Sacher, 1973) during fruit ripening and known effects of phosphate level in the soil on leaf senescence, fruit set and development, and rate of ripening of tomato fruit (Besford, 1979), we have tested the possibility that phosphate affects the production of ethylene in higher plants and in several other biological systems. In this paper the results of this study are presented and their possible significance is discussed.

Materials and methods

Fruit slices (10 mm diameter, 5 mm thickness) of tomato (*Lycopersicon esculentum*, of several cultivars) and avocado (*Persea americana* Mill cv. Nabal) were prepared as described previously (Mattoo & Lieberman, 1977). For studying IAA-induced ethylene production, root pieces of carrot (*Daucus carota* L.), segments of etiolated pea (*Pisum sativum* var. Early Frosty) seedlings and tomato shoots.(Type I target cell according to Osborne, 1977) were used. Fruit slices and carrot roots were incubated with 2.5 cm^3 of mannitol (0.4 mol dm^{-3}) and sodium or potassium phosphate buffer at concentrations indicated in the text, pH 7.2, in 50-cm^3 Erlenmeyer flasks. Each flask contained six to seven freshly prepared fruit slices or root pieces (2.0–4.0 g) and was incubated at 23°C in a shaking water bath. Pea seedling and tomato shoot segments were incubated with 1 cm^3 of mannitol (0.4 mol dm^{-3})-phosphate buffer, pH 7.2, in 8-cm^3 flat bottomed glass

tubes. Ethylene sampling and analysis have been described elsewhere (Mattoo & Lieberman, 1977).

Penicillium italicum (Wehm), *P. expansum* (Link), *Fusarium solani* (Sp.), *Diplodia natalensis* (P.E.), *Colletotrichum gloeosporioides* (Penz.), *Trichoderma viridae* (Pers. Ex Fries), *Alternaria citri* (Ellis & Pierce), *Oospora citriaurantii* (Sacc. and Syd.), *Botrytis cinerea* (Pers.) and *Aspergillus niger* (V. Tiegh) were grown either under shake or static cultural conditions on Pratt's liquid medium (pH 4.5) with varying concentrations of phosphate as previously described (Chalutz *et al.*, 1977; Chalutz & Lieberman, 1978). The concentration of phosphate (added as mono basic potassium phosphate) in the medium varied from 100 mol m^{-3}, the normally used concentration, to 0.01 mol m^{-3} or lower. Inoculation and incubation procedures, culture weight determination and ethylene sampling and analysis have been described elsewhere (Chalutz *et al.*, 1978; Mattoo *et al.*, 1979b).

Escherichia coli was cultured in 50-cm^3 Erlenmeyer flasks on the standard Davis medium (5 cm^3) with L-methionine (0.5 mg cm^{-3}) and varying concentrations of phosphate at pH 6.8. Incubation was carried out at 34°C in a shaking water bath. Growth was measured by reading the absorbance of the culture at 540 nm (A_{540}).

For tracer studies, 0.5 or 1μCi of L-[U-^{14}C]methionine (285 mCi/mmol) was added to each incubation flask containing fruit slices and 1.5 cm^3 of mannitol-phosphate buffer. Labelled ethylene produced was trapped in 0.1 mol dm^{-3} mercuric acetate in methanol and determined as described previously (Chalutz *et al.*, 1977).

For determination of the internal cellular (inter- and intracellular) inorganic phosphate, pink tomato slices, incubated in the test solutions for 0, 1, 3 and 6 h, were removed, washed 3 times with 10-cm^3 portions of 0.4 mol dm^{-3} mannitol, then boiled in 10-cm^3 of 80% ethanol and frozen until used. After thawing, the contents were homogenized and the insoluble material was centrifuged down. The supernatant was concentrated under vacuum and its inorganic phosphate content was determined by the method of Lowry and Lopez (1946).

Each experiment was carried out in triplicate or more and repeated at least twice. Further details of techniques and methods are described in legends to figures and tables.

Results

Fruit tissue

A significant inhibition in ethylene production was found to be correlated with a substantial increase in the cellular concentration of free inorganic phosphate (referred to hereafter as phosphate) (Fig. 1), when slices of tomato fruit were incubated in an isotonic solution containing different phosphate concentrations. The internal phosphate content of the fruit slices

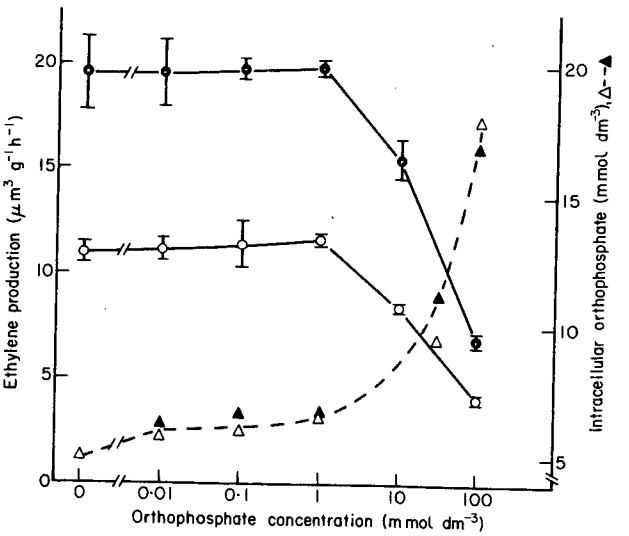

Figure 1. Accumulation of intracellular orthophosphate and time-dependent inhibition of ethylene production in pink tomato fruit slices. Fruit slices were incubated in mannitol (0.4 mol dm^{-3}) with varying concentrations of phosphate buffer, pH 7.2. Ethylene was determined at the end of the third (○) and sixth (●) sealed 1-h period of incubation. (△) and (▲) indicate the intracellular inorganic phosphate content (mol m^{-3}; 1 g tissue was taken as equivalent to 1 cm^3) of the slices following 3rd and 6th h of incubation, respectively. Bars indicate standard error.

increased only three times, from 6 to 18 mol m^{-3}, in 3–6 h while the phosphate content of the incubating medium was varied, from 1 to 100 mol m^{-3}, i.e. 100-fold. Concentrations of phosphate lower than 1 mol m^{-3} had no effect on ethylene production and very little, if any, accumulation of cellular phosphate occurred (Fig. 1). Under the conditions of phosphate inhibition of ethylene production, respiration, as measured by evolution of CO_2, was not affected (data not shown). The effect of non-inhibitory and inhibitory concentrations of phosphate was also tested after adjusting the ionic strength of the incubating medium with NaCl (checked by measuring the electrical conductivity of the solutions). The data (Table 1) indicated that inhibition of ethylene by phosphate was not a result of ionic strength. Inhibition of ethylene production by high phosphate was altered by less than 5%

Table 1. Effect of phosphate buffers with similar or different electrical conductivities on ethylene production by pink tomato fruit slices. NaCl was added to bring the ionic strength of the 0.01 mol m^{-3} phosphate buffer or of the mannitol solution to that of 100 mol m^{-3} phosphate buffer.

Phosphate concentration (mol m^{-3})	Addition of salt	Electrical conductivity of solution (μmho)	pH	Ethylene production ($\mu m^3 g^{-1} h^{-1}$)
0.01	None	0.02	6.5	42
0.01	NaCl	7.6	6.8	38
100	None	7.6	7.2	16
Mannitol only	NaCl	7.6	6.6	37

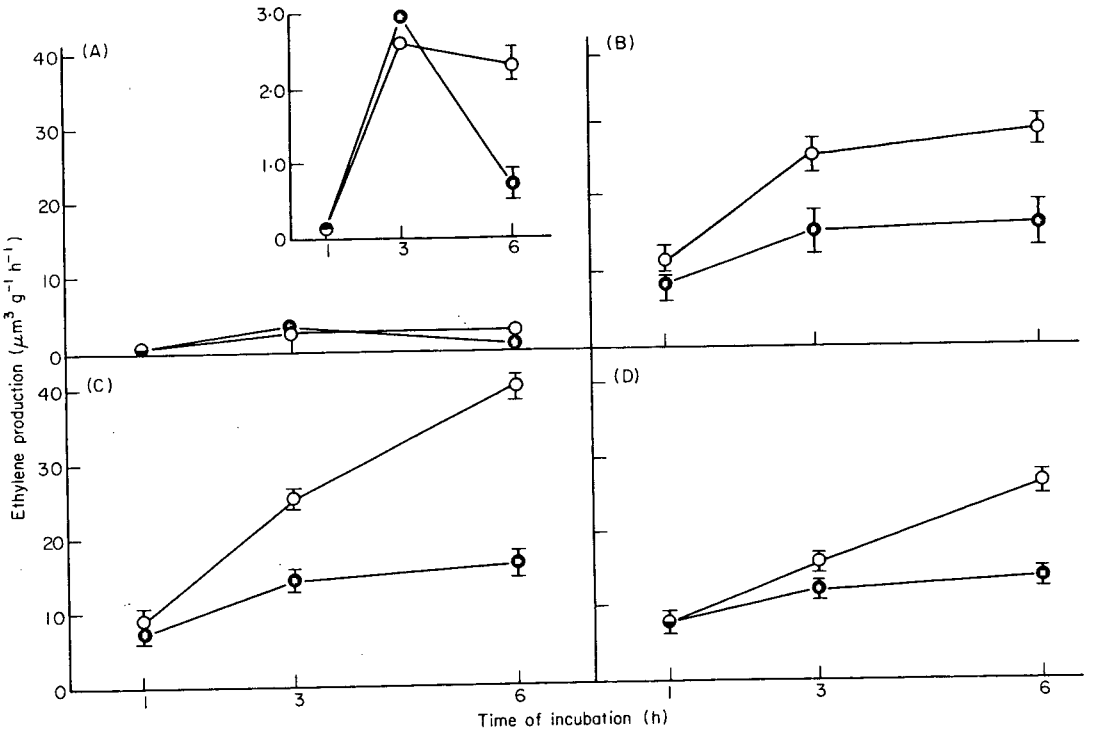

Figure 2. Sensitivity of ethylene production by green (A), breaker (B), pink (C) and red (D) tomato fruit slices to high concentration of inorganic phosphate. The developmental stages of tomato fruit were scored according to the U.S. Department of Agriculture colour chart (The John Henry Co., Michigan, U.S.A., February, 1975). 'Breaker' signifies that there is a difinite break in colour from green to tannish-yellow, pink or red on not more than 10% of the surface. Slices were incubated in high (•, 100 mol m^{-3} and low (o, 0.01 mol m^{-3}) phosphate-0.4 mol dm^{-3} mannitol buffer (pH 7.2). Bars indicate the standard error.

when the pH of incubating solution was varied between 6.5 and 8.

Phosphate-mediated inhibition of ethylene produced was found to be highest in the slices prepared from pink fruit, which produced ethylene at the highest rate and least in those from green fruit which had a relatively lower rate of ethylene production (Fig. 2); however, the inhibitory effect of phosphate was evident with slices prepared from fruit at all the stages of ripening (Fig. 2), and also in those from avocado (Table 2). Potassium salt was as effective as the sodium salt of phosphate in inhibiting the ethylene production (Table 2).

Confirmation of these results was obtained with experiments carried out on the incorporation of [U-^{14}C]methionine into labelled ethylene by fruit slices

Table 2. Effect of various buffers and of phosphate on ethylene production by tomato and avocado fruit slices. Pink tomato and early climacteric avocado fruit slices were incubated in mannitol (0.4 mol dm^{-3}) with different buffer solutions, pH 7.2, at 23°C. The flasks were sealed for the 6th h of incubation and ethylene accumulated in the atmosphere above the slices was collected and determined.

Buffer solutions	Concentration of phosphate (mol m^{-3})	Ethylene production ($\mu m^3 g^{-1} h^{-1}$) Tomato	Avocado
Tris-HCl (0.05 mol dm^{-3})	0	30	41
K-Phosphate	0.05	28	—
K-Phosphate	50	12	—
Na-Phosphate	0.05	31	44
Na-Phosphate	1	30	42
Na-Phosphate	40	20	32
Na-Phosphate	50	16	33
Na-Phosphate	80	13	—
Na-Phosphate	100	12	31

incubated with 0.01 or 100 mol m^{-3} phosphate (Table 3). At the high phosphate concentration, incorporation of the label from methionine into ethylene by slices of green and pink tomato, and climacteric avocado was inhibited by 67, 17 and 23% in 1 h, respectively, and the degree of inhibition increased on further incubation for 2 h to 74, 53 and 42%, respectively (Table 3). Radioactive ethylene production was inhibited more in the green than red tomato, while the converse was found for cold ethylene measurements.

In order to determine whether phosphate effect on ethylene production is a reversible phenomenon, pink tomato slices were immersed in mannitol-buffer containing 0.01 or 100 mol m^{-3} phosphate for 30, 60 and 120 min and the rate of ethylene produced was measured. After each of these incubation periods, slices were washed and transferred to fresh mannitol-buffer containing either 0.01 or 100 mol m^{-3} phosphate and incubated further. The results (Fig. 3) suggested a reversibility of the phosphate effect. The recovery of ethylene production from phosphate inhibition appeared to be time-dependent.

Phosphate also influenced inhibition of ethylene production by aminoethoxyvinylglycine (AVG), the inhibitor of ethylene biosynthesis in higher plants (Baker, Lieberman & Anderson, 1978; Lieberman, Kunishi & Owens, 1974; Mattoo & Lieberman, 1977) (Fig. 4). In the presence of 0.01 mol m^{-3} phosphate, AVG inhibited the production of ethylene by green and red tomato slices by 70 and 64%, respectively, whereas in the presence of 100 mol m^{-3} phosphate, it had no effect (Fig. 4).

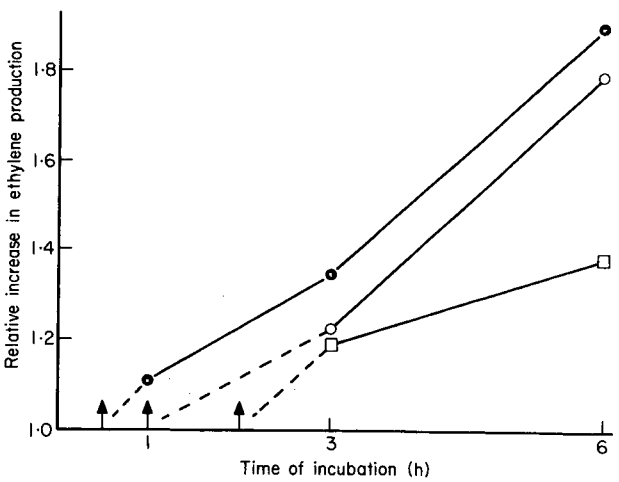

Figure 3. Reversibility of phosphate inhibition of ethylene production in pink tomato slices upon transfer to low phosphate. Pink tomato fruit slices were incubated in mannitol (0.4 mol dm^{-3})-phosphate (100 mol m^{-3}) buffer for 30 (●), 60 (○) and 120 (□) min. At the end of these incubations (indicated by arrows) fruit slices were washed three times with 20 cm^3 portions of 0.4 mol dm^{-3} mannitol. Half of these were transferred to mannitol (0.4 mol dm^{-3})-phosphate (0.01 mmol dm^{-3}) buffer and half to mannitol (0.4 mol dm^{-3})-phosphate (100 mmol dm^{-3}) buffer before incubating them further for various times. Relative increase in ethylene production is expressed as the ratio of ethylene produced by slices transferred to low phosphate-mannitol buffer to that by slices transferred to high phosphate-mannitol buffer.

Table 3. Effect of phosphate on the incorporation of L-(U-^{14}C)methionine into labelled ethylene by tomato and avocado fruit slices. Fruit slices (1.5–2.0 g) were preincubated for 30 min with 1.5 cm^3 of 0.4 mol dm^{-3} mannitol-0.01 mol m^{-3} phosphate buffer, pH 7.2, in 50-cm^3 Erlenmeyer flasks. 1 or 0.5 μCi of L-(U-^{14}C)methionine (679 cpm/pmol) was then added to each flask. The flasks were sealed for 1 h. A 2-cm^3 air/ethylene sample was then withdrawn for cold ethylene determination. The atmosphere remaining above the liquid and slices was next transferred by vacuum into 3 cm^3 of ice-cold 0.1 mol dm^{-3} mercuric acetate in methanol and radioactivity was determined. After transfer of the gases, i.e. after 30–60 s, the solution in each flask was decanted off and the slices without any solution were resealed for the second 1 h incubation period. Collection of gases for cold and labelled ethylene was then repeated.

	Ethylene production (pmol $g^{-1}h^{-1}$)			
	Buffer solution system*		No solution system*	
	Phosphate concentration (mol m^{-3})			
Tissue	0.01	100	0.01	100
Tomato				
Green	0.76	0.25	2.24	0.59
Pink	0.60	0.50	1.25	0.59
Avocado				
Climacteric	0.21	0.16	0.38	0.22

* For this nomenclature refer to Lieberman & Kunishi (1971)

Vegetative tissues

Ethylene production by vegetative tissues is known to be induced by IAA (Abeles, 1973). In carrot root and pea seedlings, this IAA-induced ethylene production was inhibited by increasing the concentration of phosphate in the inducing medium (Table 4). A similar effect of phosphate was observed on the IAA-induced ethylene production by segments of elongating tomato shoots (Type I target cell, Osborne, 1977) (data not shown).

Micro-organisms

Of all the post-harvest fruit pathogens listed in the Materials and methods section, only *P. expansum*, in addition to *P. digitatum*, produced ethylene at high rates, i.e., $\geqslant 1$ to 5 mm^3 h^{-1} when cultured on Pratt's medium. The production of ethylene by *P. expansum* was markedly dependent on phosphate content of the medium (Fig. 5). The rate of ethylene production reached 500 mm^3 h^{-1} flask within 72 h of incubation and remained so until 120 h, when the fungus was cultivated on the medium containing 0.1 mol m^{-3} phosphate. This rate was >100 times that obtained

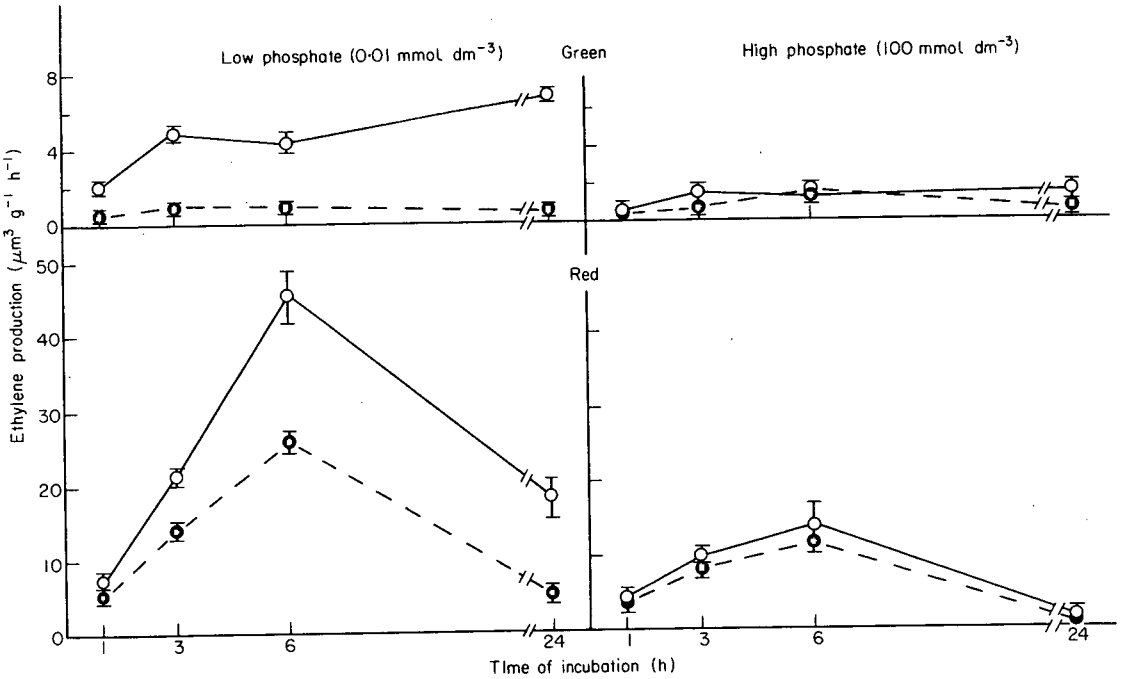

Figure 4. Effect of aminoethoxyvinylglycine (•) on ethylene production by green and red tomato slices at high and low phosphate levels. (○) indicates control levels. The concentration of aminoethoxyvinylglycine was 0.1 mol m^{-3}. Bars indicate standard error.

with 1 or 100 mol m^{-3} phosphate (Fig. 5). Mycelial weights of the culture cultivated on 0.01, 0.1 and 1 mol m^{-3} phosphate media were only one-half to one-third of that cultivated on the medium containing 100 mol m^{-3} phosphate at 48–120 h incubation.

Ethylene production by *Escherichia coli* requires induction by methionine (Primrose, 1976, 1979). When cultivated in Davis medium supplemented with methionine, *E. coli* produced ethylene at a rate which was found to be inversely related to the concentration of phosphate in the medium (Table 5). The rate of ethylene production increased by 70-fold when the concentration of phosphate in the medium was lowered from 100 to 0.1 mol m^{-3}; bacterial growth decreased by only 7-fold.

Table 4. Inhibition by phosphate of IAA-induced ethylene production by carrot roots and pea seedlings

Treatment	Concentration of phosphate (mol m^{-3})	Ethylene production* (μm^3/g fresh wt/h) Carrot roots	Pea seedlings
Buffer controls	0.01	—	1.4±0.3
	1.0	0.07±0.02	1.1±0.2
	100.0	0.07±0.01	0.5±0.1
IAA (10^{-4} mol dm^{-3})+ buffer	0.01	—	9.0±0.8
	1.0	0.80±0.07	6.5±0.6
	10.0	0.64±0.04	—
	50.0	0.36±0.03	—
	100.0	0.19±0.04	4.5±0.2

* Ethylene was determined after 17 h of incubation

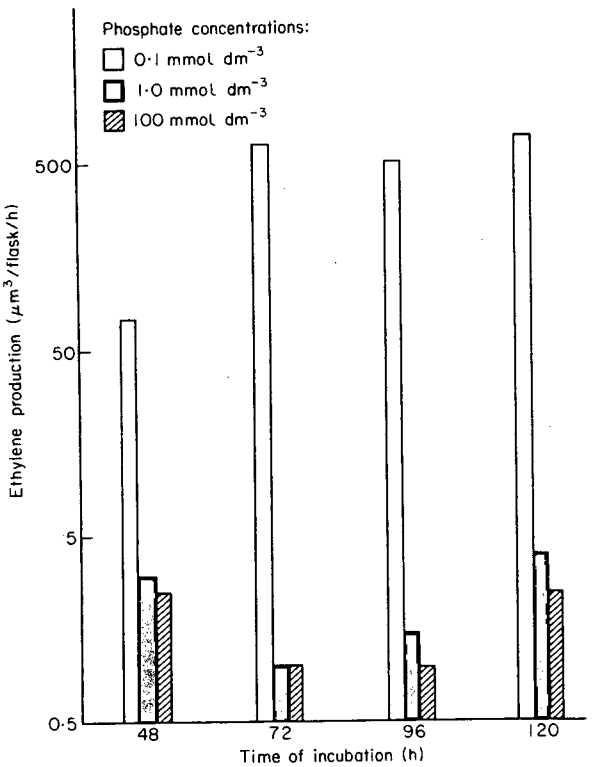

Figure 5. Effect of initial phosphate concentration of the medium on ethylene production by *P. expansum*. The fungus was cultured in shake cultures on Pratt's liquid medium with various concentrations of phosphate and without yeast extract at pH 4.2. Each day the flasks were sealed for 1 h and ethylene accumulated in the atmosphere above the culture was determined.

Table 5. Effect of initial phosphate concentration of the medium on ethylene production by *E. coli*. The bacterium was cultured as described in Materials and methods, and the gaseous atmosphere which accumulated above the liquid during the 24th and 27th h of incubation was collected and ethylene content determined. A_{540} indicates the absorbance of the culture read at 540 nm and was taken as the measure of growth.

Phosphate concentration (mol m^{-3})	A_{540}	Rate of ethylene production (μm^3/flask/h)	($\mu m^3/A_{540}$/h)
0.01	0.24	17.0	70.8
0.1	0.28	15.0	53.6
1.0	0.34	8.0	23.5
10.0	0.71	3.0	4.2
100.0	1.50	1.5	1.0

phosphate inhibition of ethylene production, together with the reversibility of this phenomenon and its confinement to only the biological systems, suggest that the phosphate effect on ethylene is not simply due to a general metabolic breakdown or to any permanent cellular damage but rather a specific one.

In addition to higher plants, control by phosphate of ethylene production was observed also in *E. coli* and *P. expansum* as with *P. digitatum* (Chalutz *et al.*, 1978; Mattoo *et al.*, 1979b), suggesting that these varied biological ethylene producing systems may share a common feature either in their biosynthetic pathway(s) or in its regulation. This suggestion finds support in the recent studies of Mattoo, Chalutz & Lieberman (1979a) who found similar effects of some membrane probes on ethylene-producing systems of apple fruit and *P. digitatum*.

Table 6. Stimulation of ethylene production by phosphate in the methionine-Cu^{2+}-ascorbate model system for ethylene production. The reaction was carried out in 50-cm^3 Erlenmeyer flasks containing 10 cm^3 buffer (pH 7.6), 1 mol m^{-3} methionine, 1 mol m^{-3} Cu^{2+} and 10 mol m^{-3} ascorbate (Lieberman *et al.*, 1965). Incubation was carried out at 25°C for 15 and 30 min.

Concentration of phosphate buffer (mol m^{-3})	0.1	0.5	1.0	5.0	10.0	50.0	100.0	500.0
Ethylene production (mm^{-3} h^{-1})	2.9	2.5	3.2	5.1	5.4	6.7	7.7	17.0

Chemical (model) system

Unlike its effect on the ethylene production in various biological systems, phosphate stimulated ethylene production in the chemical, methionine-Cu^{2+}-ascorbate model system (Table 6).

Discussion

The present study demonstrates that high concentrations of phosphate inhibit ethylene production in a number of biological systems and that this phenomenon appears to be of wide occurrence. In higher plants, relatively high concentrations of phosphate in the incubating medium were necessary to bring about a significant inhibition of ethylene production. This could be related to the fact that the internal level of phosphate may have to reach a certain elevated inhibitory level, since the degree of inhibition of ethylene production correlated with the increased internal concentration of phosphate and not with that of the incubating medium (Fig. 1), However, internal accumulation of phosphate appeared to saturate within 3 h of incubation while inhibition of ethylene continued to increase with further incubation (Fig. 1). One explanation for this could perhaps be the existence of a phosphate gradient within the cell causing differential access of phosphate to its site of action with time. The findings showing no effect of phosphate on respiration and non-involvement of ionic strength during the

An additional significant feature of the inhibition by phosphate of ethylene production in tomato fruit slices concerns its relationship to the effects of AVG. Earlier, Baker *et al.*, (1978) reported that AVG markedly inhibited ethylene production in green but not in pink and red tomato fruit slices. They suggested that an AVG-insensitive pathway of ethylene production may exist in the ripe tomato. However, in their studies, Baker *et al.* (1978) used 4.7×10^{-2} mol dm^{-3} phosphate in the incubating medium and contrary to their findings, we have shown (Fig. 4) that the inhibitory effect of AVG is obscured at high phosphate concentrations in both green and red tomato. Our results, therefore, provide one possible explanation for the relative ineffectiveness of AVG to inhibit ethylene production in pink and red tomato slices but not for the data on green slices. From the data, we emphasize that caution should be exercised in using phosphate buffers in studies on the biosynthesis of ethylene. The site of phosphate action needs to be tested in future experimentation.

Although the detailed mechanism of the phosphate effect remains to be elucidated, the present data indicate a biological significance to this effect on ethylene biosynthesis and/or regulation.

