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Ethylene-controlled Induction of Phenylalanine Ammonia-lyase in Citrus Fruit Peel[1]

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Abstract. L-Phenylalanine ammonia-lyase (PAL) activity is low in the external layers (flavedo) of intact mature grapefruit peel. Flavedo discs evince upon incubation increasing PAL activity and ethylene production. Light has no effect in enhancing PAL activity in discs. Exogenous ethylene stimulates PAL activity in the flavedo of intact mature grapefruits (half maximum stimulation at 15 ppm); such activity rapidly decreases when fruit is removed from the ethylene containing atmosphere. Carbon dioxide inhibits both ethylene production and PAL activity of discs; exogenous ethylene only partly relieves PAL inhibition. Cycloheximide inhibits both PAL activity and ethylene production by flavedo discs. The same concentration of cycloheximide also inhibits PAL activity of discs in the presence of exogenous ethylene. Protein synthesis seems therefore to be needed at both levels of ethylene evolution and enhancement of PAL activity.

Irradiation of citrus fruits results in an increase in L-phenylalanine ammonia-lyase (E.C. 4.3.1.5) (PAL) activity in the flavedo, the external colored peel layers (16). Other treatments or conditions which result in increased PAL activity in plant tissues include wounding (4, 13, 23), disease (13, 15) and light (4, 5, 6, 17, 23). In some cases the increase in PAL activity was shown to be due to *de novo* synthesis of the enzyme