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Biosynthesis of ethylene in higher plants: the metabolic site of inhibition by phosphate*

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Abstract. Phosphate inhibited endogenous as well as 1-aminocyclopropane-1-carboxylic acid (ACC)-stimulated ethylene synthesis in slices of tomato fruit, segments of carrot root and pea hypocotyls. ACC concentrations of up to 10 mol m^{-3} did not overcome this inhibition. Phosphate inhibited the conversion of ^{14}C ACC to ethylene in tomato fruit and vegetative tissue. Enzymatic conversion of ACC to ethylene by pea seedling homogenate was also inhibited by phosphate with a linear concentration dependency. The formation of ACC from S-adenosylmethionine (SAM) by extracts of pink tomato fruit was slightly, but not significantly, affected by phosphate. However, the SAM to ACC conversion was greater when extracts from tomato fruit were made in phosphate rather than in HEPES-KOH buffer. Non-enzymatic ethylene synthesis from ACC in a model system was stimulated by phosphate. We suggest that phosphate is an inhibitor of ethylene biosynthesis in higher plants and that one site of its control is the conversion of ACC to ethylene.

Key-words: Lycopersicon esculentum; Daucus carota; Pisum sativum; tomato; carrot; pea; ethylene; phosphate control; 1-aminocyclopropane-1-carboxylic acid.

Introduction

Ethylene is one of the plant growth regulators synthesized by higher plants and by some micro-organisms (for a recent review see Lieberman, 1979). In higher plants, ethylene is formed from methionine (Lieberman *et al.*, 1966). There is good evidence for the following biosynthetic sequence: methionine→S-adenosyl-methionine (SAM)→1-aminocyclopropane-1-carboxylic acid (ACC)→ethylene. It seems well established that ACC is an immediate precursor of ethylene both by *in vivo* labelling studies (Adams & Yang, 1979; Lürssen, Naumann & Schroder, 1979), and by *in vitro* conversion of SAM to ACC by a cell-free preparation from tomato fruit (Boller, Herner & Kende, 1979). A homogenate of etiolated pea shoots has also been shown to catalyse the conversion of ACC to ethylene (Konze & Kende, 1979). Following these reports on ethylene biosynthesis it is now possible to study the site of action of effectors that might specifically affect the formation of ethylene from SAM using *in vitro* systems. Inorganic phosphate is a depressor of ethylene synthesis in both micro-organisms (Chalutz *et al.*, 1978; Chalutz, Mattoo & Fuchs, 1980), and higher plants (Chalutz *et al.*, 1980). Inhibition of ethylene synthesis by phosphate in higher plants is correlated more with the increased internal cellular concentration of phosphate rather than with that of the incubating medium, and is reversible (Chalutz *et al.*, 1980). Phosphate control of ethylene synthesis in *Penicillium digitatum* involves repression of the ethylene-synthesizing system (Mattoo *et al.*, 1979) and there is an increase in the intracellular level of ATP (Chalutz *et al.*, 1978). Nothing is known, however, about the site(s) at which phosphate controls ethylene biosynthesis in higher plants. In this paper we report studies carried out on the possible site where phosphate may exert its control along the biosynthetic pathway of ethylene from SAM.

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, S-adenosyl methionine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT, dithiothreitol.

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Materials and methods

Slices of tomato (*Lycopersicon esculentum*) fruit of several cultivars and apple (*Malus sylvestris cv.* Anna), discs of carrot (*Daucus carota* L.) root, and 1 cm sub-hook segments of etiolated pea (*Pisum sativum* cv. Early Frosty) seedlings were prepared as described previously (Chalutz *et al.*, 1980; Mattoo & Lieberman, 1977). Tissues weighing 2–4 g fresh weight were incubated with 1.5 cm^3 of a medium containing 0.4 mol dm^{-3} mannitol and potassium phosphate buffer (pH 7.2) at various phosphate concentrations in 50 cm^3

Erlenmeyer flasks (for fruit tissues) or in 8 cm^3 vials (for carrot and pea tissues), on a shaking water bath at 23°C. Test substances were included in the incubation medium. The ethylene evolved during the incubation was accumulated in the containers sealed with serum caps and samples were withdrawn with air-tight syringes for ethylene determination by gas chromatography (Lieberman *et al.*, 1966). The flasks and vials were flushed with air between successive determinations. All experiments were repeated at least twice.

The developmental stages of tomato fruits were scored according to the USDA Visual Aid TM–L–1, February 1975, U.S. Department of Agriculture colour chart.

ACC was obtained from Sigma (U.S.A.). Radioactively labelled ACC was accumulated by feeding 3,4-(^{14}C)L-methionine (Research Products International Corporation, France, a gift from Dr M. Lieberman, USDA, Beltsville, MD) to apple tissue under a nitrogen atmosphere (Adams & Yang, 1979). Apple slices were incubated with 0.4 mol dm^{-3} mannitol, 100 mol m^{-3} phosphate (pH 7.2), and 37 MBq of 3,4-(^{14}C)L-methionine (s.a. 1.81 TBq mol^{-1}) in a nitrogen atmosphere for 18 h at 25°C. After incubation the slices were homogenized with a mortar and pestle in 80% ethanol containing 0·05% (v/v) mercaptoethanol (Adams & Yang, 1979). The homogenate was centrifuged at 15 000 × *g* for 15 min, and the supernatant was dried under a stream of nitrogen. The residue was then taken up in 100 mm^3 of 80% (v/v) ethanol and spotted (40 mm^3 along 13 cm) on thin layer plates (aluminium sheets, 20 × 20 cm, layered with silica gel 0.2 mm thick, ICN Pharmaceutical, Eschwege, West Germany) which were developed with phenol:water (4:1, v/v). After developing, 5 or 10 mm sections were scraped off the plates and eluted with 3 cm^3 mannitol (0.4 mol dm^{-3}) and phosphate (0.05 or 100 mol m^{-3}) at pH 7.2 for tissue feeding experiments. For chemical assay of ACC, the scraped silica gel powder was eluted with 100 mol m^{-3} phosphate buffer at pH 11.5.

Radioactive ethylene, synthesized from labelled ACC, was assayed by the following method: to 0.5 cm^3 ACC containing solution at pH 8.0, 0.1 cm^3 of 1 mol m^{-3} mercuric acetate was added and the test tube was sealed, then 0.1 cm^3 of a mixture of 2 parts of 5% NaOCl and 1 part of saturated NaOH were added, followed by an incubation in the cold for 30 min (Lizada & Yang, 1979). Ethylene was trapped in ice-cold 0.1 mol cm^{-3} mercuric acetate in methanol according to the previously described procedure (Chalutz, Lieberman & Sisler, 1977). Conversion of radioactive ACC by tissue slices to labelled ethylene was determined by feeding the radioactive precursor to the tissue in 0.4 mol cm^{-3} mannitol, in either 0.01 or 100 mol m^{-3} phosphate buffer, pH 7.2. CO_2 evolved during the incubation was adsorbed onto filter paper discs moistened with 0.2 mol dm^{-3} KOH. Radioactive ethylene was trapped by mercuric acetate as mentioned above, mixed with 10 cm^3 of Insta-Gel (Packard), and determined in a scintillation spectrometer (Packard).

Cell extracts converting SAM to ACC were prepared from pink tomato pericarp tissue in 0.1 mol dm^{-3} sodium phosphate buffer (pH 8) or in 0.1 mol dm^{-3} potassium-HEPES (K-HEPES) buffer (pH 8.0) as described by Boller *et al.* (1979). In some experiments, 5 cm^3 of the crude extract were layered on a Sephadex G-50 column (2.5 × 40 cm) previously equilibrated with either 2 mol m^{-3} K-HEPES buffer (pH 8.0) or 2 mol m^{-3} phosphate buffer (pH 8.0) each containing 0.1 mol m^{-3} DTT and 2 mmol m^{-3} pyridoxal phosphate. In other experiments, crude extract was dialyzed overnight against 500 volumes of one of these buffers (2 mol m^{-3}). The standard reaction mixture contained 0.4 cm^3 of either dialyzed or gel-filtered cell extract, 0.04 cm^3 buffer and 0.06 cm^3 of 0.5 mol m^{-3} SAM solution. Incubation was carried out for 2 or 3 h at 30°C. The ACC produced was analyzed by the method of Lizada & Yang (1979).

Ethylene formation from ACC was studied also using homogenates of etiolated pea seedlings prepared in 60 mol m^{-3} Tris-HCl or phosphate buffer at pH 7.9 as described by Konze & Kende (1979). The homogenate was centrifuged at 11 000 × *g* for 20 min and the supernatant stored overnight at 5°C. The reaction mixture contained 150 mol m^{-3} Tris-HCl or, where indicated, phosphate buffer (pH 7.9), 5 or 10 mol m^{-3} ACC and a suitable aliquot of the enzyme preparation in a total volume of 0.4 cm^3 in 8 cm^3 vials. The vials were sealed with serum-vial caps and incubated for 90 min in a water bath at 28°C. The ethylene produced was measured by gas chromatography.

Results and discussion

Ethylene production by segments of carrot roots and pea seedlings was markedly enhanced by the addition of 1 mol m^{-3} ACC in the absence of any exogenous auxin (Table 1), as was previously observed with mung bean hypocotyl segments (Cameron *et al.*, 1979). The presence of phosphate at 50 mol m^{-3} or higher

Table 1. Inhibition by phosphate of ACC-induced ethylene production by carrot roots and pea seedlings

Treatment	Concentration of phosphate (mol m^{-3})	Ethylene production: Carrot roots (m^3 g^{-1} fr. wt. h^{-1} × 10^{12})	Ethylene production: Pea seedlings (nl g^{-1} fr. wt. h^{-1})
Buffer controls	0.01	—	1.4 (0.3)*
	1·0	0.07 (0.02)	1.1 (0.2)
	100.0	0.07 (0.01)	0.5 (0.1)
Buffer + ACC (1 mol m^{-3})	0.01	—	44.0 (3.5)
	1.0	0.41 (0.03)	40.2 (5.5)
	10	0·40 (0.02)	—
	50	0.29 (0.02)	—
	100	0.20 (0.05)	16.6 (4.3)

* Numbers in parentheses indicate standard error. Details are given in the text.

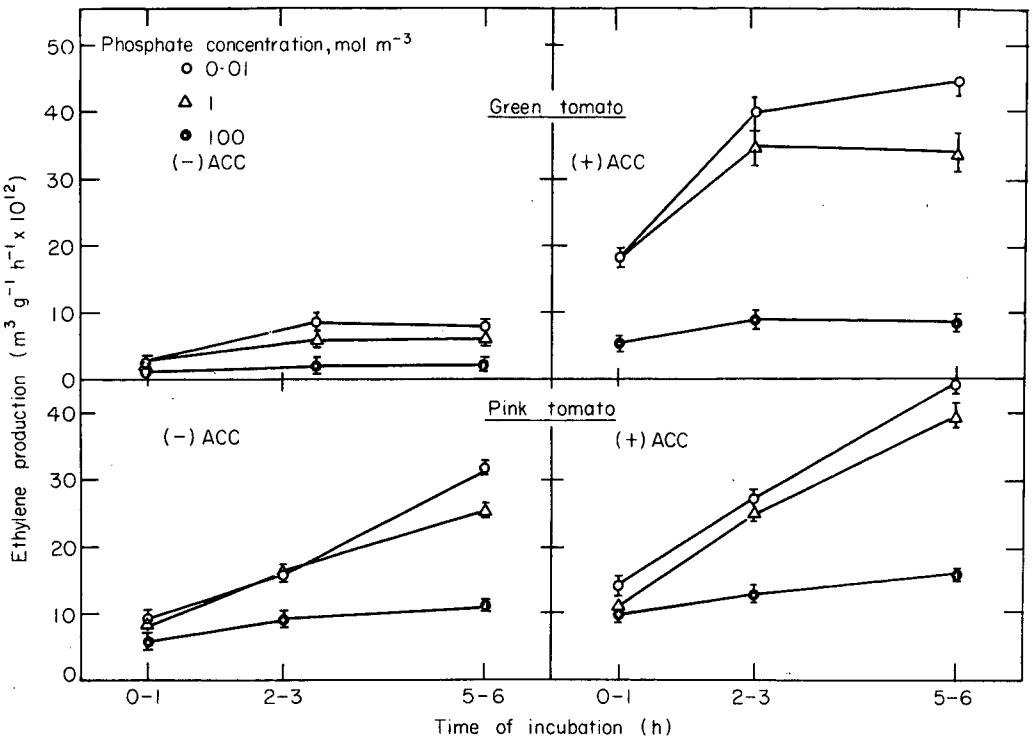

Figure 1. Inhibition by phosphate of ACC-stimulated and endogenous ethylene production by slices of green and pink tomato fruits. ACC concentration was 0.1 mol m^{-3}.

concentrations inhibited by 25–60% the response of the tissue segments to ACC (Table 1). Similarly, ACC-stimulated ethylene production by slices of green or pink tomato fruit also was inhibited by 100 mol m^{-3} phosphate (Fig. 1). Previously we (Chalutz *et al.*, 1980) had shown that this effect of phosphate is not due to an increase in the isotonicity of the medium. Furthermore, it was also shown that the rate of ethylene production decreased as the intracellular concentration of phosphate increased from about 6.5 mol m^{-3} to 10 mol m^{-3} and higher. In these studies, when the phosphate content of the incubating medium was varied from 1 to 100 mol m^{-3}, the internal phosphate content of the tomato fruit slices increased only by three times, from 6 to 18 mol m^{-3}, in 6 h of incubation.

ACC concentrations of up to 10 mol m^{-3} did not overcome the inhibition due to phosphate (Fig. 2), thereby ruling out the possibility of a direct competition between phosphate and ACC for the ACC binding site. The possibility that *in vivo* phosphate may inhibit the conversion of ACC to ethylene was tested by incubating segments of carrot roots and slices of pink tomato fruit with (^{14}C) ACC in the presence of 0.01 or 100 mol m^{-3} phosphate. Both tissues formed labelled ethylene from (^{14}C) ACC, and the rate of conversion was inhibited in the presence of 100 mol m^{-3} phosphate (Fig. 3). These data indicated that phosphate inhibits the conversion of ACC to ethylene. It did not, however, exclude the possibility of additional sites of control where phosphate may act. Therefore, we carried out *in vitro* experiments with cell free extracts of tomato fruit and etiolated pea seedlings which, respectively, convert SAM to ACC (Boller *et al.*, 1979) and ACC to ethylene (Konze & Kende, 1979).

The specific activity of enzymatic conversion of SAM to ACC by tomato cell-free preparation was higher when cell-extract was made in phosphate buffer than when K-HEPES buffer was used and the differences became 4-fold after gel-filtration of the extract using phosphate buffer (Table 2). This suggested that the enzyme binds phosphate and becomes more active. However, the enzymatic conversion of SAM to ACC

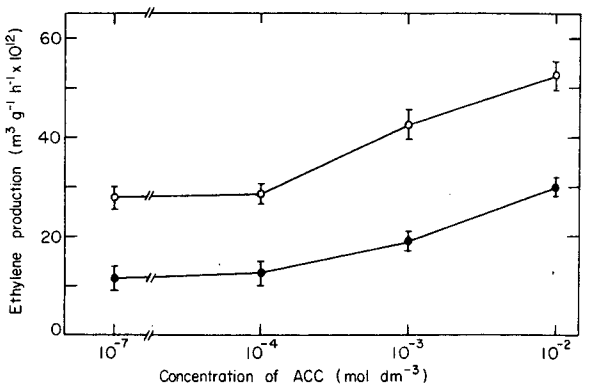

Figure 2. Effect of increasing concentrations of ACC on the inhibition by phosphate of ethylene production by pink tomato fruit slices. Phosphate: (o), 0.01 mol m^{-3}; (•), 100 mol m^{-3}.

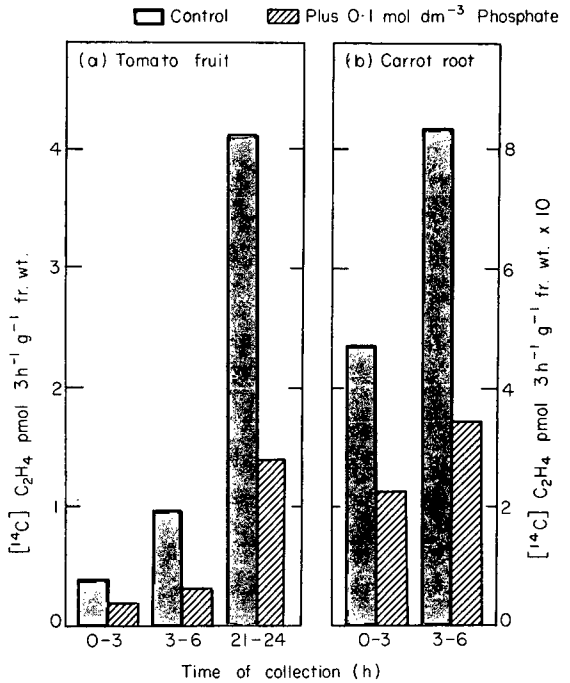

Figure 3. Conversion of labelled ACC to ethylene by pink tomato fruit slices (a) and carrot root segments (b) in the presence of 0.01 mol m^{-3} (□) or 100 mol m^{-3} (▨) phosphate. Tissue slices (2.5 g) were incubated with 0.4 mol dm^{-3} mannitol-phosphate buffer, pH 7.2, and 90 pmol of (^{14}C) ACC in a final volume of 3 cm^3. During the times indicated, flasks were sealed and the labelled ethylene formed was collected and determined.

was not significantly reduced when determined in the presence and absence of 100 mol m^{-3} phosphate using the K-HEPES extract (data not shown). Thus, phosphate was a better extractant (and perhaps a stabilizer) of the enzyme catalyzing the hydrolysis of SAM than was K-HEPES. Furthermore, gel-filtration using K-HEPES buffer seems to inactivate the enzyme compared with dialysis. This observation has not been explored further.

In contrast to these results, the conversion of ACC to ethylene by cell-free extract of pea seedlings prepared in phosphate buffer was only 40% of that obtained with cell-free extract prepared in Tris-HCl buffer. Also the conversion of ACC to ethylene by the extract of pea segments prepared in Tris-HCl buffer was inhibited by added phosphate with a concentration dependency (Fig. 4). These *in vitro* data complement those obtained *in vivo* (Fig. 3) and support the suggestion that phosphate mainly inhibits the conversion of ACC to ethylene. We consider it significant that the phosphate effect on the *in vivo* formation of ethylene from ACC was mimicked *in vitro* casting lesser doubts than put forward initially (Konze & Kende, 1979) on the fidelity of the *in vitro* system.

Further confirmation of the conclusion drawn from the above data—that phosphate regulates the step of ethylene formation from ACC—has been obtained by Even-Chen, Goren & Mattoo (unpublished results) from *in vivo* substrate feeding experiments. In their experiments, Shamouti orange peel discs incubated in air for 20 h with 3,4-(^{14}C)methionine accumulated 474 and 714 pmol (^{14}C) ACC g^{-1} fr. wt. tissue in the absence and presence of 100 mol m^{-3} phosphate, respectively. Thus, about 50% more ACC accumulated in the tissue in the presence of 100 mol m^{-3} phosphate as compared with controls incubated in the absence of phosphate. From all this data we conclude that one major site of phosphate inhibition of ethylene biosynthesis in higher plants is the step of conversion of ACC to ethylene. The mechanism of this inhibition remains to be elucidated.

ACC breaks down to ethylene non-enzymatically in chemical model systems that may utilize H_2O_2 (Boller *et al.*, 1979) or perchlorite (Lizada & Yang, 1979). Therefore, caution has to be exercised when reporting a biological conversion of ACC to ethylene. In our studies we found that phosphate stimulated the chemical breakdown of ACC to ethylene in a model system (Table 3) but inhibited the enzymatic *in vitro* and *in vivo* conversions of ACC to ethylene (Figs 3 & 4). We have previously (Chalutz *et al.*, 1980) shown that the methionine-Cu^{2+}-ascorbate model system for ethylene production was also stimulated by phosphate. Therefore, it seems that the mechanism of phosphate effect observed *in vivo* and in cell-extracts may be different from that in the chemical systems.

The significance of the data presented here is in its

Table 2. Conversion of SAM to ACC by cell-free systems prepared from tomato fruit. Cell extracts were prepared from pink tomato pericarp tissue in either 0.1 mol dm^{-3} phosphate or 0.1 mol dm^{-3} K-HEPES buffer, both at pH 8 as described in the text. 5 cm^3 of the enzyme solution was either dialysed against 500 volumes of each solution or gel-filtered as indicated.

Extraction buffer	Treatment of extract	ACC produced (μmol/mg protein)
Phosphate	Dialysis against 2 mol m^{-3} phosphate	1030
K-HEPES	Dialysis against 2 mol m^{-3} K-HEPES	858
Phosphate	Sephadex G-50 filtration with 2 mol m^{-3} phosphate	624
K-HEPES	Sephadex G-50 filtration with 2 mol m^{-3} K-HEPES	154

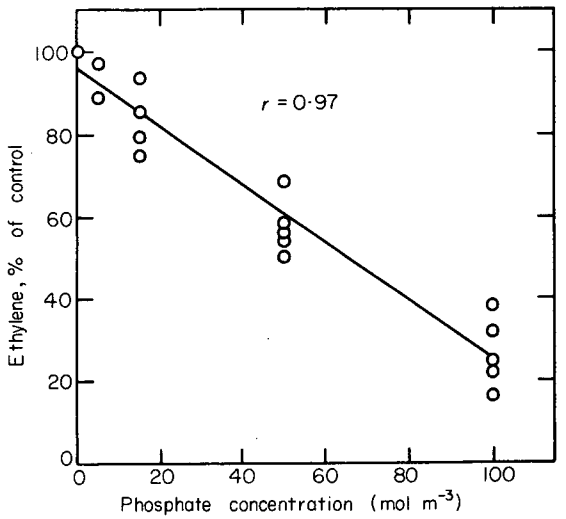

Figure 4. Concentration-dependent inhibition by phosphate of the conversion of ACC to ethylene by Tris-HCl extracts of pea sub-hook segments. The absolute value of ethylene produced in controls, taken as 100%, ranged between 5.0 and 10.3 $m^3 \times 10^{-12}$ C_2H_4 h^{-1} mg^{-1} protein. The line was fitted by a linear regression analysis. *r* represents the correlation coefficient.

Table 3. Stimulation by phosphate of ethylene production in a model system. Each reaction tube contained 1 mmol m^{-3} ACC, 0.1 mol m^{-3} mercuric acetate and 5 cm^3 of indicated concentration of phosphate at pH 7.6, in 8 cm^3 vials. The vials were sealed and 0.1 cm^3 of 1% (w/w) sodium hypochlorite was added with a syringe. After 30 min the amount of ethylene evolved was determined.

Phosphate concentration (mol m^{-3})	Ethylene production (m^3 $h^{-1} \times 10^{12}$) (nl h^{-1})
0.1	71
0.5	63
1	65
5	119
10	166
50	360
100	321
500	315

possible relationship to physiological processes of development, ripening and senescence. Inorganic phosphate content of tomato fruit has been shown to decrease throughout fruit growth and development (Stevens & Paulson, 1973), particularly when the mature fruit starts to produce ethylene at high rates. Incubation of ethylene-producing tomato fruit slices in high phosphate medium increased their internal concentration of phosphate to levels (Chalutz *et al.*, 1980) which are present in young developing fruit (Stevens & Paulson, 1973) that produce little or no ethylene. Therefore, we consider our findings of phosphate effect *in vivo* on ethylene production from ACC (Table 1, Figs 1 & 3) to be of physiological significance, although the *in vitro* system required higher phosphate concentrations for maximal inhibition of ethylene production (Fig. 4). In addition, high endogenous levels of phosphate in tomato plants have been shown to decrease leaf senescence, fruit set, development and rate of ripening of the fruit (Besford, 1979). Also, high levels of phosphate in apple fruit delay the ripening climacteric respiration (personal communication from D. Minnis & L. Brohier, Knoxfield, Australia). It is likely that some or all of these effects may be due to inhibition of ethylene production by phosphate, the phenomenon described in this paper.

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INHIBITION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE BY PHENOTHIAZINES*

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SUMMARY

Trifluoperazine (TFP), chlorpromazine (CPZ) and CPZ analogs, the phenothiazine drugs which are antagonists of calmodulin, inhibit tomato fruit 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity. The I_{50} dose ranged between 20 μM and 58 μM for various analogs. Inhibition was not influenced by light or by varying the calcium concentration. CPZ caused a hyperbolic uncompetitive type of inhibition. The enzyme showed a high affinity for long chain alkyl and ω-aminoalkyl agarose hydrophobic columns. However, the enzyme was not retained on QAE Sephadex or calmodulin-Sepharose columns which, under standard conditions, are known to bind calmodulin. Thus, although the phenothiazines inhibit ACC synthase, the mechanism probably does not involve calmodulin; rather, the inhibition may occur through fortuitous hydrophobic interactions of inhibitor and enzyme.

INTRODUCTION

In higher plants, calcium is known to delay senescence [1] perhaps by inhibiting the biosynthesis of the senescence-inducing hormone, ethylene

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✿ Deceased, January 18, 1982.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CPZ, chlorpromazine; CPZS, chlorpromazine sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether)*N*,*N*'-tetraacetic acid; EPPS, *N*-2-hydroxyethylpiperazine propane sulfonic acid; SAM, *S*-adenosylmethionine; MES, 2(*N*-morpholino)ethanesulfonic acid; TFP, trifluoperazine.

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[2—4], while lowering the microviscosity of microsomal membranes [3]. The sites in the ethylene biosynthetic pathway from methionine at which calcium exerts its influence are the conversion of *S*-adenosylmethionine (SAM) to ACC [5; Lieberman and Wang, unpublished] and the conversion of ACC to ethylene [4,6], the latter being attributed to a membrane stabilization effect of the divalent cation [3,4]. Labeling studies in vivo have shown (Adams, Wang and Lieberman, unpublished) that TFP, an antagonist of calmodulin, markedly inhibits the conversion of SAM to ACC without affecting the formation of ethylene from ACC suggesting that the former reaction may be under the control of calmodulin. In this paper we show that ACC synthase, the enzyme [7,8] catalyzing the conversion of SAM to ACC, is strongly inhibited by phenothiazine derivatives but calmodulin does not appear to be involved.

MATERIALS AND METHODS

Discs of pericarp tissue were cut from greenhouse-grown tomatoes (*Lycopersicon esculentum* Mill., var. Pik red) in the 'pink' or 'red' stage and incubated overnight (16—18 h) at 30°C in a solution (1 : 1, w/v) containing 0.6 M sorbitol and 0.01 M 2(*N*-morpholino)ethane sulfonic acid(MES)-KOH buffer (pH 6.0). The tissue was then washed with distilled water and homogenized with an equal weight of buffer A (100 mM *N*-hydroxyethylpiperazine propane sulfonic acid(EPPS)-KOH (pH 8.5), 2 mM dithiothreitol (DTT) and 12.5 μM pyridoxal phosphate). The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 14 000 $\times g$ for 20 min. Solid $MgCl_2$ was added to the supernatant fluid at 2 mg/ml (final conc.) and the solution was stirred for 30 min at 5°C. After another centrifugation at 14 000 $\times g$ for 20 min, the supernatant fluid was filtered through a column of Sephadex G-25, previously equilibrated with buffer B (2 mM EPPS-KOH (pH 8.5), 0.4 mM DTT and 2.5 μM pyridoxal phosphate). Fractions containing protein were pooled and used for the assay of the ACC synthase. In some experiments this enzyme preparation was chromatographed on various columns.

Columns of QAE Sephadex G-25 and calmodulin-Sepharose, prepared by previously published methods [9] were kind gifts from Dr. V.C. Manganiello and Ms. Ferol Lieberman (Laboratory of Cellular Metabolism, NHLBI, N.I.H., Bethesda, MD 20205). They were equilibrated with buffer C (25 mM EPPS-KOH (pH 7.5), 1 mM ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 5 mM $MgCl_2$ and 250 mM NaCl) and buffer D (25 mM EPPS-KOH (pH 7.5), 2 mM $CaCl_2$, 1 mM EGTA, 5 mM $MgCl_2$ and 250 mM NaCl), respectively. The agarose-C_n and agarose-C-NH_2 series from Hydrophobic Chromatography Kits (Miles Labs., IN) were equilibrated with buffer B.

Unless otherwise stated, a standard reaction mixture containing 100 mM EPPS-KOH (pH 8.5), 50 μM SAM, 0.25 ml of buffer B and an appropriate

aliquot of enzyme solution in a total volume of 0.5 ml, was incubated with and without test compounds at 30°C for 30 min or 60 min. SAM was omitted from blank samples. The reaction was stopped by adding $HgCl_2$ to the reaction tubes to a final concentration of 1 mM and transferring them to an ice bath. The ACC formed was assayed according to Lizada and Yang [8,10]. Protein concentration was determined by the method of Bradford [11].

TFP and the 1-, 3- and 4-chloro analogs of CPZ were generous gifts from Ms. C. Worthen (Smith, Kline & French Labs., Philadelphia, PA 19101). CPZ and CPZ sulfoxide (CPZS) were gifts from Dr. A. Manian (N.I.M.H., Rockville, MD).

RESULTS

Figure 1 shows a concentration-dependent inhibition of tomato ACC synthase by the various compounds tested: TFP, 2-chloro-10-dimethylaminopropyl phenothiazine hydrochloride (CPZ), CPZS and 1-, 3- and 4-chloro-analogs of CPZ. The concentrations of inhibitors needed for a 50% reduction in the enzyme activity were 20, 36, 38, 40, 50 and 58 μM for CPZ, 3-CPZ, CPZS, 4-CPZ, 1-CPZ and TFP, respectively. To determine the nature of the inhibition, the effect of varying the substrate (SAM) and inhibitor (CPZ) concentrations was studied. The Lineweaver-Burke plot (Fig. 2) of the data suggest an uncompetitive type of inhibition. A replot of $1/v$-axis intercept against CPZ concentration was not linear (Fig. 2, inset) indicating a hyperbolic uncompetitive type of inhibition [12], suggesting that SAM binding to the enzyme favors inhibition by CPZ.

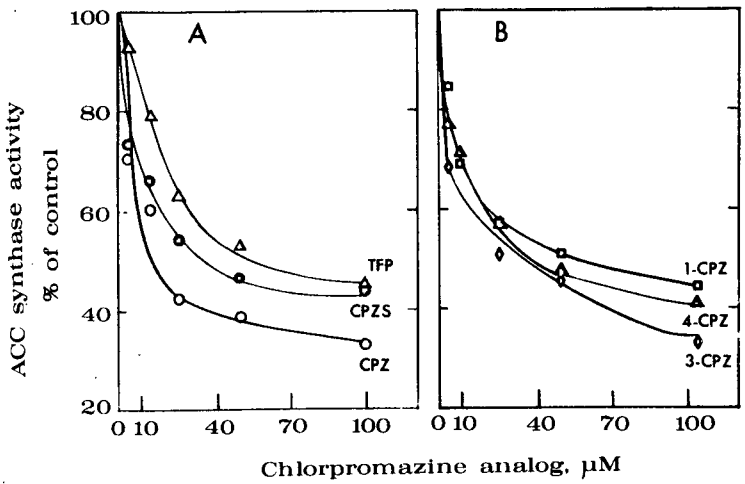

Fig. 1. Inhibition of tomato ACC synthase by various phenothiazine drugs. The enzyme activity of the controls taken as 100% was 25.8 nmol ACC formed h^{-1} mg^{-1} protein (A) and 10 nmol h^{-1} mg^{-1} (B). In B the enzyme had been frozen after the gel filtration step and thawed once.

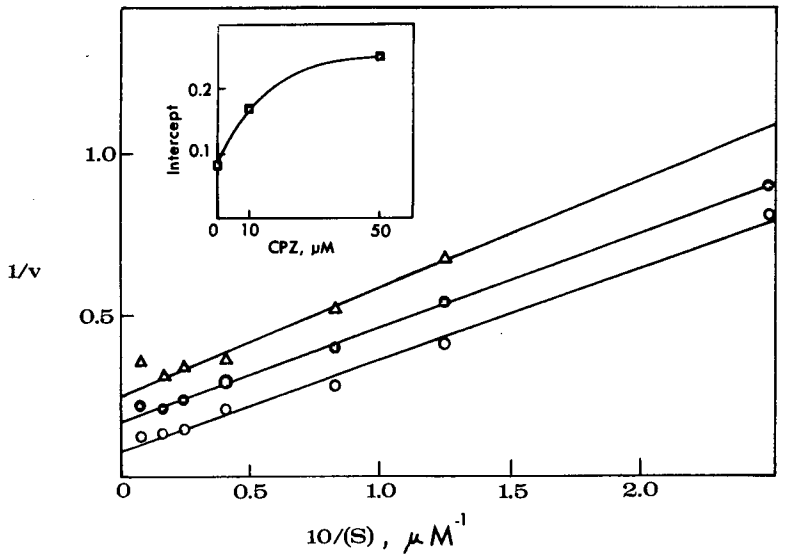

Fig. 2. Lineweaver-Burke plot of ACC synthase in the absence (○) and presence of 10 (●) and 50 (△) μM CPZ. The constants obtained from linear regression analysis of the data points were used to draw the lines. *Inset:* A replot of 1/*v*-axis intercept against CPZ concentration.

The inhibition of ACC synthase by CPZ was not affected by light (Table I) or by increasing the concentration of calcium in the reaction mixture from 10 μM to 100 μM (Fig. 3). A very slight inhibition of the enzyme activity was apparent at the higher concentrations of calcium (Fig. 3).

The inability of calcium to promote ACC synthase activity and the nature of enzyme inhibition by CPZ prompted us to ascertain if inhibition was mediated through calmodulin action. Chromatography on QAE Sephadex has been used to deplete the enzyme extracts of calmodulin and calmodulin linked to Sepharose retains several proteins that participate in calcium-regulated cellular processes [9; also references therein]. When partially

TABLE I

EFFECT OF 2-CHLORO ANALOG OF CPZ AND CPZS ON THE ACTIVITY OF ACC SYNTHASE ASSAYED IN THE DARK OR LIGHT

Incubation condition	ACC synthase activity (nmol mg^{-1} h^{-1})		
	Control	Plus 2-CPZ[a] (50 μM)	Plus CPZS (50 μM)
Dark (28°C)	7.99	3.34	4.41
Light (2000 Lux, 28°C)	8.63	3.86	4.05

[a] 2-CPZ, 2-chloro analog of chlorpromazine.

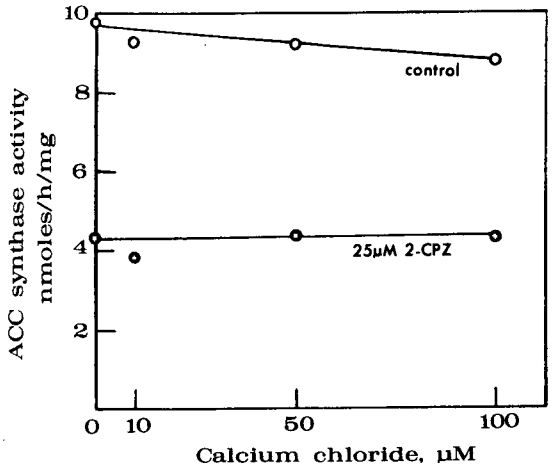

Fig. 3. Effect of calcium chloride concentration on the inhibition of ACC synthase by the 2-chloro analog of CPZ. The enzyme source was the same as in Fig. 1B.

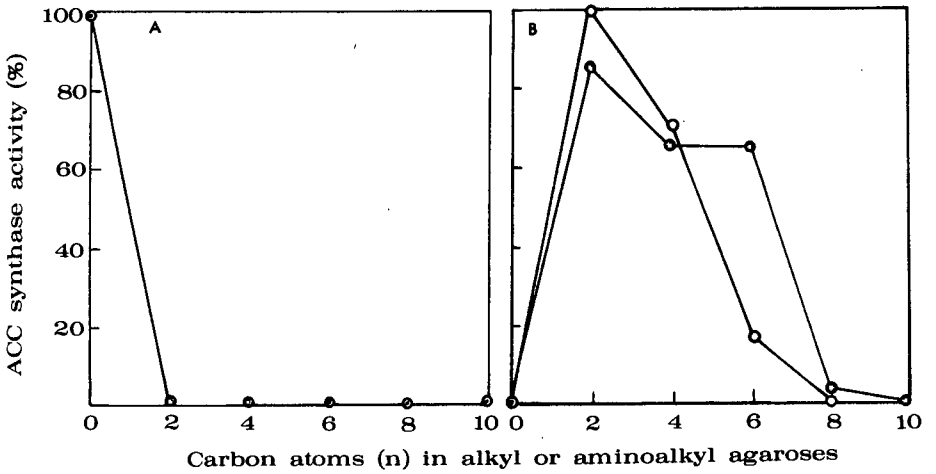

Fig. 4. Binding (A) and elution (B) of ACC synthase activity to and from agarose-C_n series (○) and agarose-C-NH_2 series (●) columns. The percentage activity in the effluent is plotted against the number of carbon atoms (n) in the alkyl chains of the columns. The enzyme activity unbound to agarose-C_0 or agarose-NH_2 was taken as 100%. In A, 0.25 ml of enzyme solution after gel filtration was applied to each 1-ml column and washed with 2 ml of buffer B. Each column was eluted with 2.5 ml of 0.5 M NaCl in buffer B. Activity in B is expressed as a percentage of that which passed through the underivatized agarose column. Slightly different elution patterns were obtained for the alkyl and aminoalkyl agarose series.

purified ACC synthase made 0.25 M with respect to NaCl was applied to a column of QAE Sephadex equilibrated with buffer C, the pass through (unbound) eluate and wash contained 85—90% of the applied enzyme activity. A part of the unbound fraction having a specific activity of 7.76 nmol mg^{-1} h^{-1} was made 2 mM in $CaCl_2$ and was then applied to a column of calmodulin-Sepharose equilibrated with buffer D. About 75—80% of the applied activity was recovered in the pass through eluate plus wash fraction. The specific activity of the enzyme in this fraction was 7.86 nmol mg^{-1} h^{-1} Therefore under standard conditions [9] when calmodulin should bind to QAE Sephadex and calmodulin-Sepharose columns, ACC synthase is not bound and retains complete enzyme activity that is still sensitive to CPZ (data not shown). These data suggest that the observed interaction of CPZ and its analogs with ACC synthase is not mediated by calmodulin but by another mechanism.

ACC synthase seems to be a hydrophobic protein having high affinity for both long- and short-chain alkyl and aminoalkyl agarose hydrophobic columns [13]. This is illustrated in Fig. 4, where the percentage activity in the effluent is plotted versus the number of carbon atoms (n) in the alkyl chains of the columns. The columns with $n \geqslant 8$ chains retained the enzyme very strongly, less than 7% activity being eluted with 0.5 M NaCl (Fig. 4B).

DISCUSSION

We have demonstrated that ACC synthase is strongly inhibited by micromolar concentrations of TFP, CPZ and CPZ analogs. The inhibition was not influenced by light or calcium and was of the partial uncompetitive type, the inhibitor apparently decreasing the V_{max} while the affinity for the substrate increased.

Our results indicated that all the analogs were inhibitory and therefore inhibition was not related to their efficacy as antipsychotic agents. For example, the phenothiazine drug with relatively weak antipsychotic action, CPZS, was just as potent an ACC synthase inhibitor as the others. Although the effectiveness of these tranquilizer drugs has been related to the inhibition of calmodulin-mediated stimulation of enzyme activity [14—17], this view has been challenged [18,19]. Our results with ACC synthase and the lack of calcium stimulation of the enzyme activity lend credence to the latter reports and suggest additional alternative mechanism(s) for inhibition of cellular processes by phenothiazines. Thus, the mechanism of their inhibitory action on calmodulin-dependent enzymes may be different from that for ACC synthase reported here. Nevertheless, it is possible that the domains on calmodulin and ACC synthase that bind the antipsychotic drugs are similar. It has been suggested that the potency of these drugs as inhibitors of calmodulin action may be related to non-stereospecific hydrophobic interactions with calmodulin [18,19]. In this regard, the tighter binding of ACC synthase to hydrophobic ω-aminoalkyl and alkyl agarose columns as the number of

carbon atoms in the alkyl chains increase is of interest. Such a property of the enzyme may facilitate its interaction with the phenothiazine drugs. Finally, our results agree with previous reports [18—20] suggesting that antagonism of calcium-dependent processes by phenothiazines is not specific and certainly not a sufficient criterion for implicating involvement of calmodulin; interpretations of such results should be made with caution.

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MEMBRANE ASSOCIATION AND SOME CHARACTERISTICS OF THE ETHYLENE FORMING ENZYME FROM ETIOLATED PEA SEEDLINGS[1]

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SUMMARY: When freshly prepared homogenates of etiolated pea (Pisum sativum L. cv Calvedon) subhook segments were fractionated by high speed centrifugation, the enzyme catalyzing the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene was found associated with the particulate fraction. However, on aging the homogenates at 5°C prior to fractionation, 50 to 75% of the enzyme activity partitioned into the soluble fraction; this solubilization led to a highly activated enzyme form. Both the particulate and soluble enzyme exhibited non-linear substrate saturation kinetics and were inhibited to similar extents by ascorbic acid, EDTA, $CoCl_2$ and limiting oxygen. However, they differed in their response to incubation with n-propylgallate, dithiothreitol, $CaCl_2$ and 100% oxygen. Calcium stimulated only the particulate form and increased both the 'low K_m' for ACC from 2.99 to 5.58 mM and apparent Vmax from 88 to 285 nl/mg protein/h.

Ethylene is a plant hormone regulating many aspects of growth, development and senescence in higher plants (see Ref. 1). The identification (2,3) of 1-aminocyclopropane-1-carboxylic acid (ACC) as an immediate precursor of ethylene, and in vitro demonstration (4,5) of its formation from S-adenosyl-methionine (SAM) have established the following metabolic sequence for the biosynthesis of ethylene: methionine → SAM → ACC → ethylene. However, the enzyme system that catalyzes the conversion of ACC to ethylene (ethylene forming enzyme) under physiological conditions has not unequivocally been isolated and characterized, although homogenates of pea shoots do form ethylene from ACC (6).

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In vivo, conversion of ACC to ethylene seems to be, in most cases, constitutive (7-9) and membrane-associated (10,11). We report here that the enzyme activity catalyzing the formation of ethylene from ACC may be associated with a microsomal fraction from etiolated pea subhook segments and is released (solubilized) on storage of the homogenates in the cold. Some characteristics of the membrane-associated and soluble form of the enzyme were determined. We also show that ACC saturation kinetics for both enzyme forms are non-linear and appear distinctively biphasic.

MATERIALS AND METHODS

Pea (Pisum sativum L. cv. Calvedon) seeds were imbibed in the dark for 24 h in running water. After sowing them in previously autoclaved vermiculite wetted by sterile distilled water, they were grown in the dark at 24°C for 6-7 days. The seedlings were harvested when they grew to a height of 8-11 cm.

Subhook segments, 1 cm long, were cut from the etiolated seedlings and homogenized at 2°C by grinding the material with a mortar and pestle using ice-cold 60 mM Tris-HCl buffer, pH 7.9 (0.4ml/g of tissue) (6). The homogenate was centrifuged at 11,000 x g for 15 min at 2°C. The residue was discarded. The supernatant was either taken immediately or after storage at 5°C for different periods of time, for further centrifugation at 80,000 x g for 1 h at 5°C. Both the residue (particulate fraction) and supernatant (soluble fraction) were saved. The particulate fraction was suspended carefully in 60 mM Tris-HCl buffer, pH 7.9, and the volume was adjusted to that of the soluble fraction. Protein was determined by the method of Bradford (12).

Enzymatic conversion of ACC to ethylene was carried out in 7-ml vials in a reaction mixture containing, unless otherwise stated, 100 µl of 300 mM Tris-HCl buffer, pH 7.9, 100 µl of the enzyme extract and 10 µl of ACC of a known concentration. Each reaction was run in triplicate. Control vials did not contain ACC. The vials were flushed with compressed air and stoppered with serum caps prior to incubation in a water bath at 28°C. After an appropriate incubation time, the atmosphere accumulated in the vials above the solution was sampled (2ml) and assayed for ethylene by gas chromatography (13). Each experiment was repeated at least twice. Additional methods, modifications or details are given in the text or under legends to figures and table.

RESULTS AND DISCUSSION

In preliminary experiments considerable variability was observed in the rate of enzymatic conversion of ACC to ethylene by homogenates prepared from subhook segments of etiolated pea seedlings. Subsequently, this variability in enzymatic activity was found to be related to the time of assay of the enzyme after preparation of the homogenate. Fig. 1 shows the results of an experiment where 11,000 x g supernatant was assayed immediately or after different times in storage on ice. The enzyme activity remained more or less constant

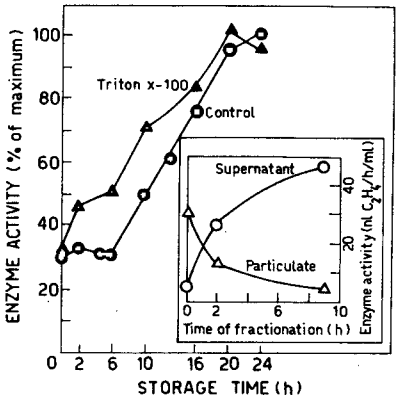

Fig. 1. Ethylene forming activity of a pea seedling homogenate stored for various times at 5°C. Aliquots of 11,000 x g supernatant (see Materials & Methods) were stored with (▲) or without (●) 1% Triton X-100 at 5°C and enzyme activity was determined at times indicated. The absolute value of the fully activated enzyme in both cases was 12.2 nl/mg/h. Inset shows change in distribution of enzyme activity as a function of storage time of the 11,000 x g supernatant incubated with 1% Triton X-100. ○, ○ - 80,000 x g supernatant; ▲, ▲ - 80,000 x g residue.

up to 6 h in storage and thereafter increased considerably (∿ 3 times), reaching a maximum at about 24 h. The lag of about 6 h in the gradual increase of enzyme activity was eliminated by addition of Triton X-100 (1% final) to the homogenate immediately after preparation and prior to storage (Fig. 1).

When a freshly obtained 11,000 x g supernatant was centrifuged at 80,000 x g for 1 h, most of the ethylene forming activity was associated with the particulate fraction (Table 1). The recovery of the enzyme activity in this fraction was ∿ 170%, suggesting the removal by centrifugation of an inhibitor from this fraction. The specific activity of the enzyme in the particulate fraction was about 16 times as much as in the soluble fraction. Similarly, distribution of enzyme activity was determined after the 11,000 x g supernatant was stored at 5°C for 20 h. As shown in Table 1 there was >50% loss in enzyme activity after high speed centrifugation of aged extracts as also observed previously (6) and the remaining activity was equally distributed between the particulate and soluble fractions. However, after aging, the specific activity of the particulate fraction decreased by about 2.5 times, while that of the soluble fraction was enriched by 4.3 times. In other experiments overnight storage resulted in 75% of the activity partitioning into the soluble fraction.

Table 1. Fractionation of the ethylene forming activity by differential centrifugation of homogenates prior to and after storage at 5°C for 20 h.

Fraction	h of storage	Total Activity (nl/h/extract)		Specific Activity (nl/h/mg protein)	
		0	20	0	20
11,000 x g supt.		77.50	226.80	5.86	17.16
80,000 x g supt.		12.43	53.65	1.04	4.48
80,000 x g residue		131.43	53.03	16.77	6.77

Also, overnight storage at 5°C of the 80,000 x g particulate fraction by itself did not show any increase in the enzyme activity; instead, it decreased (data not shown), suggesting that a soluble factor is responsible for releasing the enzyme from the particulate fraction. Thus, it appears that ethylene forming enzyme is associated with a membrane (particulate) fraction. Elimination of the lag period by Triton X-100 during the increase in enzyme activity on storage (see Fig. 1) was correlated with more of the enzyme activity being distributed in the soluble than in the particulate fraction (Fig. 1, inset). At 9 h in storage in buffer, the ratio of soluble(s)/particulate(p) enzyme activity was 1.19 whereas storage in 1% Triton X-100 within the same period resulted in a s/p ratio of 10.26. Thus, increase in the enzyme activity on storage of 11,000 x g supernatant is correlated with solubilization of the ethylene forming enzyme from the membrane fraction, the solubilization concomitantly leading to a highly activated enzyme form (14). Earlier, it was reported (6) that one-tenth of the extractable ethylene forming enzyme activity is associated with a particulate fraction. The discrepancy between the data of the previous report and those presented here may be due to the different pea cultivar used and/or to our use of, specifically, the 1-cm subhook segments and not entire shoots, as was done previously (6,15). The physiological significance of the release and activation following solubilization on storage of the ethylene forming enzyme from membrane is a matter for conjecture. However, its membrane association provides a potential for some control by substances which act on the membrane. Indeed, ethylene synthesis by intact tissues is disrupted by

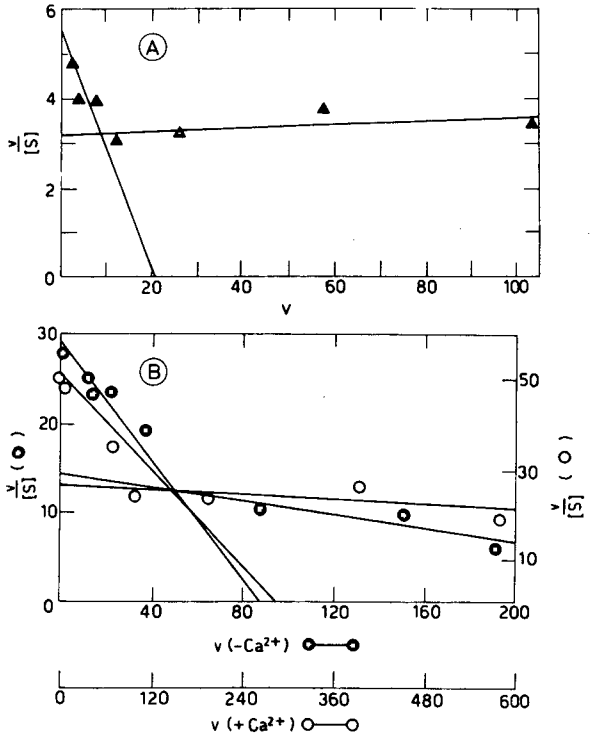

Fig. 2. v vs v/[S] plots of soluble (A) or particulate (B) ethylene forming enzyme. In B, ● minus calcium; O plus 1 mM calcium chloride. The constants obtained from linear regression analysis of the data points, separately for two distinct regions of each curve, were used to draw the lines.

treatments that interfere with membrane function (18-20), one of the metabolic sites being the conversion of ACC to ethylene (10,21).

The log-log substrate versus velocity plots (see 16) for the particulate and soluble enzyme activity showed an obvious inflection in the velocity curve for the particulate enzyme preparation while none was found for the soluble fraction, suggesting either the presence of two enzyme forms with differing affinity constants in the former preparation or the presence of complex kinetics (16). However, both enzyme preparations exhibited anomalous kinetic behaviour (Fig. 2 A,B). The exact determination of K_m values was difficult because of the gradual change in the curves. However, a lower apparent K_m for ACC was estimated to be 2.99 and 3.77 mM for the particulate and soluble enzymes, respectively. These results, in general, contrast with previous studies (6,15) which claimed that the kinetics of ACC saturation

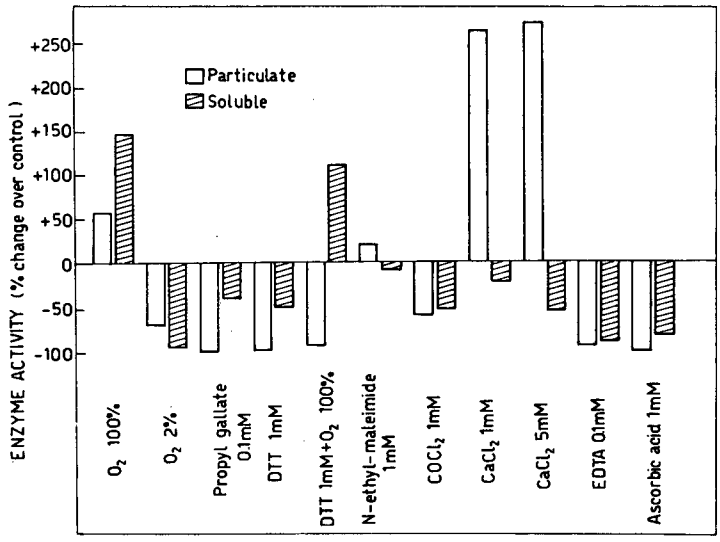

Fig. 3. Response of particulate (▫) or soluble (▨) ethylene forming enzyme to various inhibitory or stimulatory effectors.

followed classical Michaelis-Menten type. It is evident from our data that a wide ACC concentration range must be used to detect the biphasic character of the double reciprocal plots. We varied the ACC concentration up to 600-fold. The Michaelis-Menten kinetics of the enzyme reported earlier was most probably caused by working in a narrow range of ACC concentration as well as by representing the data in a Lineweaver-Burke plot, a procedure which tends to minimize any curvature in the data (16,26). Thus, the biphasic nature of substrate kinetics demonstrated here has not been recognized previously.

Ethylene forming enzyme in both membrane and soluble fractions was inhibited (Fig. 3) by oxygen deprivation (2%O_2), ascorbic acid (1 mM), EDTA (0.1 mM) and $CoCl_2$ (1 mM), known inhibitors of this reaction in vivo (2,10,17). Although the above results more or less show that the enzyme in particulate and soluble fractions share many common properties several important differences between them were also evident. The particulate enzyme was relatively more sensitive to inhibition by the free radical quencher, propylgallate, and the reducing agent, DTT. Moreover, the sensitivity to DTT of the soluble system is virtually eliminated by incubation in 100% O_2, while this was not the case for the membrane-associated enzyme. The ineffectiveness of N-ethylmaleimide

in inhibiting the enzyme suggests that inhibition due to DTT may not involve sulfhydryl groups, but may perhaps be related to involvement of free radicals in the conversion of ACC to ethylene which is supported by the inhibition of the enzyme by free radical quenchers including ascorbic acid (27) and the report of McRae et al (28). Calcium chloride (1 mM) stimulated only the particulate enzyme and increased both the 'low K_m' for ACC from 2.99 to 5.58 mM and apparent Vmax from 88 to 285 nl/mg/h. Assuming that the ethylene forming enzyme in the soluble and particulate fraction is one and the same, this observation suggests that the calcium effect on the membrane system is probably not the result of direct interaction of calcium with the ethylene forming enzyme, but may be a result of interaction of the cation with a more polar group of the membrane phospholipids (22). Further study of the calcium effect is important, particularly when calcium is known, among other effects, to stimulate ethylene production in mungbean (23) and pea (24) seedling tissue.

In addition, catalase (10 µg/ml) inhibited by 60-70% the soluble ethylene forming enzyme and had very little effect on the particulate enzyme fraction (Mattoo & Lieberman, unpublished results). Earlier it was shown (6) that catalase inhibited ethylene formation from ACC in unfractionated 11,000 x g supernatant of pea seedlings.

Finally, it should be mentioned that the major difficulty in studies of the ethylene forming system to date (6,15) is the apparently high concentration of ACC needed to saturate the enzyme. However, this may be an apparent anomaly with the enzyme systems that fulfill most, if not all, characteristics of the in vivo physiological system. Such a system may be the membrane-associated enzyme reported here. Although it showed biphasic substrate saturation kinetics, the 'low' K_m of 2.99 mM is lower by more than an order of magnitude than the 'high', almost infinite, K_m (6). Moreover, since the enzyme system used is not homogenous the presence in close proximity of other competing enzymes utilizing ACC cannot be ruled out. One such reaction could be the enzyme system that catalyzes the conjugation of ACC to malonyl-ACC, a metabolite of ACC recognized very recently (25) in higher plants.

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Variations in Adenylates and Adenylate Energy Charge During Phosphate-mediated Inhibition of Ethylene Biosynthesis in *Penicillium digitatum**)

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Summary

Changes in adenine nucleotides and adenylate energy charge in shake cultures of *Penicillium digitatum* were determined under conditions when ethylene biosynthesis was either activated or inhibited. Activation of ethylene biosynthesis under phosphate-limiting growth conditions was accompanied by a 28 % decrease in the ATP level of the mycelia. Under these growth conditions, changes in the adenylate energy charge were inversely related to the concentration of AMP but did not correlate with changes in the rate of ethylene biosynthesis or in total adenylate nucleotides. Adenine and AMP, when added individually, partially prevented the inhibitory effect of 0.01 mM orthophosphate on ethylene biosynthesis. On addition of orthophosphate, orthophosphate and adenine, or orthophosphate and AMP a rapid decrease within 2 hours in ethylene biosynthesis occurred without a striking increase in the ATP level of the cultures. In non-growing mycelia of *P. digitatum*, there was considerable modulation in the concentration of individual adenine nucleotides but only small changes in the adenylate energy charge were observed. The data indicated that levels of adenylates, glutamate, or the adenylate energy charge do not mediate or limit phosphate inhibition of ethylene biosynthesis. However, it is suggested that an orthophosphate-repressible phosphatase and/or a protein kinase may be involved in this process.

Key words: Penicillium digitatum, adenylates, adenylate energy charge, ethylene production, orthophosphate.

Introduction

Considerable information is now available on the biosynthesis of ethylene in higher plants and microorganisms (Yang, 1974; Lieberman, 1979). The most extensively studied microbial ethylene producer is *Penicillium digitatum* Sacc. Earlier

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we reported (Chalutz et al., 1977, 1978) the presence of two pathways of ethylene biosynthesis in shake cultures of this fungus. One is induced by methionine which is also the ethylene precursor. A second pathway, in which glutamate is the ethylene precursor like that described for surface cultures of this fungus (see Yang, 1974), is operative under phosphate-limiting growth conditions and is inhibited by exogenously added orthophosphate (Chalutz et al., 1978; Mattoo et al., 1979 a). This latter phenomenon occurs also in *P. expansum* (Chalutz et al., 1980) and *P. cyclopium* (Pazout et al., 1982). The microbial formation of another plant hormone, abscisic acid, has also been reported to be affected by phosphate (Griffin and Walton, 1982).

High amounts of orthophosphate are known to inhibit the production of secondary metabolites such as antibiotics and pigments (Weinberg, 1974; Drew and Demain, 1977). Thus, regulation of ethylene biosynthesis in shake cultures of fungi and of other secondary metabolites by orthophosphate may have some common features. This was also suggested by observations of a rapid increase in the intracellular ATP level after phosphate addition to cultures of *Streptomyces griseus* (Drew and Demain, 1977; Martin, 1977) and to shake cultures of *P. digitatum* (Chalutz et al., 1978), which coincided with the suppression of candicidin and ethylene production in the respective cultures. These observations offer interesting regulatory consequences in light of the recognition of a unique role of adenine nucleotides in stoichiometric coupling and energy transduction between metabolic pathways (Holzer, 1970; Chapman and Atkinson, 1977).

The manner in which energy metabolism regulates growth has been extensively studied in recent years (Neijssel and Tempest, 1976; Chapman and Atkinson, 1977; Thomas and Dawson, 1977). However, few studies have been carried out with respect to secondary metabolites. The effects of depletion of available phosphate on the concentration of adenine nucleotides and changes in adenylate energy charge in relation to secondary metabolism remain unclear (cf. Demain, 1972; Thomas and Dawson, 1977; Chapman and Atkinson, 1977). An important area which remains unknown is the relationship between the concentrations of adenylates and adenylate energy charge and the biosynthesis of secondary metabolites. The present study is a preliminary attempt towards understanding this relationship and reports on the variations that occur in adenylates and adenylate energy charge during conditions when ethylene biosynthesis in *P. digitatum* is either activated or inhibited. Also, the influence of exogenously supplemented adenine and AMP on ethylene biosynthesis and adenylates in the absence and presence of orthophosphate is reported.

Materials and Methods

P. digitatum (ATCC ≠ 10030) was grown as a shake culture (Chalutz et al., 1977, 1978) on modified Pratt's liquid medium (Spalding and Lieberman, 1965) containing various concentrations of phosphate without yeast extract. Other inoculation and incubation procedures, culture weight determination and ethylene analyses were performed as previously described (Spalding and Lieberman, 1965; Chalutz et al., 1977). Protein content of the fungal mycelium was

determined in 10 percent trichloroacetic acid precipitates using the fluorescamine method (Bohlen et al., 1973).

ATP, ADP and AMP contents of the mycelium were determined on trichloroacetic acid-soluble fractions after ether extraction of the trichloroacetic acid phase as previously described (Anderson, 1977; Chalutz et al., 1978).

Further details of techniques are described in figure legends and the table. All treatments were run with two or three replicates and each experiment was repeated at least once. Data presented are from representative experiments

Results and Discussion

Ethylene Biosynthesis, Adenylates and Adenylate Energy Charge in P. digitatum during Growth on Medium with Different Phosphate Concentrations

The effect of orthophosphate (Pi) concentration on rates of ethylene production, adenylates and adenylate energy charge is shown in Fig. 1. As was shown earlier

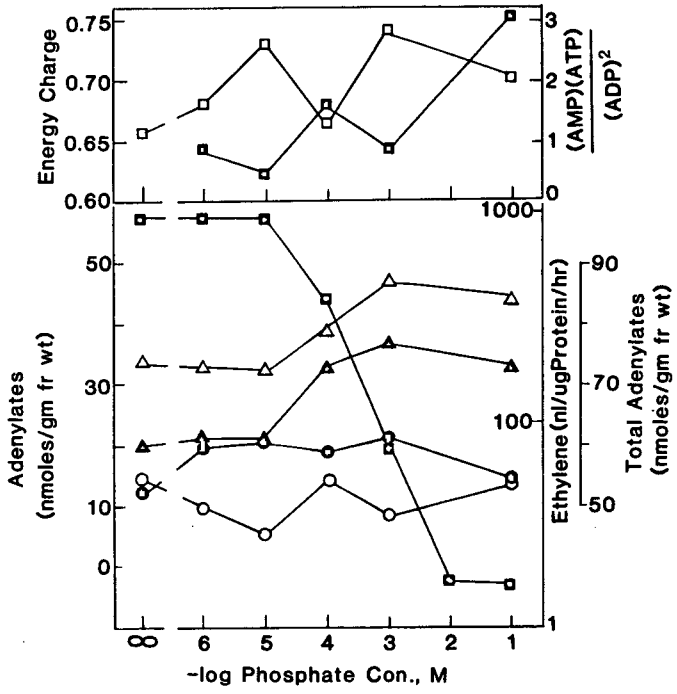

Fig. 1: Effect of phosphate concentration of the medium on the intracellular levels of AMP (○), ADP (●), ATP (△), total adenylates (▲) and on adenylate energy charge (□), adenylate kinase reaction ratio, (AMP) (ATP)/(ADP)^2 (■) and ethylene production (■) of shake cultures of *P. digitatum* after 3 days of growth. Cultures were incubated in 50-ml Erlenmeyer flasks containing 10 ml of modified Pratt's medium without yeast extract but with the indicated concentration of phosphate.

(Chalutz et al., 1978), the rate of ethylene biosynthesis at 0.01 mM Pi was ~ 55 times higher than that at 10 or 100 mM Pi. Activation of ethylene biosynthesis at low Pi concentrations was accompanied by a decrease in ATP level of the mycelia from 47 to 34 nmol · g^{-1} fresh wt. of mycelium. The changes in adenylate energy charge (Atkinson, 1969) value,

$$\frac{[ATP]+1/2[ADP]}{[ATP]+[ADP]+[AMP]}$$

did not coincide with changes in the rate of ethylene biosynthesis but appeared to be inversely related to the concentration of AMP. ADP level was 20 nmol · g^{-1} fresh wt. between 1 to 0.001 mM Pi (Fig. 1). These results obtained with cultures growing on different Pi levels show that low levels of AMP favor conversion of ADP to ATP, increasing the ATP/ADP ratio, as dictated by the above equation. These data suggest that metabolic regulation occurs under the described conditions in growing mycelia of *P. digitatum*, and that a rapid decrease in energy charge is not accompanied by a corresponding decrease in the sum of the total adenylate nucleotides.

Ethylene Biosynthesis and ATP Concentration in the Mycelia of Non-growing P. digitatum in the Presence of Orthophosphate, Adenine and AMP

Our earlier work showed (Chalutz et al., 1978; Mattoo et al., 1979 a) that the addition of 100 mM Pi to high ethylene producing *P. digitatum* cultures strongly inhibited ethylene production. This inhibition was accompanied by a concomitant rise in ATP content of the mycelium suggesting that ATP could be an intracellular effector of the Pi-mediated control of ethylene biosynthesis. A similar explanation was offered for the phosphate control of candicidin biosynthesis (see Martin, 1977). To test this hypothesis, the effect of addition of adenine and AMP, the easily transportable ATP precursors, in the absence and presence of Pi, was examined on ethylene biosynthesis and on intracellular ATP level. Results of short term kinetic experiments (Fig. 2 A) showed that adenine and AMP when added individually caused only a 10 to 20 percent inhibition of ethylene biosynthesis. However, when added in the presence of Pi, they partially prevented the inhibitory effect of 0.01 mM Pi.

Changes in ATP concentration of the mycelium under the above conditions are illustrated in Fig. 2 B. It was observed that the rapid decrease in ethylene biosynthesis after 2 hours occurred without a significant increase in the ATP level of cultures to which either exogenous Pi, Pi and adenine, or Pi and AMP were added. In most cases, ATP levels increased only after prolonged incubation, i. e. after 4 hours (Fig. 2 B). In addition, 0.01 mM Pi resulted in only a 13 percent increase in ATP level when ethylene biosynthesis decreased by 65 percent after 2 hours. Also, in the presence of adenine and Pi, an increase in ATP level of 24 percent was accompanied by only 27 percent inhibition of ethylene biosynthesis (Fig. 2 B).

These data suggest that ATP may not mediate the inhibition by Pi of ethylene biosynthesis. It may be pointed out that the marked increase in ATP level reported

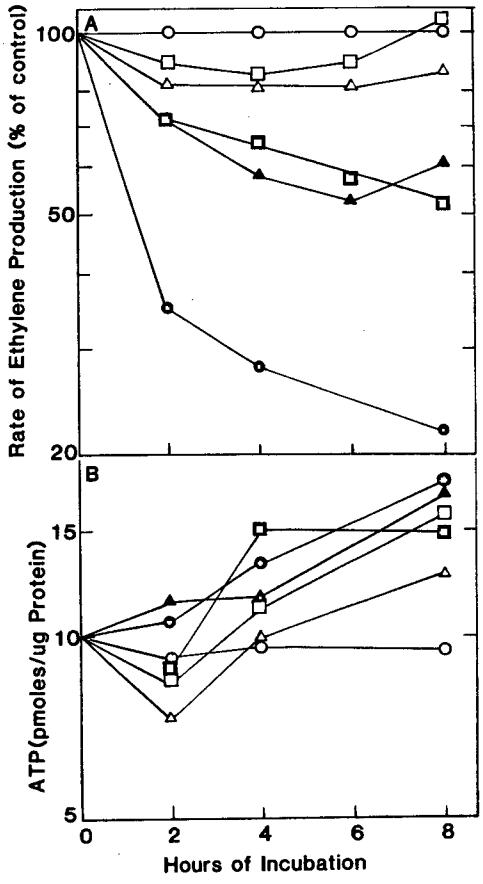

Fig. 2: Effect of addition of adenine (△) and AMP (□) in the absence (open symbols) and presence (closed symbols) of phosphate (0.01 mM) (●) to the medium of high-ethylene producing, shake cultures of *P. digitatum* on the subsequent rate of ethylene production (A) and ATP content (B). Procedure was similar to that described in the legends of Fig. 1 and Table 1. The concentration of each tested compound was 0.01 mM. Ethylene production in water controls (○) and experimental flasks was determined after closing the flasks for several, 1-hr periods following the addition of each compund. The ATP content was determined in separate flasks. The absolute value for ethylene production in water controls at time 0, before addition of water, was 728 nl · μg^{-1} protein hr^{-1}.

earlier by us (Chalutz et al., 1978) was observed in the presence of 100 mM Pi and not 0.01 mM used here. The lower concentration of added Pi used in the present study is a more physiologically relevant concentration and may cause relatively slower changes (kinetically) in metabolism.

Adenylates, Adenylate Energy Charge, and Adenylate Kinase Reaction in the Mycelia of Non-growing P. digitatum in the Presence of Orthophosphate, Adenine and AMP

The above data prompted us to study the changes in ATP, ADP and AMP, and consequently in adenylate energy charge during inhibition by Pi of ethylene biosynthesis in non-growing cells of *P. digitatum*. The results (Fig. 3) showed that the intracellular steady-state AMP and ATP levels of the phosphate-limited, non-growing mycelia (control) varied from 5 to 10 pmol · μg^{-1} protein while the level of ADP

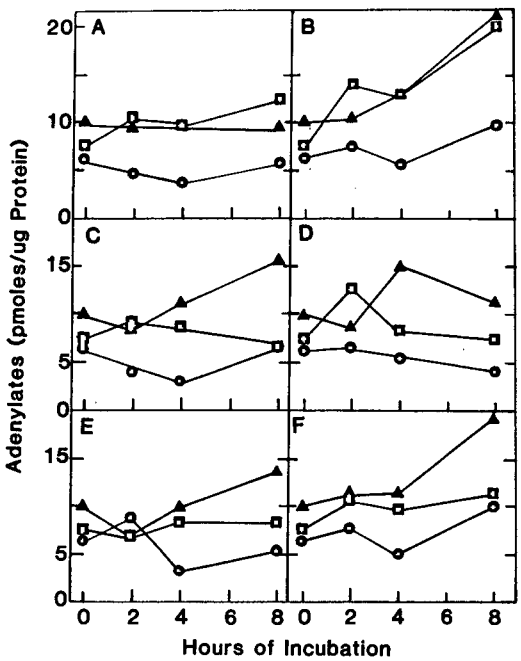

Fig. 3: Effect of addition of phosphate alone (B) and of AMP (C, D) or adenine (E, F) with (D, F) or without (C, E) phosphate to the medium of high-ethylene producing shake culture of *P. digitatum* on the intracellular levels of AMP (○), ADP (□) and ATP (△). Water controls (A) were analyzed simultaneously. The concentration of each tested compund was 0.01 mM. Other details are given in legends to Figs. 1 and 2.

increased from 7.5 to 12.5 pmol · μg^{-1} protein respectively over a period of 8 hours (Fig. 3 A). Upon the addition of Pi to the ethylene-producing cultures, the intracellular ADP level increased by 100 percent within 2 hours (Fig. 3 B) when ethylene production decreased by 65 percent (Fig. 2). A marked rise in intracellular ATP level was also evident (Fig. 3 B) but only 4 hours after Pi addition. Initially ADP level also increased (67 percent) in cultures to which Pi was added in concert with AMP (Fig. 3 D).

Although there was considerable fluctuation in the concentrations of individual adenine nucleotides under the above conditions, only small changes in the adenylate energy charge were observed (Table 1). Marked increases in adenylate energy charge occurred only in those cultures to which AMP was added. The response of the energy charge to Pi, adenine, and AMP was therefore much smaller than the increase in ADP concentration after 2 hours and in ATP after 4 hours. However, the level of ADP in controls after 8 hours was similar to the level seen 2 hours after the addition of Pi (Fig. 3 A, B), and did not correlate with the rate of ethylene production (see

Table 1: Adenylate energy charge, adenylate kinase reaction ratio and ethylene production in low phosphate-grown shake cultures of *Penicillium digitatum* following the addition of Pi, adenine and AMP.

Addition to low Pi growing culture*)	Ethylene $nl \cdot mg^{-1}$ protein h^{-1}			Adenylate energy charge			(AMP)(ATP) (ADP)			ATP/ADP		
	Time after addition, Hours											
	0	2	4	0	2	4	0	2	4	0	2	4
None (Control)	728	728	720	0.59	0.60	0.66	1.25	0.40	0.39	1.44	0.89	1.02
Pi		246	202		0.55	0.62		0.41	0.45		0.75	1.04
Adenine		583	576		0.50	0.66		1.39	0.43		1.07	1.19
AMP		671	518		0.80	0.68		0.40	0.43		0.92	1.27
Pi + Adenine		517	417		0.56	0.63		1.16	0.98		1.07	1.46
Pi + AMP		523	471		0.54	0.66		1.66	0.74		0.69	1.76

*) Shake cultures of *P. digitatum* were incubated in cotton-plugged, 50-ml Erlenmeyer flasks containing 10 ml of modified Pratt's medium without yeast extract and with 0.001 mM phosphate. After 3 days of growth, cotton plugs were removed and the indicated compounds added to the culture medium so that final concentration of each compound was 0.01 mM. Water was added to the control flasks. At regular intervals, samples were analyzed. Other details are described in the legend to Fig. 2 and in the text.

Fig. 2 A). Therefore, the level of ADP does not seem to be an important regulatory factor.

Pi level did not significantly affect the ratio, (AMP) (ATP)/$(ADP)^2$, which is a measure of adenylate kinase reaction (Table 1).

Furthermore, limitation of the availability of the precursor, glutamate, does not seem to be the reason for the low amount of ethylene produced under excess Pi conditions because (a) exogenously added labeled or unlabeled glutamate to such cultures did not increase ethylene synthesis (Mattoo et al., 1979 a) and (b) high-Pi cultures contain 3 times as much intracellular glutamate (0.88 ± 0.19 $\mu mol \cdot g^{-1}$ fresh wt.) as low Pi cultures (0.31 ± 0.11 $\mu mol \cdot g^{-1}$ fresh wt.) on the 3rd day of growth when the maximal rate of ethylene biosynthesis occurs under Pi-limiting conditions (Chalutz et al., 1978; Mattoo et al., 1979 a).

Thus, it appears that inhibition of ethylene production by orthophosphate may not involve regulation by energy charge or adenylate levels. However, it is clear that the Pi-mediated effect is directed at a step(s) in the conversion of glutamate to ethylene and not in the availability of glutamate. In addition to the previously implicated control of ethylene biosynthesis at transcriptional and translational levels by Pi, regulation involving post-translational machinery of the cell may also exist. This could involve a Pi repressible phosphatase and/or a protein kinase (Mattoo et al., 1979 a), particularly in view of the close correlation between the level of acid phosphatase activity and rate of ethylene production in *P. digitatum* (Kapulnik et al., 1983). Such a mechanism was proposed earlier (see Drew and Demain, 1977) for the inhibition by Pi of streptomycin biosynthesis. Our results also support their sugges-

tion that regulation of secondary metabolites by Pi may be mediated through different mechanisms.

Furthermore, as suggested earlier (Kapulnik et al., 1983) phosphate levels may also directly affect the ethylene-producing enzyme system in *P. digitatum* as in the case of other biochemical pathways (Mason et al., 1981) and enzymes (Schramm and Hochstein, 1971; Schramm and Leung, 1973; Mattoo and Shah, 1974; Curdova et al., 1976; Behal et al., 1977; Majmudar et al., 1978; Mattoo et al., 1979 b).

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The Relationship Between the Production of Ethylene by Certain Fungi, Their Enzyme Constitution, and Their Ability to Accumulate Intracellular Phosphate*)

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Summary

An ethylene-producing isolate of a wild type of *Penicillium digitatum* was compared to a non-ethylene-producing isolate of this fungus with regard to various biochemical parameters. The intracellular acid-soluble phosphate content was markedly higher in the non-ethylene-producing isolate and in several other non-ethylene-producing fungi than in the ethylene-producing isolate whether cultures were grown in medium containing high (100 mM) or low (0.1 mM) phosphate. Ethylene production by the ethylene-producing isolate increased markedly in low phosphate growth medium and correlated with an increase in acid phosphatase activity but not with changes observed in the activities of glucose-6-phosphate dehydrogenase, pyruvate kinase, malate dehydrogenase or succinate dehydrogenase. However, these latter enzymatic activities were significantly different in the two isolates when cultivated in high phosphate medium. The data indicated that the ability of the fungi to produce ethylene may be related to high phosphatase activity and to their capacity to maintain low phosphate intracellularly.

Key words: Penicillium digitatum, ethylene production, orthophosphate, phosphatase.

Introduction

Ethylene, a plant hormone, is produced by higher plants and by a wide variety of microorganisms in culture (Ilag and Curtis, 1968; Lynch, 1974; Primrose, 1976; Lieberman, 1979). Since enhanced ethylene production has often been observed in diseased plants, the possible involvement of this gas in pathogenesis has been suggested (Pegg, 1976). The biosynthetic pathway of ethylene has been studied extensively in both higher plants and microorganisms (Chou and Yang, 1973; Yang, 1974; Lieberman, 1979). In higher plants, ethylene is derived from methionine through a biosynthetic pathway that involves S-adenosylmethionine (SAM) and l-

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aminocyclopropane-l-carboxylic acid (ACC) (Adams and Yang, 1979). In *Penicillium digitatum*, the most extensively studied microbial ethylene producer, the immediate precursor(s) of ethylene were unambiguously shown to be 2-oxoglutarate and glutamate (Chou and Yang, 1973).

Production of ethylene by shake cultures of *P. digitatum* and by other microorganisms is affected by the concentration of inorganic phosphate in the growth medium (Chalutz et al., 1978; Mattoo et al., 1979 a; Chalutz et al., 1980; Pazout et al., 1982). Reducing the concentration of phosphate – but not of any other component of the medium – very markedly stimulates the production of ethylene. This increased production is strongly inhibited by exogenous addition of orthophosphate to the low-phosphate medium.

Recently, a non-ethylene producing isolate of *P. digitatum* was reported which did not produce ethylene in culture or during infection of its host – citrus fruit (Chalutz, 1979). This isolate, however, is similar to the wild type of the ethylene-producing fungus in both morphological and physiological characteristics, as well as in its pathogenicity and virulence to citrus fruits (Chalutz, 1979). In the present work we compared the non-ethylene-producing isolate to the wild type, ethylene-producing isolate as well as to some other fungi with regard to several biochemical parameters that could be related to the ability of the fungi to produce ethylene.

Methods

Organisms and growth conditions. Single spore cultures of the fungus *Penicillium digitatum* Sacc. (American Type Culture Collection 10030), the ethylene-producing isolate (P), and the non-ethylene-producing isolate (NP) (obtained from green mould-infected citrus fruits; Chalutz, 1979), were grown on yeast-extract-free, modified Pratt's liquid medium (Pratt, 1944) containing per litre: 18.0 g glucose; 4.0 g NH_4NO_3; 1.23 g $MgSO_4 \cdot 7H_2O$; 0.02 g $FeCl_3 \cdot 6H_2O$; 0.22 mg $ZnSO_4 \cdot 7H_2O$ and deionized distilled water. The concentration of phosphate (KH_2PO_4) in the medium was varied from 100 mM (high phosphate), the optimal concentration for fungal growth (Pratt, 1944), to 0.1 mM (low phosphate) and the pH was adjusted to 4.5 with 0.5 M NaOH before autoclaving for 20 min at 15 psi. The fungus was incubated on a shaking (100 strokes/min) water bath at 23 °C. Inoculation and other incubation procedures, culture weight determinations, and ethylene analysis were performed as described previously (Spalding and Lieberman, 1965; Chalutz et al., 1977).

Determination of protein and acid-soluble phosphate. Protein and acid-soluble phosphate contents of the fungal mycelium were determined after hand homogenization of washed mycelium with acid-washed sand and ice-cold, 5% TCA in a mortar and pestle. Cell breakage (more than 90%) was checked under a light microscope. The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was analyzed for acid-solubilized phosphate by the method of Lowry and Lopez (1946). The residue obtained after centrifugation was suspended in 0.1 M NaOH for 24 h at 25 °C to dissolve the precipitated protein. After centrifuging down the residue, the protein content of the supernatant was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzymatic assays. For enzyme activity determinations the mycelium was homogenized in ice-cold 0.05 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride (Sigma), 1 mM Na_2 EDTA, and 5 mM β-mercaptoethanol. The homogenate was centrifuged at 30,000 *g* for 20 min and the pellet was discarded. The supernatant was dialyzed at

4 °C for 24 h against 100 volumes of 0.05 M Tris-HCl buffer, pH 7.5, and then used as an enzyme preparation.

Acid phosphatase activity was determined by the method of Brandenberger and Hanson (1953). One unit of enzyme activity was defined as the amount that hydrolyzed 1 μmol of o-carboxyphenyl phosphate per min. Glucose-6-phosphate dehydrogenase (G6PDH) was assayed by the method of Caldrider and Gottlieb (1963). One unit of G6PDH activity was defined as a change of 0.001 in absorbance at 340 nm per min. Pyruvate kinase (PK) was assayed by the method of Kapoor and Tronsgaard (1972). One unit of PK activity was similar to that defined for G6PDH. Malate dehydrogenase (MDH) activity was assayed by the method of Courbright and Henning (1970). One MDH unit was defined as that amount of enzyme that reduced 1 μmol of 3,4,5-dimethylthiazolyl-2-2,5 diphenyl tetrazolium bromide per min. Succinate dehydrogenase (SDH) activity was assayed by the method of Courbright and Henning (1970) and one unit of activity was defined as that amount of enzyme that oxidized 1 μmol NADH per min.

All experiments were repeated at least three times.

Results and Discussion

Ethylene production and phosphate content of fungal mycelium. As reported earlier (Chalutz et al., 1978; Mattoo et al., 1979 a), ethylene production by the shake cultures of a wild type isolate (P) increases as the concentration of phosphate in the cultivation medium is decreased. However, the NP isolate did not respond similarly and produced negligible ethylene when cultivated in a medium containing varying amounts of phosphate (Table 1). In this regard, the NP isolate resembles other fungi, viz. *P. expansum* (Link) Thom., *P. italicum* Wehmer and *Geotrichum candidum* Lk. & Pers. that produce very little ethylene (Table 2).

Table 1: Production of ethylene by two isolates of *P. digitatum* when cultivated in Pratt's liquid medium containing different concentrations of orthophosphate. Ethylene production was determined on the fourth day of cultivation after 1 h of sealed period.

Phosphate content of the medium	Ethylene production	
	P isolate	NP isolate
(mM)	($nl \cdot h^{-1} \cdot g^{-1}$ FW)	
100	1	0.5
1	350	0.7
0.1	1000	0.5
0.01	1200	0.3
0.001	1300	0.7

Since ethylene production by the P isolate was markedly affected by the phosphate content of the cultivation medium, we analyzed the mycelial acid-soluble phosphate content of this fungus, of the NP isolate (of *P. digitatum*) and of the non-ethylene-producing fungi listed above. The P isolate maintained the lowest intracellular acid-soluble phosphate content relative to all the other isolates or fungi tested (Table 2). At the time of maximal ethylene production by the P isolate, its intracellular phosphate content was about 8 times lower than in the NP isolate (0.18 mg per g FW

Table 2: Intracellular acid-soluble phosphate content and rate of ethylene production of several fungi on the fourth day of cultivation in high or low-phosphate medium.

	Phosphate content of the medium (mM)			
	100		0.1	
	Acid-soluble phosphate	Ethylene	Acid-soluble phosphate	Ethylene
	($mg \cdot g^{-1}$ FW)	($nl \cdot h^{-1} \cdot g^{-1}$ FW)	($mg \cdot g^{-1}$ FW)	($nl \cdot h^{-1} \cdot g^{-1}$ FW)
P. digitatum				
P isolate	1.10	2.0	0.18	1000.0
NP isolate	6.44	0.5	1.42	1.0
P. expansum	11.30	2.5	1.32	3.0
P. italicum	5.70	1.0	1.87	1.5
G. candidum	5.82	0.1	2.34	0.1

compared to 1.42 mg per g FW). A similar relationship was obtained when the data were expressed either on a fresh weight or on a protein basis.

Enzyme levels. Earlier studies with transcriptional and post-transcriptional inhibitors led us to suggest (Mattoo et al., 1979 a) that phosphate-mediated control of ethylene production involved *de novo* protein synthesis. Also, we showed that the levels of alkaline phosphatase and protein kinase activities were higher in low than in high phosphate-grown cultures. In order to check if phosphate levels differentially affect some other key enzymes in cellular metabolism of the P isolate compared to the NP isolate, we determined the activities of pyruvate kinase (representing the glycolytic pathway), glucose-6-phosphate dehydrogenase (representing the pentose monophosphate pathway), malate and succinate dehydrogenases (representing the Krebs cycle), and acid phosphatase. The activities of acid phosphatase (Weinberg, 1974; Ezhov and Santesevich, 1976), other phosphohydrolases (Mattoo et al., 1979 b, 1980), malate dehydrogenase, and glucose-6-phosphate dehydrogenase (Savant et al., 1982) have been shown in other organisms to be inversely proportional to the phosphate level of the medium. Table 3 lists the levels of the various enzyme activities tested in the P and NP isolates cultivated on low and high phosphate medium. In low phosphate grown cultures, when the P isolate produces ethylene at high rates, the levels of glucose-6-phosphate dehydrogenase and acid phosphatase were 61 and 26 times higher, respectively, than those in cultures grown in high phosphate medium. On the other hand, the levels of pyruvate kinase, malate dehydrogenase, and succinate dehydrogenase were considerably lower in the low phosphate than in the high-phosphate-grown culture (Table 3). In contrast to the enzyme makeup of the P isolate, in the NP isolate the levels of glucose-6-phosphate dehydrogenase and acid phosphatase were respectively only 4.6 and 2.4 times higher in low-phosphate-grown cultures than in the high-phosphate-grown fungus. Moreover, instead of decreased levels of pyruvate kinase, malate dehydrogenase, and succinate dehydrogenase as shown in the low-phosphate grown P isolate, the activities of all these enzymes were higher in the low-phosphate-grown NP isolate compared to their levels in the high-phosphate-

Table 3: Pyruvate kinase (PK), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), succinate dehydrogenase (SDH) and acid phosphatase (AP) activities of P and NP isolates of *P. digitatum* cultivated in low or high phosphate medium. The activities were determined in mycelia obtained from 4-day old cultures.

Enzyme assayed	Phosphate level (mM)	P isolate		NP isolate	
		100	0.1	100	0.1
		(units $min^{-1} \cdot mg^{-1} \cdot$ Protein)			
PK		11.86	2.91	1.28	2.68
G6PDH		0.01	0.61	0.18	0.82
MDH		0.76	0.32	0.02	0.26
SDH		0.96	0.45	0.06	0.41
AP ($\times 10^3$)		0.80	21.00	0.08	0.19

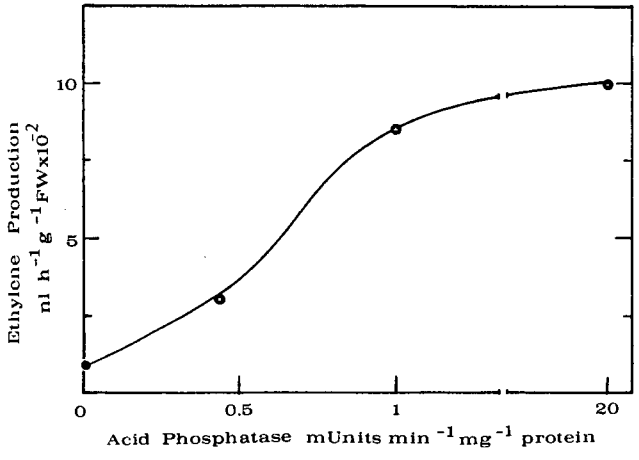

Fig. 1: The relationship between the activity of acid phosphatase and the rate of ethylene production in the P isolate of *P. digitatum*.

grown culture. However, when a direct comparison is made between the levels of enzyme activities in the P and NP isolates cultivated in low phosphate or in high phosphate, marked differences in the enzyme activities were observed only when the cultures were cultivated in high phosphate medium (Table 3). However, in low-phosphate-grown cultures the activity of acid phosphatase was 110 times higher in the P compared to the NP isolate.

The levels of acid phosphatase activity determined in the P isolate on different days of cultivation correlated closely with the increase in ethylene production (Fig. 1). This difference in acid phosphatase activity between the P and the NP isolates grown in low phosphate may be significant in the light of the observation that the activity of none of the other enzymes tested changed so markedly in low phosphate cultures and

that the P isolate, under these conditions, produced ethylene while the NP isolate did not (Table 1). This could be a reflection of the difference in the intracellular concentration of acid-soluble phosphate which was approximately 8 times higher in the NP isolate than in the P isolate as shown above (Table 2).

The results presented indicate that, unlike the P isolate of *P. digitatum*, the NP isolate did not respond by producing ethylene in low phosphate medium (Table 1). Thus, the NP isolate differs from the wild type isolate in its inability to produce ethylene, not only during infection of citrus fruit (Chalutz, 1979) but also when cultivated under conditions that stimulate ethylene production in the P isolate.

The inability of the NP isolate to produce ethylene may be related to its relatively high intracellular phosphate content. Even when the NP isolate was cultivated in low phosphate medium, it maintained more intracellular phosphate than did the P isolate when cultivated in high phosphate medium (Table 2). Similarly, other fungi tested maintained a higher intracellular phosphate content than the NP isolate, and none of them produced ethylene in any considerable amounts. Thus, it is possible that the ability of *P. digitatum*, and perhaps of other microorganisms as well, to produce ethylene may be related to a critical low intracellular phosphate concentration. Such a phenomenon was recognized earlier with secondary metabolites (Weinberg, 1974), each of which is inhibited by a certain specific phosphate level of the medium. However, in these earlier studies intracellular phosphate content was not measured.

The data presented in this work indicate clear differences between the P and NP isolates of *P. digitatum* not only in their ability to accumulate acid-soluble phosphate intracellularly but also in the levels of various enzyme activities, particularly acid phosphatase activity. These observations may be related to the inability of the NP isolate and of some other fungi, to produce ethylene. One of the possible mechanisms of control of ethylene in fungi by intracellular phosphate levels may involve phosphorylation/dephosphorylation of the ethylene-producing enzyme system mediated through changes in protein kinase activity (Mattoo et al., 1979 a) and acid phosphatase activity as shown here. Low concentrations of phosphate lead to the derepression of acid phosphatase in other microorganisms as well (Nyc, 1967; Weinberg, 1974; Boutin et al., 1981) and may activate the ethylene-producing enzyme system in *P. digitatum* as in the case of other biochemical pathways in microorganisms (Mason et al., 1981). However, a direct testing of such a suggestion has to await the isolation of the ethylene forming system in fungi, attempts at which have not been successful to date.

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Effect of Wounding on 'Fuerte' Avocado Ripening[1]

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Additional index words. wound ethylene, firmness, polygalacturonase, *Persea americana*

Abstract. **Wounded mature 'Fuerte' fruit (*Persea americana* Mill.) ripened faster than non-wounded fruit when stored at 14°C. Significant differences were not observed in respiration or ethylene production between wounded and non-wounded fruit when stored at 20° but the former softened faster and showed greater polygalacturonase activity. Wounded and non-wounded fruit ripened at similar rates when stored after wounding for 10 days at 5° and thereafter transferred to either 14° or 20°. No "wound ethylene" could be detected immediately after wounding at any temperature and is not the earliest event occurring during ripening. Effects of wounding in metabolic processes of ripening are observed better at a moderate continuous storage temperature of 14° than at 20°.**

It is well known wounding in various fruit enhances physiological processes which lead to faster ripening and decay (3, 4, 10, 11). It has been suggested (3) the response of fruit to wounding is complex, apparently differing among cultivars and even in an individual fruit depending upon the extent of the wound or the conditions under which it was inflicted. More recently, it was reported that removal of a pulp plug from avocado fruit and covering of the wound immediately with lanolin did not affect the general pattern of respiration and ethylene production during ripening (1).

Avocado harvesting is an expensive and difficult operation, leading people to try cheaper and faster ways of harvesting fruit. Tree-shaken harvest has been attempted (7) and effects of snap-harvest of avocado upon ripening and weight loss have been studied (5). Some of the fruit are wounded in the above-mentioned operations and during packing and transportation; therefore, it was of interest to study the effects of wounding upon the ripening processes and shelf life of avocado fruit.

Mature 'Fuerte' avocado fruit were harvested when they contained 15% oil, held for one day at 14° or 20°C and 85 to 90% relative humidity, then wounded by excising 1 cm^3 of tissue including the peel and pulp at the widest part of the fruit.

Five fruit from each treatment were tested daily, each in a 2-liter jar for exactly 1 hr; 10 ml of gas from the head space was then sampled with a syringe for ethylene and CO_2 determination by gas chromatography (6). The limit of detection was 20 ppb ethylene/ml.

Firmness was determined without removing the peel, with a machanized Chatillon pressure tester using a conical tip 34.6mm in diameter (12). The beginning of softening could be sensed by hand when the resistance to penetration was about 3.5 kg. Three fruit per treatment were tested at each determination, each at 2 points, then sampled for polygalacturonase (PG) activity. The latter was determined as described previously (13); 50 g fruit pulp was ground, 1 part tissue and 3 parts 1 M NaCl, in an Osterizer blender for 3 min. The filtered extract was incubated for 30 min with 1% aqueous solution of citrus pectin (Yakin, Israel) at a ratio of 1:10, at pH 5.0 and 30°C. PG activity was expressed as percent loss of viscosity using an Ostwald Viscometer (Volac No. 150). Similar studies were carried out during 3 consecutive harvest seasons, however in this communication representative data of only 1 season of studies are presented.

Wounding avocado fruit accelerated their ripening, this effect being more clearly observed at 14° then at 20°C. Respiration rates and ethylene production were similar in both wounded and non-wounded fruit at 20°. Peak respiration rate on the 5th day and that of ethylene production on the 4th day after wounding (Fig. 1A & B). Rates and levels of CO_2 and ethylene production were lower at 14° and their peaks in wounded fruit preceded those in non-wounded fruit by 2 days. The ethylene peak in sound fruit occurred on the 12th day after wounding and that of respiration on the 13th day; whereas the respective peaks were observed in wounded fruit on the 10th and 11th days (Fig. 1A & B). Awad and Young (1) recently showed that the general pattern on avocado ripening processes was not altered by wounding. No increase in ethylene evolution was observed either immediately after wounding or 2, 6, and 24 hr later at either temperature (Fig. 1B). It is in contrast to reports about "wound ethylene" produced by other fruits (8, 9). The fact that no "wound ethylene" could be observed after wounding is in line with the suggestion that ethylene may not be the initiator of

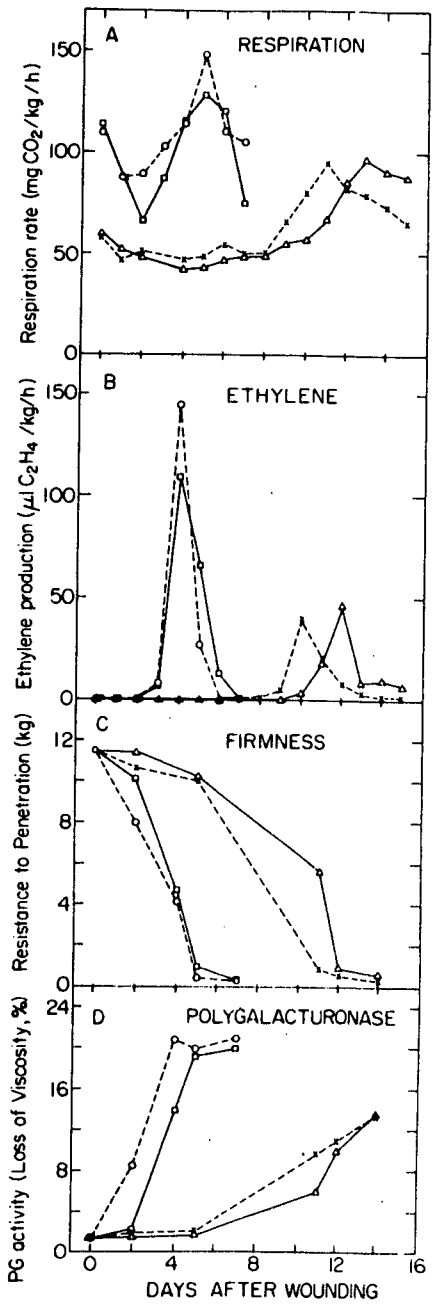

Fig. 1. The effect of wounding of avocado fruit on respiration rate (A), ethylene production (B), softening (C) and polygalacturonase activity (D), during storage at 14° and 20°C. Respiration and ethylene were measured in the same fruit; softening and polygalacturonase activity are averages of 6 determinations. Intact fruit at 20° —○—; Wounded fruit at 20° —●—; Intact fruit at 14° —△—; Wounded fruit at 14° —▲—.

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natural ripening but rather a product of ripening processes (2). It seems possible that, in wounded avocado fruit, the hydrolytic enzymes are activated or released directly by the act of cell breaking (wounding) or are indirectly activated by some agents other than ethylene.

Softening was accelerated by and PG activity increased following wounding (Fig. 1C & D). A difference could be observed only on the 11th day at 14°C, but it was obvious already by the 2nd day after wounding at 20°. Final PG activity in sound and wounded fruit reached similar levels at both temperatures similar to what was reported by Awad and Young (1); however the final level of PG activity was lower at 14° (Fig. 1D). There were no differences between sound and wounded fruit in softening rates when fruit were stored at 5° for 10 days (9.5 Kg resistance to penetration) before being transferred to either 14° or 20°. However, fruit, both sound and wounded, become soft (0.5 kg) 7 days after it was transferred to 14° and after only 4 days at 20° (0.4 kg). Wounding the fruit did not cause any decay development during softening under our conditions.

It appears that when ripening processes are accelerated by storage at a temperature such as 20°C small differences cannot be observed; however, by slowing these process at 14° the relatively small differences become more obvious as the time scale of ripening is stretched out.

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Fungal infections suppress ethylene-induced phenylalanine ammonia-lyase activity in grapefruits†

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(*Accepted for publication December 1982*)

Ethylene-induced phenylalanine ammonia-lyase (PAL) activity in yellow grapefruit peel was inhibited by inoculation of the fruit with *Penicillium digitatum*, not only at a place close to the infection site but also in areas farther away from it. The degree of inhibition was related to the distance of the fungus from the assayed region. Other fungi tested such as a non-ethylene producing isolate of *P. digitatum*, an isolate of *P. italicum* and an isolate of *Geotrichum candidum* had a similar effect. This phenomenon was also found in orange fruit peel. PAL was not induced in the green fruit by ethylene and also not induced during natural infection of mature fruits by *P. digitatum* in spite of the production of ethylene during infection.

These findings indicate a mechanism whereby fungi are able to prevent the defence response of host cells to invasion.

INTRODUCTION

Increase in phenolic compounds, derived from phenylalanine, may occur in many plants after infection with pathogens, particularly in the case of incompatible host–pathogen interactions [*7, 8, 13*]. Phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5) is a key enzyme in the secondary metabolism of plants. The product of the enzyme reaction, cinnamic acid, is the precursor of many compounds, e.g. *p*-coumaric, caffeic, ferulic acids and lignins [*4*] thought to be involved in the defence mechanisms of some plants [*7, 8, 13*]. Increase in PAL activity has been found after wounding and following inoculation [*4, 8, 9, 11, 17*], and increased amounts of *p*-coumaric acid residues were also found in lignin formed in wheat plants following inoculation [*16*].

Healing of wounds in citrus fruit is an active biological process involving the induction of PAL activity. Such increase in enzymatic activity is probably responsible for the increase in lignin and free phenolic constituents in injured citrus flavedo [*11*]. It has been suggested that lignin deposition provides a mechanical barrier blocking penetration by fungal germ tubes [*2, 3, 10*]. In addition, free phenolic compounds may directly act as fungitoxic compounds [*7, 13*].

PAL activity in citrus fruits can be induced by wounding [*11, 17*] and by ethylene applications [*17*] but it is not induced by light [*17*]. Whether or not infections of citrus fruits by some of their important pathogens such as *Penicillium digitatum* Sacc., *P. italicum* Wehmer or *Geotrichum candidum* Lk. ex Pers. affect PAL activity is

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Fungal infections suppress ethylene-induced phenylalanine ammonia-lyase activity in grapefruit

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(Accepted for publication [illegible])

Ethylene-induced phenylalanine ammonia-lyase (PAL) activity in yellow grapefruit peel was inhibited by inoculation of the fruit with *Penicillium digitatum*, not only at a place close to the infection site but also in areas further away from it. The degree of inhibition was related to the distance of the fungus from the assayed region. Other fungi tested, [illegible] producing isolate of *P. digitatum*, an isolate of [illegible] and an isolate of [illegible] had a similar effect. This phenomenon was also found in [illegible] that PAL was not induced in the green fruit [illegible] also [illegible] during natural infection of [illegible] fruits by *P. digitatum* in spite of the production of ethylene during infection.

These findings [illegible] a mechanism [illegible] by fungi [illegible] able to prevent [illegible] the defence response of host cells to invasion.

INTRODUCTION

Increases in phenolic compounds, derived from phenylalanine, may occur in many plants as a reaction to pathogens, particularly in the case of incompatible host-pathogen interaction [?, 5, 13]. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a key enzyme in the secondary metabolism of plants. The product of the enzyme reaction, cinnamic acid, is the precursor of many compounds, e.g. flavonoids, isoflavonoids and lignins [4] thought to be involved in the defence mechanisms of the plant [7, 8, 13]. Increases in PAL activity were found after wounding and following inoculation [?, ?, 14, 17] and increased amounts of *p*-coumaric acid residues [illegible] in wheat plants following inoculation [16].

Healing of wounds in citrus fruit is an active biological process involving the induction of PAL activity. Since an enzymatic activity is [illegible] responsible for the increase in [illegible] phenolic constituents in injured citrus peels [?], it has been suggested that [illegible] provides a mechanical barrier blocking penetration by fungal germ tubes [?, 8, ?]. In addition, the phenolic compounds may directly [illegible] [?, 13].

PAL activity in citrus fruit peel is induced by ethylene [11, ?] and [illegible] by light [?]. Whether or not infections of citrus fruit by one of their important pathogens such as *P. digitatum* or [illegible] affect PAL activity is

[illegible]

not clear. Ismail & Brown [*11*] reported recently that PAL activity in injured orange fruits was inhibited after inoculation of the injured area with *P. digitatum*.

We report here on an inhibition of PAL activity by fungal inoculation not only at the infection site, but also in peel areas distant from the site of infection.

MATERIALS AND METHODS

Fungi

The fungi used in this study were (1) an ethylene-producing isolate (EP) of *P. digitatum* Sacc. (American Type Culture Collection 10030), (2) a non-ethylene-producing isolate (NP) of *P. digitatum* obtained from infected citrus fruits, (3) an isolate of *P. italicum* Wehmer and (4) an isolate of *Geotrichum candidum* Lk. ex Pers. All fungi were obtained as single spore cultures and kept on potato-dextrose agar (PDA, Difco) until used.

Plant material

Ripe grapefruit (*Citrus paradisi* Macf., "Marsh Seedless") were hand picked twice during the season (January and April, unless otherwise indicated). Only fruits, uniform in size, from the shadowed centre of the trees were used in these tests. "Shamouti" oranges [(*Citrus sinensis* (L.) Osbeck)], were picked in January. The fruits were stored at 11 °C at 90% rh until used.

Fruit inoculation

In general, dense spore suspensions obtained from PDA-grown, 4 to 7-day-old fungal cultures, to which 0·1% Tween 80 was added, were used for inoculation. Fruits were inoculated near the stem-end by placing a drop (40 µl) of the spore suspension on the surface of the fruits and then piercing once with a needle. Inoculated fruits were placed inside 6-l glass jars, generally three fruits in each jar. The jars were kept at 18±1 °C in the dark except when fruits were inoculated with *G. candidum*. In this case, the fruits were kept in the dark at 25 °C and at high humidity (95 to 100% rh) during the whole experiment to promote better infection and development of the fungus. When fungi developed on the fruits to a visible area of either 2 or 4 cm in diameter, each jar was connected for 48 h to a flow system through which an ethylene-air mixture (100 ppm ethylene) was passed at a constant rate of 30 ml min^{-1}. The fruits were then removed from the jars, the flavedo of seven to nine fruits at the area to be examined was carefully cut out and immediately frozen at −18 °C until assayed for PAL activity.

PAL extraction and determination of its activity

The enzyme was prepared from frozen flavedo essentially as described by Chalutz [*5*] and Rahe *et al.* [*15*]. Ten grams of the flavedo were ground in a Waring Blender for 1 min with 100 ml acetone at −18 °C The blended flavedo was filtered through Whatman No 1 filter paper and the powder blended again with acetone. This step was repeated three times. The acetone powder was then dried in the air at room temperature for 3 h, collected and stored at −18 °C until used. For enzyme assay, 500 mg of the dry acetone powder was added to 10 ml cold 0·1 M borate buffer, pH 8·8, and stirred for 1 h at 4 °C. The suspension was centrifuged twice at 12 100 ***g*** at

4 °C and the clear yellowish supernatant was then dialysed at 4 °C against 2 l of 0·2 M borate buffer, pH 8·8, for 48 h and then taken for PAL determinations. The reaction mixture contained 1·5 ml enzyme preparation, 2.5 ml 0·1 M borate buffer, pH 8·8, and 1·0 ml 0·05 M L-phenylalanine (Aldrich Chemical Inc.). Tubes containing the reaction mixture were incubated for 1 h at 40 °C. In some experiments Tris–HCl buffer was substituted for borate buffer with no differences between the level of activity obtained [*14*]. The reaction was stopped by the addition of 0·1 ml 5 N HCl. Then, 7 ml of diethyl ether (spectroscopic, Merck) were added to the reaction mixture and thoroughly mixed in a Vortex apparatus. Since a thick gel was formed in the upper ether fraction the mixture was centrifuged at 12 100 ***g*** for 5 min and 3·5 ml of the clear ether supernatant was taken for direct measurements, in a spectrophotometer at 269 μm, of the cinnamic acid formed.

Scanning the ether fraction of controls stopped at time 0 of incubation, showed an absorption peak at 283 μm. However, the addition of pure cinnamic acid to this preparation shifted the peak from 283 to 269 μm. Thus it seemed that the presence of pre-existing substance(s) in controls with an absorption peak near that of cinnamic acid could interfere with enzyme readings at low enzyme activities and so this question was investigated further. Three millilitres of ether fractions of controls (without cinnamic acid) and of controls to which cinnamic acid had been added, were dried at 40 °C and dissolved in 0·5 ml ether. These were then loaded on silica gel 60 tlc plates containing a F_{254} fluorescent indicator (E. Merck). Plates were run in a solvent mixture of toluene:ethyl-acetate:90% formic acid (50:40:5 v/v). After development the plates were air-dried and three spots could be visualized under a short-wave uv lamp in samples containing cinnamic acid. One spot having a R_f similar to authentic cinnamic acid (R_f=0·6) was removed from the plates, resuspended in ether and as expected showed maximum absorbance at 269 to 270 μm. The other two spots (R_f 0·90 and 0·95 respectively) were present in all samples and found to have maximum absorbance at 283 μm. This tlc method was subsequently adapted to check spectrophotometric determinations of cinnamic acid by using a relationship which was determined between cinnamic acid concentrations, diameter of spots on tlc plates and absorbance.

Ethylene determination

Individual fruits inoculated or not inoculated with *P. digitatum* were placed inside 1-l glass jars kept at 18 °C. Every day the jars were sealed for 1 h prior to sampling of the air above the fruit for ethylene determinations as described previously [*6*, *18*].

RESULTS

Influence of fruit age on PAL induction by ethylene

Grapefruits were tested monthly from September until February in order to determine their ability to respond to ethylene treatment with increased PAL activity. In September fruits were still deep green and about 7·0 cm in diameter. In December the fruits reached full size and were fully coloured. The results showed (Fig. 1) that treating fruits for 48 h with 100 ppm ethylene did not induce PAL activity as long as the fruits were not completely yellow. Therefore all our experiments were carried out with fruits picked from January onwards.

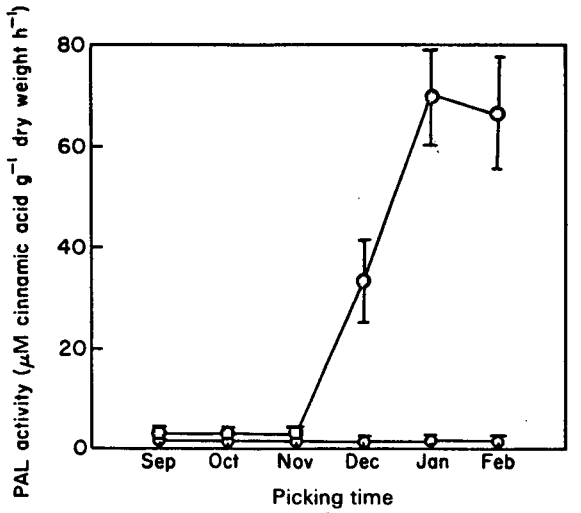

FIG. 1. Ethylene-induced PAL activity in the flavedo portion of the peel of harvested grapefruits as affected by fruit development on the tree. Control – no ethylene (○); ethylene treated (●). The bars represent the standard deviations about the means of three experiments.

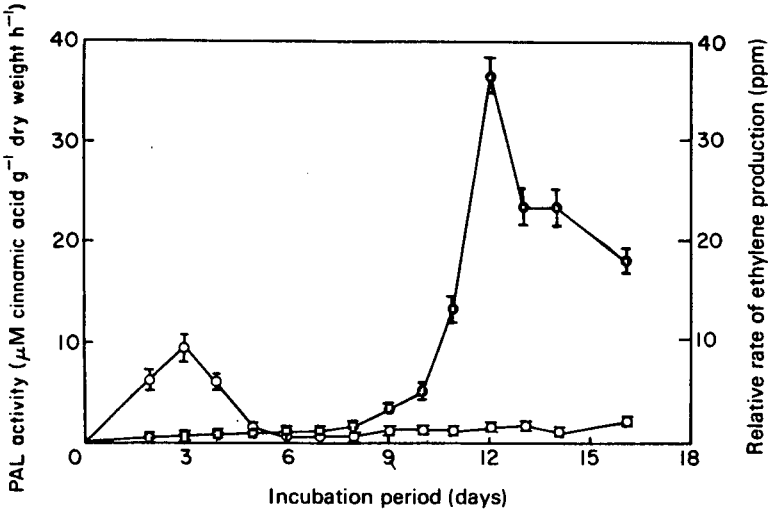

FIG. 2. PAL activity and ethylene production in grapefruit inoculated with *P. digitatum*. PAL activity was assayed at the inoculation site (○). Values of ethylene production by the whole inoculated fruit (●) were obtained daily. The bars represent the standard deviations about the means of three experiments.

PAL activity in grapefruit inoculated with P. digitatum

Since ethylene is known to induce PAL activity in citrus fruits [*17*] and since inoculation by *P. digitatum* results in ethylene production [*6*], PAL activity in grapefruit peel inoculated by this fungus was examined. Different peel areas of the inoculated fruits were tested for PAL activity: the inoculation site, the water soaked area surrounding the inoculation site, the region surrounding the water-soaked area and an additional area 2·5 cm away from the inoculation site. PAL activity was low in all these areas throughout the infection process. Figure 2 shows the value obtained for PAL activity at the inoculation site and for ethylene production during this period. It is possible that injuries made at the time of inoculation were responsible for the small peaks in activity found during the initial incubation period. Maximum ethylene production (37·0 ppm) was detected 12 days after inoculation when fruits were already completely covered by the fungus.

Inhibition of PAL activity at a fruit site opposite to the inoculated area by different fungi

Grapefruits were inoculated by the EP isolate of *P. digitatum* near the stem-end, kept until the lesion developed to either 2·0 or 4·0 cm in diameter. The ethylene treatment did not affect the growth rate of the fungus in the fruit peel. Non-inoculated fruits were used as controls either pierced once or not pierced, and with or without treatment with ethylene. An additional control included inoculated fruits not treated with ethylene. Our previous results (Fig. 2) indicated that 6 days following inoculation, as a result of fungal infection, fruits were exposed to a very minute concentration of ethylene which was not sufficient to induce PAL activity. Following the 100 ppm ethylene treatment, the flavedo tissue from a 1 cm wide strip at the equatorial part of the fruit and a 2·5 cm wide strip at the styler side of the fruit opposite to the inoculated area were cut and examined for PAL activity. Table 1 shows that as the fungus progressed over the fruit ethylene-induced PAL activity was inhibited, inhibition being higher in peel regions close to the inoculated area.

TABLE 1

PAL activity in the flavedo tissue of grapefruit peel inoculated with P. digitatum *and subsequently treated with ethylene*

Fruit treatment	PAL activity (μM cinnamic acid g^{-1} dry $weight^{-1}$ h^{-1}) Days after inoculation 4	6
Non-treated	Trace	Trace
Inoculated only:		
Equatorial	Trace	Trace
Stylar-end opposite to inoculation site	Trace	Trace
Ethylene treated only	63·5±12·1	68·5±15·57
Inoculated and ethylene treated:		
Equatorial	18·5±7·43	5·0±4·18
Stylar-end opposite to inoculation site	50·0±9·38	17·5±5·12

Ethylene treatment (100 ppm) was applied at two different stages of the fungus development, after 2 and 4 days of growth. Each result is the mean of five experiments ± s.d.

Inhibition of PAL activity seemed also to take place at regions of the peel far away from the infected area. Other fungi, e.g. the NP isolate of *P. digitatum*, an isolate of *P. italicum* and an isolate of *G. candidum* showed similar inhibitory effects on PAL activity (Table 2).

TABLE 2

PAL activity in the flavedo tissue of grapefruit peel inoculated with several fungi and subsequently treated with ethylene

Fruit treatment	PAL activity (μM cinnamic acid g^{-1} dry weight h^{-1})
Non-inoculated	75·0±16·1
Inoculated with:	
P. digitatum (NP)	16·0±6·60
P. italicum	15·0±5·0
G. candidum	22·0±7·50

Ethylene treatment was at 100 ppm. PAL activity was assayed at the stylar-end of the fruit opposite to the inoculated site after 6 days incubation. Non-ethylene treated fruits, inoculated or non-inoculated by these fungi, showed only traces of PAL activity. Each result is the mean of three experiments ± s.d.

In order to determine if the inhibition by fungal infection of ethylene-induced PAL activity is independent of the site of fungal inoculation, we also inoculated *P. digitatum* at a point in the equatorial region of the fruit. In these fruits as well, PAL activity assayed at the site opposite to the inoculated area was inhibited approximately fourfold by fungal infection.

Inhibition of ethylene-induced PAL activity in orange fruits by P. digitatum

To check whether the inhibitory effect of fungal infection on PAL activity was restricted to grapefruits only, similar tests were carried out with oranges. The results obtained showed that in oranges also fungal infection inhibited PAL activity at an area far away from the inoculation site. Control, ethylene treated non-inoculated fruits showed PAL activity of 70·0 μM cinnamic acid g^{-1} dry weight h^{-1}, whereas in the ethylene treated inoculated fruits PAL activity decreased by fourfold on the 6th day after inoculation.

DISCUSSION

PAL is the first enzyme of the phenylpropanoid pathway and may have an important role in the defence mechanism of plants [*8*]. In spite of the fact that during its development on grapefruit peel, *P. digitatum* is "wounding" the tissue and ethylene is produced, we did not observe an increase in PAL activity (Fig. 2) as was also reported by Ismail & Brown [*11*] who mechanically injured the tissue prior to inoculation of the injured site. Thus, in mechanically injured peel PAL activity is increased [*11*] while this is not so in inoculated tissue. A possible explanation for this fact is that mechanical injury kills only a small part of the cells and the living adjacent ones react by increased PAL activity. Fungal injury, on the other hand, probably kills most of the cells in the tissue and therefore an increase in PAL activity could not take

place. In addition, the ethylene produced during this host–parasite interaction may reach amounts which can induce PAL activity [*17*] only when most of the fruit is infected by the fungus. Therefore, ethylene in this case has little or no effect on the induction of the enzyme (Fig. 2).

Increased PAL activity has been reported to occur in plants during the resistant reaction [*8*]. Mature grapefruit and oranges are not resistant to any of the fungi used in this investigation and therefore the negligible increase in PAL activity found during pathogen development on the fruit (Fig. 2) is not unexpected. Furthermore, green grapefruit which are also susceptible to fungal attack did not respond to ethylene treatment by increased PAL activity (Fig. 1).

However, the most interesting effect of PAL inhibition by the fungus was observed at areas far away from pathogen development (Tables 1 and 2). This phenomenon was observed when fungal inoculations were performed at different sites on the fruits, when several other fungi were used to inoculate grapefruits, or when oranges were used instead of grapefruits. The absence of fungal mycelium in areas which are 2·0 cm or more away from the visible water soaked area was verified (Achilea, Fuchs & Chalutz, unpublished results).

The phenomenon of "killing in advance" the fungus then growing in the already dead tissue is well-known [*1*, *12*]. However, we report a finding which indicates that the fungus is apparently able to "neutralize" cell responses far away from the site of its development. Thus, it may prevent, in advance, the formation of unfavourable substances by the host cells before destroying the whole fruit. Further investigations on the phenol and lignin levels may indicate the ability of the fungus to suppress these two other main constituents of the plant's defence systems.

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INDUCTION OF ETHYLENE BIOSYNTHESIS IN TOBACCO LEAF DISCS BY CELL WALL DIGESTING ENZYMES[1]

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SUMMARY: Cellulysin induces ethylene production in tobacco leaf discs by initiating the formation of 1-aminocyclopropane-1-carboxylic acid. Induction occurred within 30 to 60 min of incubation and was inhibited by aminoethoxyvinylglycine, and the antiproteases, PMSF and soybean trypsin inhibitor. Cycloheximide (CHI) at 2.8 µg/ml and chloramphenicol (CAP) at 100 µg/ml did not inhibit this induction although incorporation of the label from (3,4-^{14}C)methionine into the acid-insoluble fraction was inhibited by 57%. At 14 µg/ml CHI, and CAP, ethylene production was inhibited by 25% while protein synthesis was inhibited by 75%. We suggest that either the low amounts of protein synthesis that appear to be insensitive to CHI is sufficient to induce ethylene biosynthesis or that Cellulysin activates a preexisting but inactive form of ACC synthase to promote ethylene biosynthesis. Also, induction of ethylene production by microbial enzymes that digests plant cell walls may be an initial protective response of plants that serves to combat microbial infection.

Ethylene, the gaseous plant hormone, is produced, to varying degrees, by most higher plants and by microorganisms (1). Auxin treatment as well as mechanical or chemical wounding of higher plants lead to a considerable enhancement in the rate of ethylene production, particularly in those plant tissues that normally produce very little of this hormone (1-4). This type of stimulation of ethylene biosynthesis in higher plants has been related to the induction of 1-aminocyclopropane-1-carboxylic acid (ACC) accumulation as a result of enhanced conversion of S-adenosylmethionine (SAM) to ACC in the following metabolic sequence: methionine $\longrightarrow$ SAM $\longrightarrow$ ACC $\longrightarrow$ C_2H_4 The mechanism of this stimulation remains obscure, although it is apparently related to de novo synthesis of ACC synthase (3).

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[4]Deceased.

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In contrast to our knowledge of hormone- and wound-induced ethylene production in higher plants, little is known about the enhanced ethylene biosynthesis common to most host-parasite interactions during disease development. In the latter, the actual contribution of the host, or the pathogen, or both in stimulating ethylene biosynthesis is not yet established. However, one of the first events during the contact of a pathogen with the host plant involves secretion by the pathogen of enzymes that degrade plant cell walls (5). Fungi secrete a variety of extracellular plant cell wall digesting enzymes that are routinely used to obtain protoplasts from plant cells (6). In this context, we found it interesting that the fungal, cell wall degrading enzyme mixture, Cellulysin, markedly induced the production of ethylene in tobacco leaf discs. We report here that Cellulysin-induced ethylene biosynthesis is rapid, related to a considerable increase in the synthesis of ACC, and insensitive to low concentrations of the protein synthesis inhibitors, chloramphenicol and cycloheximide.

MATERIALS & METHODS

Tobacco plants (Nicotiana tobacum L. cv. Xanthi) were grown in a greenhouse under natural light at temperatures ranging between 20 and 30°C for 1-2 months. Leaves were washed with distilled water, surface-sterilized by soaking for 10 sec in 70% ethanol, and rinsed several times in sterile distilled water. Sterile technique was used in all subsequent handling of the tissue and glassware.

Discs (1 cm in diameter) were cut from leaf blades of fully expanded, deep-green leaves and were floated abaxially down under cool-white fluorescent light (1750 lux) for about 1 h in petri dishes containing, unless otherwise indicated, filter-sterilized basal medium containing 700 mM sorbitol, 10mM MES(pH 6.0), 10 mM $CaCl_2$, 50 μg/ml streptomycin sulfate and 50 units/ml penicillin G in the absence and presence of Cellulysin (Calbiochem). Six discs (70-100 mg fresh weight) were floated on 1 ml of filter-sterilized test solutions in 25-ml Erlenmeyer flasks. The flasks were sealed with rubber serum stoppers and incubated at 25°C in darkness.

Ethylene was allowed to accumulate for 1 to 2 h and quantified by gas chromatography (7). Between each sampling, the flasks were flushed with sterile fresh air. Radioactive ethylene produced by leaf discs incubated with (3,4-^{14}C)methionine was absorbed in 1 ml of ice-cold, 100 mM mercuric acetate (in methanol) and assayed in a liquid scintillation counter.

For ACC determination, leaf discs were homogenized in 70% ethanol, and the homogenate was centrifuged at 10,000 x g for 20 min. The supernatant was evaporated to dryness under vacuum at 40°C and the residue suspended in 1 ml of water. The solution was used directly or passed through a cation-exchange column (Dowex 50, H^+). In the latter case, ACC was then eluted with 2N NH_4OH and concentrated as before. ACC was then degraded to ethylene by the method of Lizada and Yang (8).

With a few exceptions, three replicates were used routinely. Experiments were repeated at least twice and gave reproducible results. Representative experiments are presented.

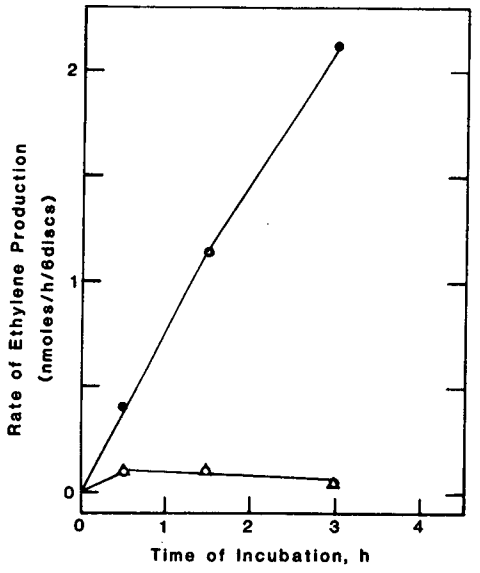

Fig. 1. Induction by Cellulysin of ethylene production in tobacco leaf discs. Leaf discs (6 per flask) prepared as described in Materials and Methods were floated on 1 ml of basal medium containing 600 mM sorbitol, 10mM $CaCl_2$, 10 mM MES, pH 6.0, 50μg/ml streptomycin sulfate and 50 U/ml penicillin G in the absence (○) and presence of either 1.3% Cellulysin (●) or 0.05mM IAA (△). Rate of ethylene production is shown in relation to the time of incubation.

RESULTS

Previously Aharoni and Lieberman (9) reported that the rate of endogenous ethylene production by tobacco leaf discs, floating on water in the dark, was very low, taking as many as 7-8 days to reach 40-80 pmoles/h/g. A rapid induction of ethylene production was observed when tobacco leaf discs were treated with Cellulysin (Fig. 1). The Cellulysin-dependent ethylene production occurred in less than 1 h and rates (2120 pmoles/h/6 discs) which were over 17-fold greater than the control were reached within 3 h(Fig. 1). In the same time period, boiling Cellulysin or supplementing IAA in the absence of Cellulysin were ineffective in stimulating ethylene production (Fig. 1). In some experiments, a lag of less than 0.5 h was observed in Cellulysin-induced ethylene production. The induction of ethylene production occurred prior to the detection of protoplasts in the medium, using a light microscope. Over the 2 years during which this investigation was carried out variation was found in the rate of ethylene production induced by Cellulysin. Much of this variability was related to the age of the leaf, from which discs were prepared.

Table 1. Inhibition of Cellulysin-induced ACC formation and ethylene production by aminoethoxyvinylglycine(AVG) in tobacco leaf discs

Additions to the incubation medium*	C_2H_4 nmoles/h/g fr wt.	ACC nmoles/h/g fr wt.
None	0.25	0.12
Cellulysin	3.03	1.83
Cellulysin + 0.1 mM AVG	0.20	0.07

*The concentration of Cellulysin was 2%. Ethylene production was measured between 0-2h. ACC was isolated from the leaf discs after a 6 h incubation period and the concentration determined as described in Materials and Methods.

The marked increase in ethylene production by tobacco leaf discs in contact with Cellulysin was accompanied with an increase in the formation of ACC (Table 1), suggesting that the conversion of SAM to ACC is the target of Cellulysin action. This conclusion is supported by the inhibition of both the Cellulysin-induced ethylene production and ACC formation by aminoethoxyvinylglycine (AVG) (Table 1), a potent inhibitor (10,11) of ACC synthase which catalyzes the SAM $\rightarrow$ ACC reaction.

Cycloheximide(CHI) (10 µM), an inhibitor of cytoplasmic protein synthesis, did not block Cellulysin-mediated induction in ethylene biosynthesis (Table 2), shedding doubts on the involvement of de novo protein synthesis in this induction. This was further tested in experiments using (3,4-^{14}C)methionine supplemented with 1mM ^{12}C-methionine (to adjust for pool saturation) as a substrate for ethylene biosynthesis as well as protein synthesis.

Cellulysin induced the conversion of (3,4-^{14}C)methionine into $^{14}C_2H_4$, which was comparable to the rise in total ethylene production over the controls (Table 2). Under these conditions, Cellulysin caused 22% inhibition in the uptake of radioactive methionine and 42% inhibition in the incorporation of radioactivity into acid-insoluble material. Inclusion of CHI (10µM) and chloramphenicol(CAP) (100µg/ml) along with Cellulysin in the incubation medium further inhibited uptake of radioactive methionine by 14% and its incorporation into acid-insoluble (protein) radioactivity by 57%, but neither total ethylene production (+10%) nor ^{14}C-methionine incorporation

Table 2. Effect of chloramphenicol(CAP), cycloheximide(CHI), isopropanol and PMSF on Cellulysin-induced ethylene production and on protein synthesis*

Addition to the basal medium	Total C_2H_4 nmoles	$^{14}C_2H_4$	Protein (PCA-insoluble)	^{14}C-met Uptake	Ratio $^{14}C_2H_4$/ ^{14}C-protein
		----------nCi----------			
None	0.11	0.04	3.84	93.0	0.01
Cellulysin	1.43	0.66	2.21	72.6	0.30
Cellulysin + CAP + CHI (10μM)	1.57	0.60	0.96	69.9	0.63
Cellulysin + CAP + CHI (50μM)	1.07	0.26	0.56	65.3	0.46
Cellulysin + isopropanol	1.18	0.43	1.79	82.4	0.24
Cellulysin + PMSF	0.56	0.22	1.50	76.2	0.15

*Each data point is the mean of 2, 6 leaf disc replicates, prepared as described in the "Materials & Methods". Discs were preincubated in 1 ml of filter-sterilized basal medium containing 1μCi L-(3,4-^{14}C)methionine (50μCi/μmol),1μmole ^{12}C-methionine and additions listed in the table. After 3 hr, total C_2H_4 and $^{14}C_2H_4$ were determined. Discs were then rinsed with basal medium and stored at -20°C until extraction with perchloric acid. The concentrations of various additives were: Cellulysin, 1.2%; CAP, 100 μg/ml; CHI, as indicated; PMSF in isopropanol, 1mM; isopropanol control for PMSF, 10μl.

into $^{14}C_2H_4$ (-8%) were affected (Table 2). Only at much higher concentrations (50μM) was CHI effective in inhibiting both the total ethylene production (by 25%) and radioactive ethylene (by 61%), when 75% of radioactivity into acid-insoluble material was inhibited. Therefore, under conditions when 57% of protein synthesis is inhibited neither total ethylene nor radio-labeled ethylene formation were affected. Indeed, the ratio of $^{14}C_2H_4/^{14}C$-protein in Cellulysin-treated tissue, which was 0.3, increased to 0.63 in the presence of 10μM CHI and CAP, and to 0.46 when 50μM CHI was used (Table 2).

It was suspected that Cellulysin-induced ethylene production may involve a modification of the already present but inactive ACC synthase, e.g. by a protease-mediated activation phenomenon observed elsewhere (12,13)in other systems. Therefore, ethylene production and ACC levels were analyzed in tobacco leaf discs after incubation with Cellulysin in the absence and presence of known protease inhibitors. The results (Table 3) show that

Table 3. Effect of protease inhibitors on Cellulysin-mediated increase in ACC level and rate of ethylene production*

Addition to the basal medium	Ethylene Production (nmoles/h/g fr. wt.)	ACC (nmoles/h/g fr. wt.)
None	0.25	0.11
Cellulysin	3.10	1.84
Cellulysin + PMSF	0.40	0.05
Cellulysin + trypsin inhibitor(1 mg)	1.83	0.50
Cellulysin + Pepstatin A (0.1 mg)	3.10	1.39

*Details are given in the text and in the legend to Table 1.

phenylmethanesulfonic acid (PMSF) and soybean trypsin inhibitor, but not Pepstatin A (an inhibitor of carboxyl proteases), markedly inhibit ethylene production induced by Cellulysin and that the inhibition parallels a corresponding decrease in the level of ACC. There was some inhibition of ACC formation but no inhibition of ethylene production by Pepstatin A (Table 3). This experiment, however, did not rule out the possibility that the serine-type protease inhibitors could also prevent any contaminating protease present in Cellulysin from acting on the leaf discs, if, in fact, that was the mechanism of induction of ethylene production by Cellulysin. To test this, Cellulysin was preincubated with PMSF for 30 min to inactivate any serine-type protease or esterase. The precipitated protein was centrifuged down and supernatant gel-filtered through a column of Sephadex G-25 (30 ml bed volume) to remove any free PMSF present and tested for ethylene-producing activity in tobacco leaf discs. PMSF-treated Cellulysin was as effective in stimulating ethylene biosynthesis as the untreated enzyme mixture (Table 4), while the addition of PMSF to the incubation medium inhibited Cellulysin-induced ethylene production. PMSF inhibited production of both the total and radioactive ethylene from (3,4-^{14}C)methionine in the presence of Cellulysin (Table 2) confirming effective inhibition of Cellulysin-induced ethylene production by PMSF. During the short incubation periods used, CHI, PMSF and

Table 4. Pretreatment of Cellulysin with PMSF does not prevent Cellulysin-induced ethylene production*

Addition to the basal medium	Ethylene production (nmoles/2h/6 discs)
None	0.08
Cellulysin	1.64
Cellulysin + isopropanol	1.37
Cellulysin + PMSF	0.83
Isopropanol-treated Cellulysin after gel filtration	1.67
PMSF-treated Cellulysin after gel filtration	1.54

*For details see text and the legend to Table 1. Incubation period was 2 hr.

Pepstatin A did not affect the conversion of exogenously applied ACC to ethylene, while trypsin inhibitor slightly stimulated this conversion (data not shown).

DISCUSSION

We have demonstrated a rapid induction of ethylene biosynthesis in tobacco leaf discs by Cellulysin treatment. This induction appears to be related to a tremendous increase in the activity of ACC synthase as judged by the formation of ACC and inhibition by AVG. In this respect, these results are similar to stress- (3,14) and auxin-induced (4) ethylene production where SAM → ACC conversion has been invoked as the rate-limiting step. Such an increase in the activity of ACC synthase has been attributed to de novo synthesis because of its inhibition by relatively high concentrations of CHI (3). However, doubts about this conclusion have been expressed (14,21) and no direct proof of actual de novo synthesis of ACC synthase has been reported.

In the phenomenon described here, either the low amount of protein synthesis that occurs in the presence of CHI is sufficient to induce ethylene biosynthesis at the level of ACC synthase activity or, alternatively, in certain situations, the increase in ACC levels and ACC synthase can occur by a

post-translational modification, involving modification of a pre-existing, enzymatically inactive protein. The absence of lag, specificity of inhibition of both Cellulysin-mediated ethylene production and ACC formation by a serine protease inhibitor, PMSF(since the carboxyl protease inhibitor, Pepstatin A, was not inhibitory), and ineffectiveness of CHI to inhibit this process support our contention that a post-translational modification/activation of an "inactive" ACC synthase may be involved. Also, we have observed (15) that PMSF inhibits the increase but not the activity of ACC synthase in wounded tomato fruit slices. However, elucidation of the activation mechanism alluded to above awaits preparing antisera to pure ACC synthase and analyzing the content of this protein in tissues unactivated and activated for ethylene production.

Cellulysin-mediated increase in ethylene biosynthesis demonstrated here has other implications. For example, the marked increase in ethylene production during host-parasite interactions (16,17) may be caused by an interaction between cell wall degrading enzyme(s) secreted by the pathogen and the host plant tissue in the same manner as reported here. Such a rapid induction of ethylene production by a pathogenic organism could be a defense mechanism involving a hypersensitive reaction (18) by which plants eliminate infected areas and/or possibly a mechanism which enhances the pathogencity or virulence of the parasite.

Finally, ethylene is known to cause abscission of leaves and fruits by inducing cellulase and polygalacturonase activity in the abscission zone (19,20). It remains to be understood whether this induction of cellulase and polygalacturonase activities is a mechanism by which the initial trigger caused by ethylene leads to continued, self-activation of ethylene production via the stimulation of ACC synthase by cellulase and/or polygalacturonase. This phenomenon would generate a cyclic process until the leaf or fruit abscisses.

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Enhancement by Ethylene of Cellulysin-Induced Ethylene Production by Tobacco Leaf Discs[1]

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ABSTRACT

Cellulysin-induced ethylene production in tobacco (*Nicotiana tabacum* L.) leaf discs was enhanced several-fold by prior exposure of the leaf tissue to ethylene. This enhancement in the response of the tissue to Cellulysin increased rapidly during 4 and 8 hours of pretreatment with ethylene and resulted from greater conversion of methionine to ethylene. On treatment with Cellulysin, the content of 1-aminocyclopropane-1-carboxylic acid (ACC) in leaf discs not pretreated with ethylene markedly increased while that of the ethylene-pretreated tissue was only slightly higher than in the tissue incubated in the absence of Cellulysin. Ethylene-treated tissue, however, converted ACC to ethylene at a faster rate than air controls. These data indicate that ethylene stimulates Cellulysin-induced ethylene production by stimulating the conversion of ACC to ethylene. Data are also presented on a possible relation of this phenomenon to ethylene produced by the tobacco leaf upon interaction with its pathogen, *Alternaria alternata*.

Ethylene is produced by higher plants as well as microorganisms and its role as a plant hormone is well established (1, 15, 16). In most diseased plants, ethylene production is stimulated and the involvement of ethylene in pathogenesis has been suggested (6, 21, 25). Ethylene may be involved in disease resistance by induction of enzymes or by formation of antifungal compounds (8, 10, 11, 21, 28), while it may also promote sensitivity of higher plants to external stimuli by accelerating senescence (1). Little is known about the mechanism of production and roles of ethylene during the interaction of host and parasite. There is also no clear knowledge of the contribution of host or pathogen to ethylene produced during disease or of the site of ethylene production (21). However, an early event in the interaction between the host and its pathogen is the secretion, by the pathogen, of cell-wall degrading enzymes (5) including cellulase (26). It was therefore of considerable interest to us that a fungal cell wall digesting preparation, 'Cellulysin,' induced ethylene biosynthesis in tobacco leaves (4) by causing a rapid formation of ACC[3], the immediate precursor of ethylene. We report here on further studies of this phenomenon, its enhancement in tissues pretreated with ethylene, and its possible relationship to ethylene produced by the tobacco leaf upon interaction with its pathogen, *Alternaria alternata*.

MATERIALS AND METHODS

Three cultivars of tobacco (*Nicotiana tabacum* L.) plants were used, viz. Burly Mammoth, Maryland 609, and Xanthi. Materials and methods used for the preparation of leaf discs and ethylene determination were described earlier (4).

Tobacco leaves were pretreated in air or ethylene in 3.8-L desiccators. Each leaf was divided in half by cutting along its mid-rib and each half was placed on filter paper, moistened with water, in individual desiccators. A vial containing filter paper soaked with 2 ml of 0.25 M mercuric perchlorate was placed in the 'air control' desiccator to absorb traces of ethylene. Similarly, in some experiments, whole, potted tobacco plants were pretreated with ethylene. Four plants were placed inside a 2-ply (3 mil), 45-L polyethylene bag for 16 h with the desired ethylene concentration. Unless otherwise indicated, three leaf discs (1 cm in diameter, weighing 50 mg) were incubated in 25-ml Erlenmeyer flasks with 0.5 ml of the basal medium containing 700 mM sorbitol, 10 mM Mes (pH 6.0), 10 mM $CaCl_2$, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin G in the absence or presence of Cellulysin (Calbiochem) (4). Cellulysin was desalted before use by ultrafiltration with an Amicon PM-10 membrane (3). Ethylene was allowed to accumulate for 1 h and quantified by GC (17). Between each sampling, flasks were flushed with sterile fresh air.

Radioactive ethylene produced by the leaf discs upon incubation with [3,4-^{14}C]methionine was accumulated for 1 h in the incubation flask. A 12-ml gas sample from the atmosphere above the discs was then injected (9) into a 485-ml jar which contained a filter paper wetted with 0.6 ml of 0.25 M mercuric perchlorate in a scintillation vial. After 2 h incubation on ice, 10 ml of a toluene-based scintillation cocktail (25% Triton X-100 in Liquifluor, New England Nuclear) was added to each vial. Radioactive ethylene absorbed by the filter paper was counted in a liquid scintillation counter (9). Uptake of [^{14}C]ACC by the leaf discs was checked by incubating the leaf discs for 15, 60, or 120 min with 0.5 ml of [2,3-^{14}C]ACC (3754 dpm/nmol). Aliquots of the incubation solution were sampled at the indicated times and counted to estimate the residual radioactivity. After each incubation, the discs were removed from the incubating solution, washed, and further incubated in water for 2 min to release the

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[2] On leave from Division of Fruit and Vegetable Storage, ARO, The Volcani Center, Bet Dagan, Israel.

[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; SAM, *S*-adenosylmethionine.

Table I. *Effect of Different Pretreatments on the Cellulysin-Induced Ethylene Production by Tobacco Leaf Discs*

Tobacco (cv Burly Mammoth) leaf tissue was pretreated in ethylene (60 μl/l) or in air (less than 8 nl/l of ethylene) for 16 h at 25°C at high (95%) RH under cool-white fluorescent light (1750 lux). Ethylene production was measured during the 3rd h of incubation after the addition of Cellulysin (1.2%, w/v). Discs without Cellulysin addition produced ethylene at rates lower than 45 nl/g fresh wt·h.

Treatment	Ethylene Production
	nl/g·h
Freshly cut (no pretreatment)	160
Pretreated as leaf (halves)	
Air	185
C_2H_4	550
Pretreated as discs	
Air	495
C_2H_4	760

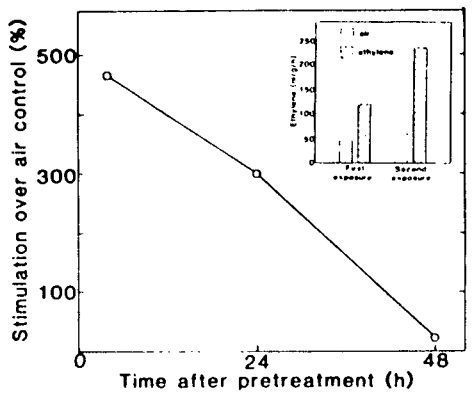

FIG. 1. Effect of the time elapsing between pretreatment in ethylene and Cellulysin applications on the Cellulysin-induced ethylene production by tobacco (cv Xanthi) leaf discs. Potted plants were pretreated in 60 μl/l ethylene for 16 h. Cellulysin was then applied to leaf discs cut from the treated plants at the times indicated. Absolute values of air controls, ranged from 100 to 150 μl C_2H_4/g fresh wt·h. Inset shows the response of leaf discs to Cellulysin after a second, 16-h exposure of the leaf to ethylene, 48 h after the first exposure, as compared to the response after the first exposure.

Table II. *Production of Ethylene by Cellulysin-Treated Leaf Discs of Several Tobacco Cultivars*

Procedures were similar to those outlined in Table I. Control discs incubated without Cellulysin produced ethylene at less than 25 nl/g·h for Burley Mammoth and less than 6.5 nl/g·h for all other cultivars.

Cultivar	Ethylene Production		
	Freshly Cut	Pretreatment	
		Air	Ethylene
	nl/g·h		
Burly Mammoth	160	200	575
Maryland 609	30	55	275
Xanthi	23	50	130
Yellow Mammoth	34	40	73
TI 102	35	38	36

label from intracellular spaces. Thereafter, the tissue was frozen and homogenized with 0.8 ml of 80% ethanol. Aliquots of the homogenate were then sampled and counted. Total ACC and [^{14}C]ACC in the tissue after incubation with [3,4-^{14}C]methionine were determined in extracts homogenized with 80% ethanol as

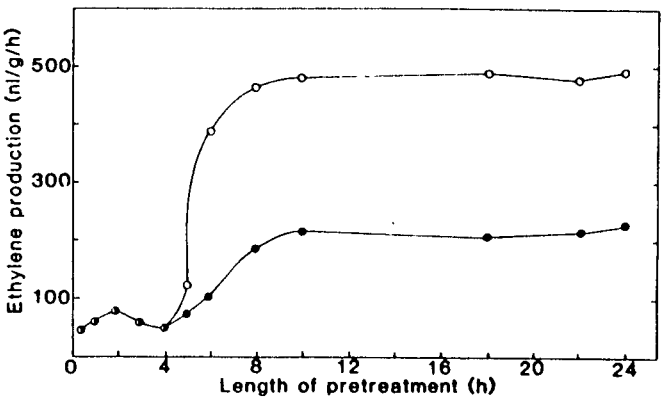

FIG. 2. Effect of the length of the pretreatment period on the Cellulysin-induced ethylene production in tobacco (cv Burly Mammoth) leaf discs. (●), Half leaf pretreated in air; (○), half leaf pretreated in ethylene (60 μl/l). Values are for the 3rd h of incubation after the addition of Cellulysin.

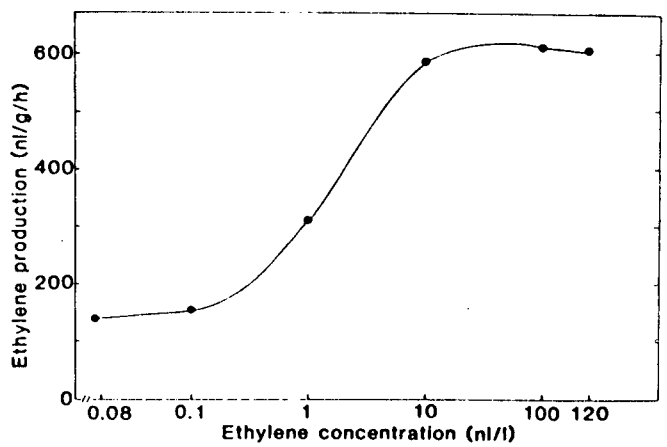

FIG. 3. Effect of the concentrations of ethylene during pretreatment on the Cellulysin-induced ethylene production in tobacco (cv Burly Mammoth) leaf discs. Procedures were similar to those outlined in Table I.

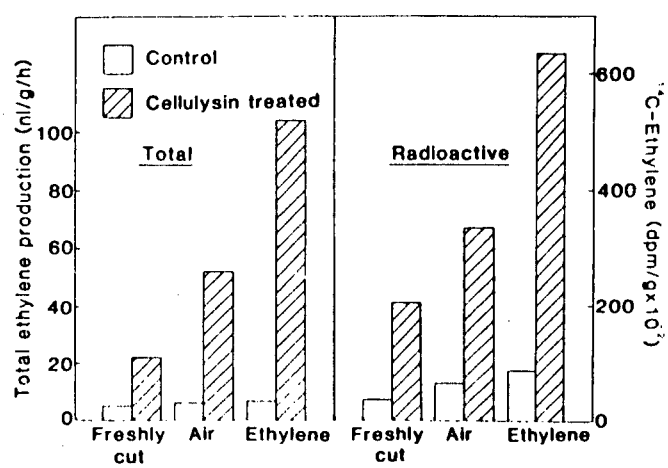

FIG. 4. Comparison between rates of total and labeled ethylene production from [3,4-^{14}C]methionine by tobacco (cv Xanthi) leaf discs. Leaves were pretreated in air or ethylene, then discs were cut and treated with Cellulysin. The values represent average rates of ethylene production during the first 2 h of incubation after the addition of Cellulysin.

Table III. *Total and Labeled ACC Content and Ethylene Produced by Tobacco (cv Xanthi) Discs Incubated with [3,4-^{14}C]Methionine in Relation to Different Pretreatments*

Leaf discs, either freshly cut or after the indicated treatments given to whole leaves, were incubated with the basal medium (6 discs/ml) containing 1952 dpm/nmol methionine.

Leaf Tissue, Treatment	Cellulysin Addition	C_2H_4[a]		ACC[a]		[^{14}C]ACC/[^{14}C]ESF[b]
		nmol	*dpm* × 10^3	*nmol*	*dpm/nmol*[c]	× 10^3
Fresh	−	0.15	3.80	1.40	2714	1.45
	+	0.65	14.30	26.95	531	8.06
Pretreated in air; 16 h	−	0.20	3.50	1.05	3333	1.04
	+	1.50	7.30	30.10	243	6.24
Pretreated in C_2H_4; 16 h	−	0.20	3.45	1.05	3286	1.38
	+	3.25	2.85	7.70	370	1.46

[a] Values are for rate, g^{-1} h^{-1}.
[b] ESF ethanol-soluble fraction.
[c] Specific activity.

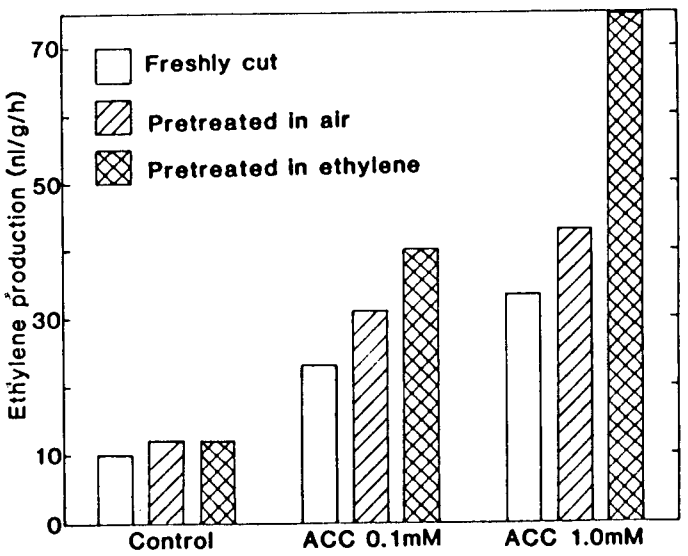

FIG. 5. Effect of pretreatment of tobacco (cv Xanthi) leaves in ethylene on their subsequent conversion of ACC to ethylene, in the absence of Cellulysin. The procedure was similar to that described in Table I. Values of ethylene production are for the 1st h of incubation following the addition of ACC, in the presence of 1 mM AVG.

described earlier (18).

Inoculation of tobacco leaves with *A. alternata* (Fr.) Keissl was carried out by the method of Spurr (24). Leaf discs, 1 cm in diameter, were inoculated by dipping them for 1 min in a spore suspension (10^3 spores/ml). Uninoculated control discs were dipped in water. Eight discs were then transferred into each 25-ml Erlenmeyer flask, placed on moist filter paper, and incubated at 25°C in the dark. Ethylene produced by the inoculated and control discs was measured by GC.

Three replicates were routinely used and experiments were repeated at least twice and gave similar pattern of results. However, due to the variability of the greenhouse-grown plants, results of typical experiments are presented.

RESULTS

As shown previously (4), tobacco leaf discs incubated in a medium containing Cellulysin respond by increased ethylene production (Table I). This response of leaf discs to Cellulysin was further enhanced several-fold by pretreating the detached leaf in ethylene (Table I). However, when leaf discs instead of whole leaves were pretreated in air, their subsequent response to Cellulysin was similar to that of the discs cut from ethylene-treated leaves. Ethylene-treated leaf discs produced ethylene in response to Cellulysin at a rate 1.5 times higher than discs treated in air (Table I). This response of ethylene-treated leaf discs or single leaves to Cellulysin was also evident when whole, potted plants were pretreated in ethylene. Under these conditions, the effect of the treatment was maintained for at least 24 h after the plants were removed from ethylene (Fig. 1). When leaves of the treated plants were detached and pretreated for a second time in ethylene, 48 h after the first treatment, the subsequent response of discs cut from these leaves to Cellulysin was 50% more than that of discs cut from leaves pretreated in ethylene for the first time (Fig. 1, inset).

Of the tobacco cultivars studied, Burly Mammoth produced the highest rates of ethylene in response to Cellulysin; cultivar differences occurred whether freshly cut leaves or leaves pretreated in ethylene were used, although some cultivars (*i.e.* TI 102) did not respond to the ethylene pretreatment (Table II). The maximal response of the leaf to ethylene pretreatment was reached between 4 and 8 h of incubation. Periods longer than 10 h of incubation did not further increase the subsequent response of the discs to Cellulysin (Fig. 2). The optimal concentration of ethylene for maximal response was between 10 and 100 μl/l (Fig. 3).

To verify that the increased production of ethylene by the discs from ethylene-pretreated leaves in the presence of Cellulysin was due to *de novo* synthesis instead of release of absorbed or bound ethylene from the tissue, discs from leaves pretreated in ethylene were incubated with [3,4-^{14}C] methionine and Cellulysin. Total and labeled ethylene were then assayed. The results (Fig. 4) showed that Cellulysin-induced ethylene production in ethylene-treated tissue resulted from the conversion of methionine to ethylene. Furthermore, these data showed close similarities in the pattern of total and labeled ethylene produced and in the specific radioactivity of ethylene produced by Cellulysin-treated discs from freshly cut or ethylene-pretreated leaves suggesting a common biosynthetic pathway.

Cellulysin caused a marked accumulation of ACC in the treated tissue (Table III), a phenomenon observed earlier (4). However, this effect was pronounced in the freshly cut or air-pretreated discs and was relatively inconspicuous in discs pretreated in ethylene (Table III). The lower content of ACC in the ethylene-treated discs in the presence of Cellulysin could result from a higher rate of conversion of ACC to ethylene. To test this

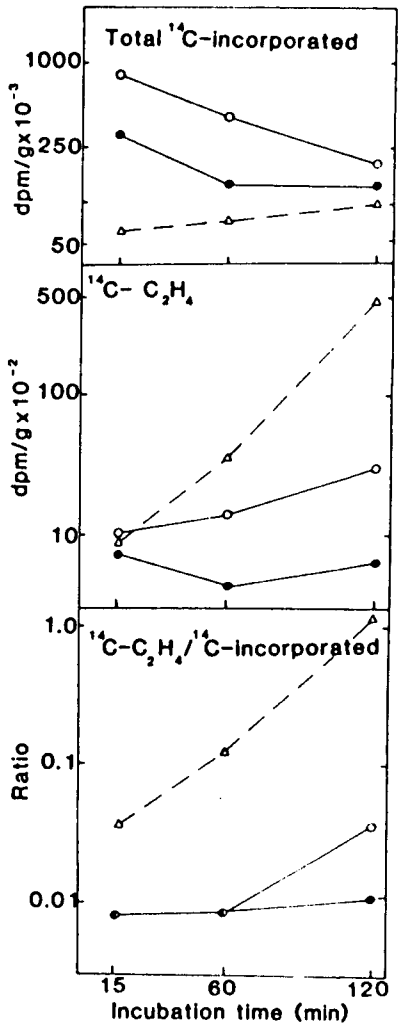

FIG. 6. Incorporation of [2,3-^{14}C]ACC and labeled ethylene production by tobacco (cv Burly Mammoth) leaf discs following pretreatment in air or ethylene, in the absence of Cellulysin. Pretreatment conditions were similar to those described in Table I. (●), Freshly cut discs; (○), discs cut from air-pretreated leaves; (△), discs cut from ethylene-pretreated leaves.

possibility, we compared the rates of ethylene production by freshly cut leaf discs as well as by discs cut from air- or ethylene-pretreated tissue in response to exogenously added ACC (without Cellulysin) while blocking the formation of endogenous ACC by including 1 mM AVG in the incubation medium. The results (Fig. 5) confirmed previous observations in *Nicotiana* (19) and other systems (14, 22) that aging of leaf tissue increases their capacity to produce ethylene from ACC. Our data further indicated that the conversion of exogenously added ACC to ethylene was twice as much in ethylene-treated than in air-treated leaf tissue while the uptake of labeled ACC by the ethylene-treated discs was lower than that by the air-treated or freshly cut leaf discs (Fig. 6).

Tobacco leaf discs inoculated with the pathogen, *A. alternata*, produced ethylene at higher rates than did uninoculated controls (Fig. 7). Moreover, inoculated discs cut from leaves pretreated in ethylene produced higher rates of ethylene than those cut from fresh tissue or from leaves pretreated in air.

DISCUSSION

Enhancement by ethylene of the Cellulysin-induced ethylene production in tobacco leaf discs as evidenced here exhibited

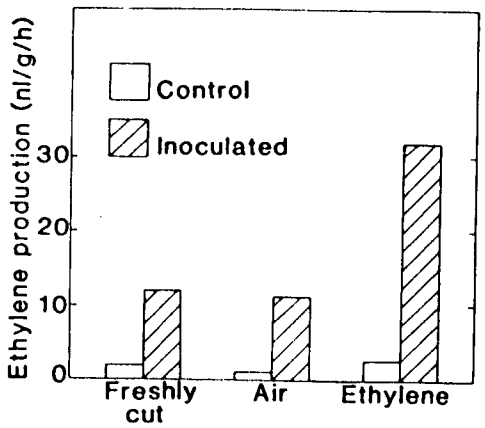

FIG. 7. Effect of pretreating tobacco (cv Burly Mammoth) leaves i air or ethylene on the subsequent ethylene production, 24 h after ino ulation of leaf discs with *Alternaria alternata*. Freshly cut leaf discs we inoculated by dipping them for 1 min in a spore suspension of 10^3 spore ml.

characteristics common to many other physiological effects o ethylene (1, 16), viz. concentration-dependence during pretrea ment (Fig. 3), a several-hour lag period (Fig. 2), and a require ment for the continuous presence of ethylene for maximal re sponse (Fig. 1). The greater response of tobacco leaf discs t Cellulysin following a second exposure of the leaves to ethylen (Fig. 1, inset) also seems to be a common effect of ethylene o plant tissues. Such a response was reported earlier (10) for th ethylene-induced formation of the phytoalexin, isocoumarin, i carrot roots, and recently (23) for the induction by ethylene o ethylene biosynthesis in citrus leaf discs. Such responses t ethylene could be observed only in tissues where an initia exposure of the tissue to ethylene does not evoke the autocatalyti production of ethylene common to climacteric-type fruits (1 22). A possible explanation of such a phenomenon was offere earlier (10) based on the data obtained on the isocoumari formation. There it was suggested that the ethylene inductio may be a two-step process: the first, activation of the biosynthet system through the induction of enzymes. This process is quan titatively dependent upon the length of the initial ethylene in duction period. The second step involves the actual synthesis o the phytoalexin and also requires the presence of ethylene (10) The relatively slow rate of decline in the response of tobacco t Cellulysin (Fig. 1) following removal of ethylene may be due t a higher rate of ethylene production by ethylene-treated leave than by those pretreated in air. Thus, ethylene may be present in or near these leaves at slightly higher concentrations than in or near control leaves. Our findings (Fig. 4, controls) support this suggestion as do those of Aharoni and Lieberman (2) who showed that tobacco leaf discs pretreated in ethylene for 24 h produced ethylene at a slightly higher rate for at least 1 to 3 d after the treatment compared to control discs.

The mode of action of ethylene in enhancing the response of tobacco leaves to Cellulysin may be complex. The following findings suggest an explanation: (a) pretreatment of leaf discs in air could partially substitute the effect of pretreatment in ethylene of whole leaves (Table I); and (b) pretreatment of leaf tissue in air also slightly, but consistently, enhanced the response of the tissue to Cellulysin (Table I; Figs. 2 and 5) as compared to freshly cut leaves. On this basis, we suggest that ethylene could be involved in the enhancement of the tissues' response at extremely low concentrations (*i.e.* less than 8 nl/l). Alternatively, these findings may suggest that other factors, in addition to ethylene, may be involved in the process. Such a suggestion was offered by Geballe and Galston (13) who studied wound-induced resist-

ance to cellulase in oat leaves and reported that ethylene was a factor in this process (12). A similar phenomenon was observed earlier in prune tissue (27). However, since exogenously applied ethylene could only partially substitute for the wounding effect, Geballe and Galston suggested (12) that the induction of resistance may require a wound signal in addition to ethylene.

Whether or not ethylene is the direct factor in the enhancement of the Cellulysin-induced ethylene biosynthesis in tobacco leaves, the results presented here clearly indicate that higher rates of ethylene production are not merely the result of the release of ethylene from the tissue. Rather, it originates from enhanced biosynthesis from methionine (Fig. 4). Also, these data indicate that in all leaf treatments, *i.e.* freshly cut, air-, and ethylene-pretreated, a common precursor and biosynthetic pathway lead to the production of ethylene (Fig. 4). However, following incubation with Cellulysin, the ACC content was much lower in discs from ethylene-treated leaves than from freshly cut or air-pretreated discs (Fig. 6). We interpret these findings to suggest that while Cellulysin induces the formation of ACC, presumably due to increased activity of ACC synthase (4), ethylene treatment of leaves causes a higher rate of conversion of ACC to ethylene, thus increasing the utilization of newly formed ACC in the ethylene-treated tissue. This suggestion is supported by the findings that discs from ethylene-treated leaves converted exogenously applied ACC to ethylene at a higher rate than discs from freshly cut or air-pretreated leaves (Fig. 5), irrespective of the differences in the uptake of [^{14}C]ACC (Fig. 6).

In a recent report, Riov and Yang (23) reported that a 24-h exposure of the citrus leaf discs to ethylene induced ethylene production by the discs. The stimulatory effect of ethylene resulted, 36 to 48 h after exposure to ethylene, from an increased formation of ACC as well as conversion of ACC to ethylene. In some respects, this effect of ethylene, and a similar observation reported recently for preclimacteric cantaloupe (14) resembles the enhancement by ethylene of the Cellulysin-induced ethylene production reported here. However, unlike the requirement of 36 to 48 h by citrus leaf discs to exhibit increased ACC formation and ethylene production in response to ethylene, the enhanced response of tobacco leaf to ethylene as exhibited by measuring Cellulysin-induced ethylene production occurs within 4 to 8 h. Also, in our studies the faster response of the tissue to ethylene was on the ethylene-forming enzyme and not on ACC synthesis. This delineation was possible since ACC synthesis is induced by Cellulysin within 1 h of incubation (4) and thus is not rate limiting.

Since the ethylene-forming enzyme seems to be membrane associated (20, 29) and ethylene is known to cause changes in membrane permeability (1), it is possible that increased conversion of ACC to ethylene in ethylene-treated tissue may be mediated through a change in the membrane milieu of this enzyme.

Earlier it was suggested (4) that Cellulysin-induced ethylene production may be a hypersensitive response of the tissue normally elicited under conditions of stress or infection. Our preliminary findings (Fig. 7) indicate that the interaction between tobacco leaves and the fungal pathogen *Alternaria alternata* can cause increased ethylene production. An enhanced response of the leaf tissue to a cellulase, to other hydrolytic enzymes (7), or to another component of fungal origin, following exposure to ethylene, may thus create an autocatalytic-like process of ACC induction and ethylene production during fungal growth in the leaf. This may predispose healthy leaf tissue to the advancing fungal mycelium by promoting premature senescence. Alternatively, it may be a process by which the plant tissue maintains its ethylene-induced defense reactions aimed at combating infection. At the present time, we are examining the possibility that induction of ethylene by *A. alternata*, during its growth on tobacco leaf tissue (Fig. 7), may involve the action of a fungal elicitor.

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Ethylene Biosynthesis and Related Physiological Changes in *Penicillium digitatum* - Infected Citrus Fruit

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Ethylene Biosynthesis and Related Physiological Changes in *Penicillium digitatum* - Infected Citrus Fruit.

Abstract

Biochemical and physiological changes and their relation to ethylene biosynthesis were studied in grapefruit (*Citrus paradisi* Macf. cv. Marsh Seedless) peel, 5 to 6 days after inoculation of the fruit with *Penicillium digitatum* Sacc. In both the albedo and flavedo tissues of the peel, fungal invasion was associated with an increase in the content of galacturonic acid and with a decrease in the pH and in soluble protein content of the tissue. The extent of these changes was related to the distance of the sampled site from the maceration front. Two parallel and distinct maceration fronts could be defined in the peel, the one in the albedo consistently preceding that in the flavedo. Fungal hyphae were present in the apparently healthy region of the fruit up to 15 mm from the maceration front.

Fungal invasion was associated also with an increase in the content of 1-aminocyclopropane-1-carboxylic acid (ACC) and ethylene production. On the other hand, the ability of the tissue to convert ACC to ethylene decreased with the development of the infection. Since this fungus is capable of producing ethylene at high rates, we propose that both the peel and the fungus were involved in the enhanced ethylene production of the diseased fruit. An early relatively low rate of ethylene production seems to originate mostly from the fruit while a later and higher rate of ethylene production originated mostly from the fungus.

INTRODUCTION

Citrus fruits are usually classified as nonclimacteric fruits. This is based on the fact that mature fruits do not exhibit a rise in either respiration or ethylene production throughout their normal pre- and post-harvest life (6,28). However, when these fruits are infected by the common postharvest pathogen Penicillium digitatum Sacc., high rates of ethylene are produced (11,27). These could be associated with the ability of the fungus to produce ethylene in culture (10,17). Increased production of ethylene is a common feature of diseased plants (22, 29); while ethylene may originate from the fungus it may also be produced partly or entirely by the plant as a response to the infection (12). Stress conditions have been reported to be accompanied by increased levels of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene in higher plants (1). Generally, a lag period is observed between the increase in ACC levels and the subsequent increase in the rate of ethylene production. However, in several plant systems accumulation of ACC is not always followed by a pronounced increase in ethylene production, as in post-climacteric avocado fruit (14). Although some microorganisms can utilize ACC as a sole nitrogen source (15), it is not clear whether P. digitatum can utilize ACC for ethylene production.

During infection of citrus fruits, P. digitatum apparently can produce a pectolytic enzyme, characterized as an exo-polygalacturonase (poly-1, 4-D-galacturonide glycanohydrolase, E.C. 3.2.1.15) (4,5), This enzyme hydrolyses pectin to D-galacturonic acid monomers, resulting in a loss of physical stability of the infected tissue. D-galacturonic acid in turn causes swelling of cell walls and collapse of their cytoplasm, leading eventually to tissue maceration (5). Barmore and Brown (5) found a maximal level of 12 mg g^{-1} of D-galacturonic acid in the peel of P. digitatum - infected Hamlin and Valencia oranges, while Miyakawa (20) detected a level of more than 2% in Satsuma oranges during the early stage of infection

with P. digitatum.

We report here the results of a study of several physiological changes caused in P. digitatum-infected citrus fruit and their relationships to ethylene production by this host-pathogen interaction.

MATERIALS AND METHODS

Plant and fungal material

Grapefruits (Citrus paradisi Macf. cv. Marsh Seedless) used for this work were harvested in mid-season, and stores at 11 to 14°C (80 to 90% rh) until use. For inoculations, a single-spore culture of Penicillium digitatum Sacc. (ATCC # 10030) was used. The culture was propagated on potato dextrose agar slants. Prior to inoculation, the fruits were washed with water and surface sterilized with 70% ethanol. They were inoculated by introducing a 5x5x1 mm piece of inoculum into a V-shaped 3 mm deep cut, made 20 mm away from the stem-end of the fruit. The wound was then covered with masking tape, and the fruit was stored at 20 to 23°C in a ventilated dark chamber.

For the various tests, samples of peel were removed with a scalpel or a cork borer, and the albedo and the flavedo parts were separated. Representative peel samples were taken from several defined regions of the inoculated fruit (Fig. 1A). A minimum number of nine fruits was used as replicates for the determination of each parameter studied.

Biochemical and physiological assays

The quantitation of fungal mycelium or spores in the fruit peel was based on the determination of glucosamine (13), the monomer of chitin - a compound present in fungal cell wall but not in higher plant tissues. The method of Netzer, Kritzman and Chet (21) was used in order to quantitatively relate glucosamine content to the amount of fungus present in the tissue. A slight modification of this method involved the extending of the acid

hydrolysis of the sample from 6h to 9h to increase the sensitivity of the method.

The degradation rate of the peel pectic substances was determined by the assay of D-galacturonic acid (7). One g tissue was homogenized with 10 ml of 70% ethanol at $4^{o}C$ for 1 min using an Ultra-Turrax. The homogenate was then centrifuged at 27000xg for 10 min and the supernatant used as the tissue extract. In the same way water extracts of the albedo and flavedo parts of the peel were prepared for the determination of the pH, soluble proteins (9) and ACC content (18).

Production of ethylene by the peel tissue was determined as follows: Peel discs, 12 mm in diameter were cut from the fruit with a cork-borer. Each disc was separated into the albedo and flavedo parts. Samples from five discs were then weighed and placed inside 21 ml glass vials. One h later, the vials were sealed with rubber serum caps and kept closed for 60 min at $24^{o}C$ in the dark. A 2 ml sample of the atmosphere above the discs was then withdrawn by a hypodermic syringe, and its ethylene content determined by a Packard gas-chromatograph equipped with an alumina column and a flame ionization detector. Under these conditions, production of wound-induced ethylene could not yet be detected.

In some experiments, the excised discs in the vials were incubated for relatively long periods of up to 32 h. Ethylene production by these discs was determined periodically at 2,8,16,24 and 32 h after excision of the discs. Between each sampling period the vials were aerated and than resealed.

Six days after fungal inoculation, several, well defined zones could be observed on the fruit surface. These were defined as follows (Fig.1A): Green spores zone (SZ); a zone 50 to 80 mm in diameter around the inoculation site, where mature green fungal spores had developed; white mycelium zone (WMZ), which consisted of an area approximately 13 mm wide, surrounding the SZ and easily distinguishable by the dense white

mycelium which developed on the peel; maceration zone (MZ), which consisted of a belt approximately 17 mm wide, surrounding WMZ and easily distinguishable by the soft peel, and the vague appearance of oil glands on its surface; a healthy, non-infected area of the peel (HZ), consisting of the rest of the fruit, where no infection symptoms were visible; the maceration front (MF), a distinct line between the MZ and the HZ.

Since the distance of any part of the fruit peel from the MF was found to be useful as a reference point reflecting the extent of fungal invasion and various physiological changes taking place in the infected fruit, we expressed the data in relation to this value (Fig. 1A). MF is referred to as the zero point. Various sites in the HZ were referred to as +X, while sites on the infected zones were referred to as -X, where X indicates the distance (in mm) of the site from the MF.

All results were expressed on the peel tissue fresh weight basis, except those of glucosamine content which were expressed on the basis of dry weight.

RESULTS

Fungal presence in the peel

The distribution of fungal mycelium in the various regions of the fruit peel is shown in Fig. 1B. The fungus was found not only in the zones showing symptoms, but also in the adjacent apparently healthy tissue. Small amounts of glucosamine were found as far as 15 mm away from the MF in the flavedo of the HZ. The total content of the fungus increased at sites closer to the inoculation site. The most distant site in the albedo where glucosamine could be detected in the apparently healthy zone was at +5. Glucosamine in the albedo increased similarly as in the flavedo, but its amounts were considerably less. This difference was most obvious in the WMZ and in the SZ. In the latter region, glucosamine content in the albedo evinced a slight decrease.

Physiological changes associated with the infection

Fungal infection markedly affected the D-galacturonic acid content of the peel (Fig. 1C). The healthy albedo and flavedo parts contained only 1 to 2 mg g^{-1} of galacturonic acid. However, when the assayed site was closer to the MF than +10 in the albedo or +5 in the flavedo, the values increased to about 14 mg g^{-1} in the MZ and the WMZ, and then slightly decreased in the SZ. Galacturonic acid content was generally higher in the albedo than in the flavedo of macerated regions. Anatomic examinations in the MF region revealed that the albedo tissue was already macerated underneath the flavedal front. Therefore, there existed two parallel maceration fronts, and the inner albedo one was preceding the external flavedo front by 5 to 10 mm.

Considerable changes were evident also in the pH of the water extract of the peel, as a result of the infection (Fig. 1D). A moderate but consistent decline was evident in the pH of albedal water extract produced from healthy fruit, ranging from 5.5 at the stylar-end to 5.2 at the stem-end. Similar values were found in the healthy zone of a P. digitatum-infected fruit. However, a sharp decline in the pH value, from 5.1 to 3.7 occurred within the narrow region of +20 to -8, with no further changes throughout the infected region. A similar pattern of pH changes was found also for the flavedo tissue.

The content of water-soluble protein in the peel of healthy and P. digitatum-infected grapefruit is shown in Fig. 1E. In healthy fruit, a moderate and consistent decline in protein content of the flavedo tissue was evident, from 3.52 mg g^{-1} at the stylar-end to 2.87 mg g^{-1} at the stem-end. Somewhat higher levels of proteins (4.02 mg g^{-1}) were found throughout the flavedo of the healthy zone of a P. digitatum-infected fruit. However, a sharp decline in the water-soluble proteins content, from 4.11 to 0.73 mg g^{-1} occurred in the region of +20 to -8. The same low value was maintained in the whole infected region. A similar pattern

of water-soluble protein content was found also for the albedo tissue but all the values (except in the region -8 to -70 in the infected fruit) were substantially lower. A constant level of soluble proteins was maintained in the healthy fruit in all regions.

To test whether the decrease in pH of the water extract (Fig. 1D) could be related to the decline in the water solubility of the peel proteins (Fig. 1E), we examined the effect of pH adjustments of the water extract of the peel of a non-infected fruit on its protein content (Table 1). Only within a pH range of 5.4 to 4.5 was a correlation found between the pH value and the protein content.

Ethylene production and the infection process

The effect of fungal infection on ACC content of the peel is shown in Fig. 1F. The basal level of ACC in healthy fruit as well as in the distal parts of the HZ of an infected fruit was low (0.27 to 0.32 nmol g^{-1}). ACC content increased in both peel tissues due to the infection process. The increase occurred first in the albedo tissue where it reached a level of 8 nmol g^{-1} at the site of +8, and 23 nmol g^{-1} at the site of -25, as compared to 3 and 29 nmol g^{-1} in the flavedo, respectively. The highest ACC values were found in both tissues at the center of the SZ.

The rate of ethylene production by healthy albedo and flavedo discs, shortly after excision from the fruit, was very low (0.5 nl $g^{-1}h^{-1}$) Fig. 1G). Albedo discs maintained this low rate at the distal HZ as well as at the MZ and at the WMZ of the infected fruit. A small but clear increase in ethylene production occurred at the proximal HZ and in the SZ (Fig. 1G). Both peaks were relatively low and did not exceed the rate of 2.3 nl $g^{-1}h^{-1}$. In the flavedo tissue, a similar pattern of ethylene production was found in all the regions from +70 to -8. However, a marked increase in ethylene production was evident starting in the WMZ and reaching a peak of 18 nl $g^{-1}h^{-1}$ at the center of the SZ (Fig. 1G).

A more detailed study was done to further characterize ethylene production patterns in the infected fruit (Fig. 2). The data which were collected during a period of 32 h of incubation after excision, indicated similar changes with time in ethylene production by the albedo and flavedo tissues, when they were in a simila r level of infestation. However, the rate of ethylene production by infected flavedo discs was always higher than that of the infected albedo discs (Fig. 2 A to D). Rates of ethylene production changed similarly throughout the incubation time in the +70 discs from either heatlhy or infected fruit (Fig. 2 A). It increased in the albedo discs from 0.3 to 26 nl $g^{-1}h^{-1}$ during the first 24 h of incubation and subsequently increased to 2.2 nl $g^{-1}h^{-1}$. This pattern was found typical for both albedo and flavedo tissues. Similar pattern was found also in the ethylene production by +8 infected flavedo (Fig. 2B) but not by albedo discs which produced ethylene at the low rate of 3.6 nl $g^{-1}h^{-1}$ or lower throughout the incubation period. A very low rate of ethylene production (0.6 nl $g^{-1}h^{-1}$) by albedo or flavedo discs cut from -8 infected fruit was typical for the first 16 h of incubation (Fig. 2C). The following 16 h of incubation were characterized by an increase in ethylene production which was moderate in the albedo and very large in the flavedo. Ethylene production by discs from the -70 region of an infected fruit increased rapidly throughout the 32 h of incubation from 18 to 120 nl $g^{-1}h^{-1}$ for the albedo (Fig. 2D). Dense mycelium and spores developed on the surface of -70 discs of both tissues during the incubation period.

The ability of the fruit peel tissue to convert exogenously added ACC to ethylene was tested in discs cut from the different zones of the infected fruit (Table 2). Ethylene production by ACC-treated discs cut from the HZ (+70) increased markedly (nearly 1000% of control) while ACC-treated discs cut from the SZ (-70) or from the MZ (-8) did not produce ethylene at a higher rate than did the control discs. ACC was readily taken up by all discs (Table 2).

DISCUSSION

The data presented clearly show that in P. digitatum-infected grapefruit, fungal mycelium is present not only in the macerated regions, but also in the regions where no infection symptoms are visible. The slightly higher glucosamine content of the flavedo, as compared to the albedo tissue in the HZ may indicate that the fungal mycelium advances somewhat faster in the flavedo than in the albedo part of the peel. Autolysis of mature mycelium (23,24,26) could account for the slight decrease in glucosamine content of the albedo in the SZ.

The marked increase of D-galacturonic acid (Fig. 1C), may be regarded as a direct result of the fungal attack, probably because of hydrolysis of pectic substances by the fungal exo-polygalacturonase enzyme (4,5). The fact that the increase in galacturonic acid content at the infected site occurred in the albedo before the flavedo, may imply that the extent of maceration depends not only on the quantity of fungal mycelium present in the host tissue (Fig. 1B), but also depends on the host response. While the level of galacturonic acid found in the macerated tissue was similar to that reported previously (5,20), the decrease in galacturonic acid content in the SZ could perhaps be due to the substantial fungal presence and intensive metabolism at this region. The lower protein content (Fig. 1E) in regions where the pH of the water extracts decreased (Fig. 1D) may be only partially related to the change in pH (Table 1). The further decrease in the content of soluble proteins in the regions where the fungus was established could be related to a direct degradation of fruit proteins by the fungus.

We found the ACC content of the peel in healthy grapefruit as well as in the HZ of an infected grapefruit to be slightly higher than the values reported by Yu and Yang (30) and by Hyodo and Nishino (16) for healthy Valencia and Citrus hassaku, respectively. However, this does not explain how the infection induced a 23 and 109 fold increase in the content of ACC,

at the SZ of the flavedo and the albedo, respectively. One possible inducer of ACC could be the galacturonic acid which changed in the infected tissue in a similar pattern as did the ACC content of the peel tissue (Fig. 1C and F). However, direct application of galacturonic acid (50-400 mM) to albedo discs, did not increase their ACC content.

Another possible mechanism by which the fungus may increase ACC content in the peel tissues is related to the high rate of ethylene production evinced by the invading fungus (Fig. 1G), and to its autocatalytic effect on ACC synthesis as shown recently by Riov and Yang (25) for citrus leaf discs. Exogenous ethylene treatment of healthy grapefruits, however, did not cause any increase in the ACC content of the peel and its level remained low, during 48 h of exposure to ethylene (our data, not shown).

Pathogen infection may enhance ACC synthesis by the wounding effect that the invading fungal hyphae impose on the peel cells or by an inducer of fungal origin (2). Mechanical and biochemical stresses are known to initiate ACC synthesis (8,16,30); and Barmore and Brown (5) have shown plasmolyzed cells, and swollen cell walls near the invading hyphae, even in the apparently healthy zone (site +8), where fungal hyphae do not penetrate cell walls.

Whatever may be the specific signal to produce ACC, the accummulation of ACC in the infected peel region (Fig. 1F) and a similar phenomenon reported recently during virus infection of tobacco leaves (12) suggest that pathogen infection may affect differently the formation of ACC on one hand and its conversion to ethylene on the other. Indeed, we showed that in the infected region of the fruit, the tissue gradually lost its capacity to convert exogenously added ACC to ethylene (Table 2). Also in the infected fruit the level of ACC (Fig. 1F) probably is not limiting its conversion to ethylene.

Further information on the processes taking place in the infected fruit can be derived from the data obtained in the 32 h incubation experiments

(Fig. 2). Ethylene production by healthy peel discs was induced by the wounding and the mechanical stress of the excision (16). It may peak at high rates during 24 h of incubation (Fig. 2A) but, when the fungus is present, even at low amounts, suppression of plant ethylene production takes place as shown for +8 albedo in Fig. 2B and for -8 flavedo during the first 16 h of incubation - in Fig. 2C. This suppression of host ethylene by the developing fungus probably results from the impairment of the conversion step of ACC to ethylene while ACC is still being formed and therefore accummulates(Fig. 1F). Thus,the impairment of the ethylene producing machinary of the host seems to affect mostly one step of the biosynthetic pathway - the conversion of ACC to ethylene - which is associated with the cell membrane (19). It does not seem to result, however, from the general death of the tissue since,on one hand, this tissue continues to form ACC and,on the other,it respires at high rates (27). The accummulation of ACC in the infected tissue reported here and by De Latt and Van Loon, who showed accummulation of ACC in virus-infected tobacco leaves (12), may be similar to an earlier observation on ACC accummulation in postclimacteric avocado fruit (14). In both the diseased and the overriped tissues, the accummulation of ACC and the lower rates of ethylene production, are probably related to the disintegration of cell membranes. Thus, since the host ethylene producing mechanism is suppressed at the advanced stages of fungal infection of the fruit, but ethylene is still being produced at high rates (Fig. 1G and 2D), ethylene is likely to originate mostly from the fungus (17) at these stages of infection.

Based on our findings to date we suggest the following hypothesis as to the origin of the ethylene produced during the infection of grapefruit by P. digitatum. At the initial phases of the infection (+8 region), the enhanced production of ethylene originates from the peel cells and is ACC mediated. This biosynthetic pathway is apparently impaired

by the infection (region - 8 to -70) since the tissue exhibits a decreased ability to convert ACC to ethylene and ACC accummulates. The increased production of ethylene at this stage is likely, therefore, to be of fungal origin and does not use ACC as the precoursor, This hypothesis is in agreement with earlier suggestions (11,27) indicating that the pathogen is responsible for the high rates of the ethylene produced in this interaction. At present, the investigation is continued with the aim of obtaining direct evidence for the contribution of host and pathogen to the ethylene produced.

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Table 1 The influence of the pH of the water extract of the peel on the solubility of its proteins.

Peel Tissue	Water soluble proteins in the extract		
	Non-treated (mg g^{-1})	Modified to pH 4.5 (% of non-treated)	Modified to pH 3.5 (% of non-treated)
Albedo	1.87 $\pm$ 0.062	82.4 $\pm$ 6.6	80.0 $\pm$ 4.6
Flavedo	3.15 $\pm$ 0.073	80.9 $\pm$ 3.9	75.9 $\pm$ 5.9

Water extracts were prepared from peel tissues of healthy grapefruits. The pH of the extracts was either non-treated or modified (by HCl) to 4.5 or 3.5. The extracts were then incuabted at $0^{o}C$ for 8 h and water-soluble proteins were determined. The original pH of the extracts of the albedo and the flavedo were 5.2 $\pm$ 0.05 and 5.4 $\pm$ 0.06, respectively. Each value is a mean of 9 replicates with S.D.

Table 2 ACC content and conversion of ACC to ethylene by albedo discs, cut from different locations of P. digitatum - infected grapefruit.

Assayed site	Control		ACC treated
	ACC content (nmol g^{-1})	Ethylene production (nl $g^{-1}h^{-1}$)	Ethylene production (% of control)
+70	0.5 ± 0.03	0.2 ± 0.06	994 ± 130
+8	8.8 ± 2.7	2.3 ± 0.8	142 ± 31
-8	18.1 ± 3.0	0.2 ± 0.08	102 ± 24
-70	32.4 ± 4.2	2.0 ± 0.6	91 ± 17

Albedo discs were excised from the indicated sites (see Fig. 1A) of P. digitatum - infected grapefruit. Five discs of each site were treated with either water (control), or with ACC (5mM) and incubated at 24°C for 6 h in the dark. ACC content and ethylene production were then determined. ACC content of the ACC-treated discs ranged from 141 to 180 nmol g^{-1}. Each value in the table is a mean of 5 replicates with S.D.

Figure 1 Some biochemical and physiological changes in the peel of P. digitatum-infected grapefruit in relation to ACC content and ethylene production.

A: Definitions of regions on a 6-days old infected grapefruit and the method of their graphic presentation as a function of their distance from the maceration front. B to G: Changes in biochemical and physiological parameters, in an infected fruit as related to the distance from the maceration front. B: Glucosamine content. C: Galacturonic acid. D: pH of water extract. E: Water-soluble protein content in water extract. F: ACC content. G: Ethylene production.

All assays were carried out on peel tissues samples taken from healthy or P. digitatum-infected grapefruits, 6 days after inoculation near the stem-end of the fruit. The fruits were incubated at 21°C in the dark. Assay procedures are given in "Materials and Methods". Bars indicate S.D.

o————o:Healthy fruit albedo. Δ--------Δ:Healthy fruit flavedo.

●————●:Infected fruit albedo. ▲--------▲:Infected fruit flavedo.

Figure 2 Time-course of ethylene production by albedo and flavedo discs, cut from different locations of healthy or P. digitatum-infected grapefruit.

Ethylene production by peel discs cut from different sites of the fruit, as defined in Fig. 1A: +70(A); +8(B); -8(C); and -70(D).

o————o: Healthy fruit albedo. Δ--------Δ: Healthy fruit flavedo.

●————●: Infected fruit albedo. ▲--------▲: Infected fruit flavedo.

Five peel discs, 12 mm in diameter were excised from each site of nine fruits. The discs were separated into the albedo and flavedo parts, weighed and placed inside a 21 ml glass vial. The vials were incubated

in the dark at 24°C. One h accumulations of ethylene were determined at various intervals during the following 32 h. Prior to every closure, the vials were aerated. The data obtained from healthy control discs are not given for Fig. 2B,C and D, since they were similar to those shown in Fig. 2A. Vertical bars indicate S.D.

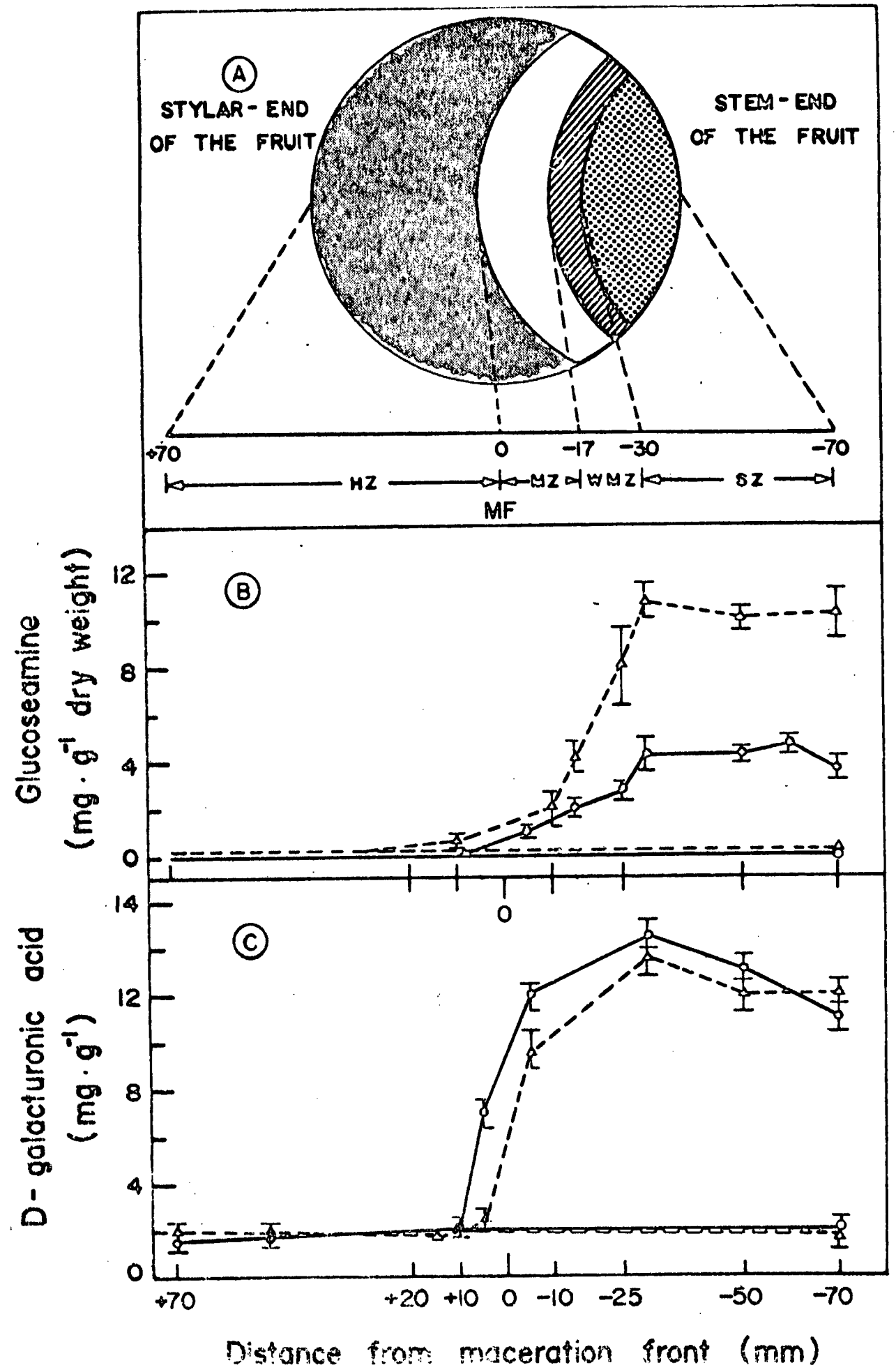

A
STYLAR-END OF THE FRUIT
STEM-END OF THE FRUIT
+70
0
-17
-30
-70
HZ
MZ
WMZ
SZ
MF
B
Glucoseamine (mg·g⁻¹ dry weight)
12
8
4
0
0
C
D-galacturonic acid (mg·g⁻¹)
14
12
8
4
0
+70
+20
+10
0
-10
-25
-50
-70
Distance from maceration front (mm)

Figure 1

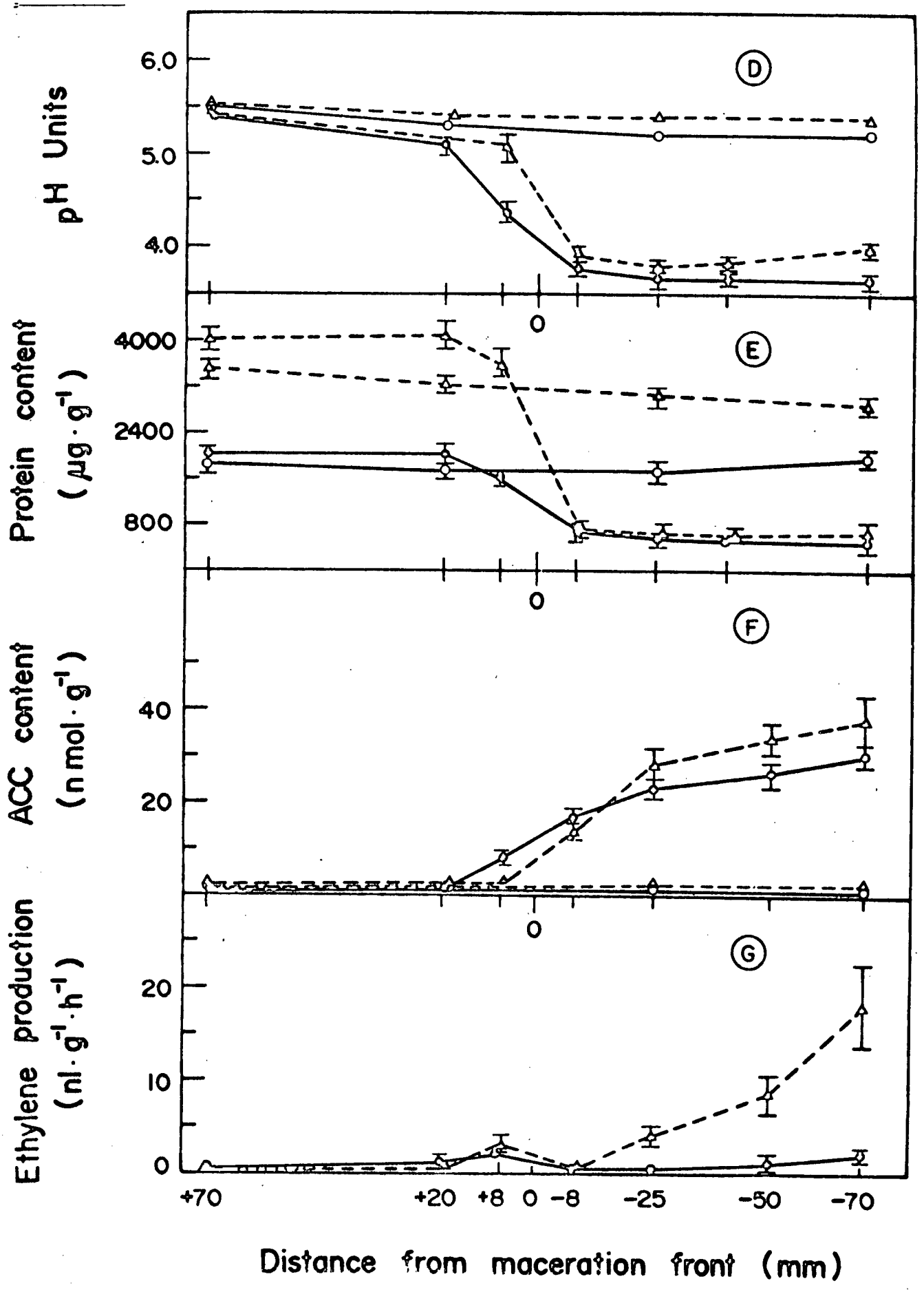
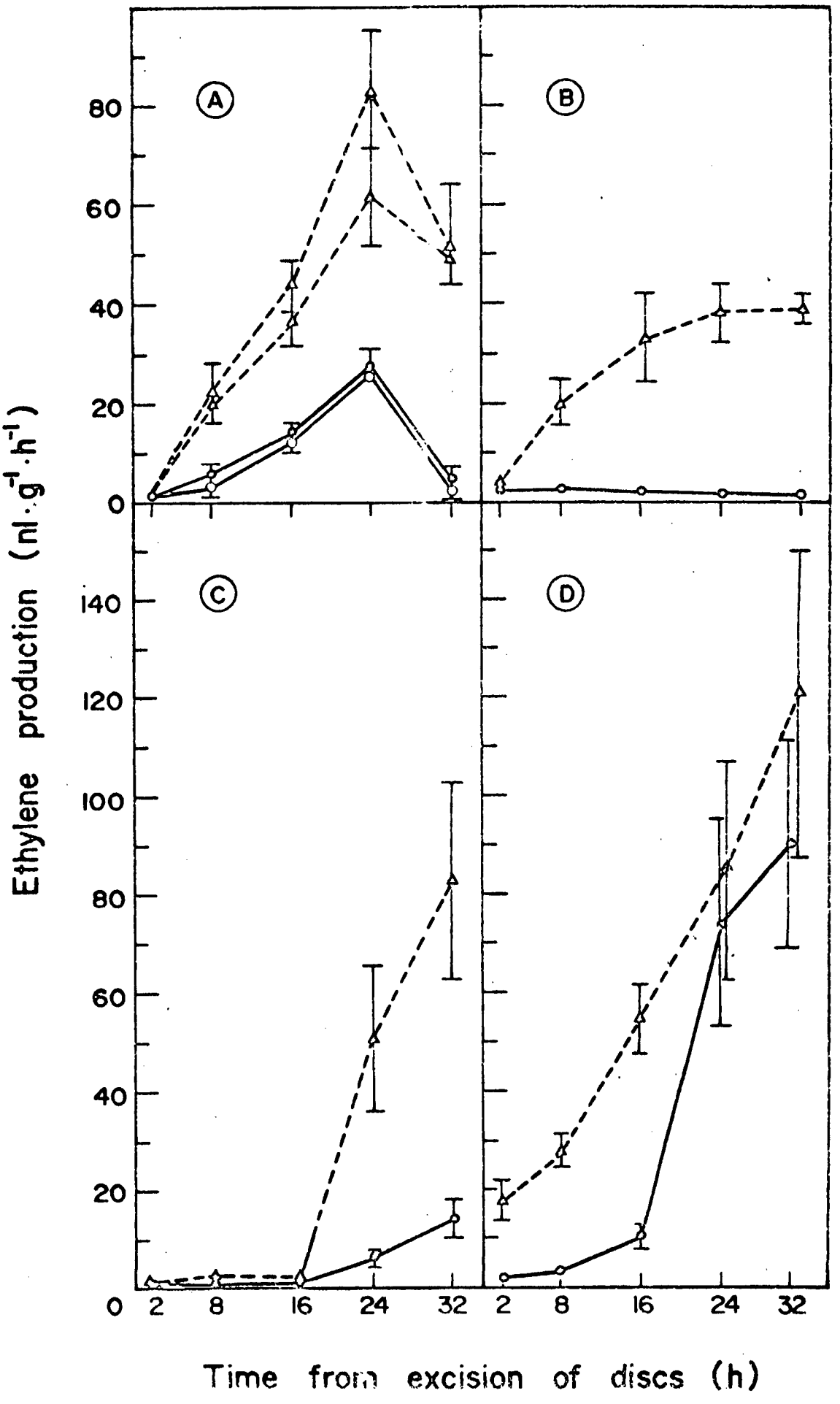

pH Units
6.0
5.0
4.0
D
0
Protein content (µg·g⁻¹)
4000
2400
800
E
0
ACC content (n mol·g⁻¹)
40
20
F
0
Ethylene production (nl·g⁻¹·h⁻¹)
20
10
0
G
+70
+20
+8
0
-8
-25
-50
-70
Distance from maceration front (mm)

Figure 2


A
B
C
D
Ethylene production (nl·g⁻¹·h⁻¹)
0
20
40
60
80
100
120
140
2
8
16
24
32
Time from excision of discs (h)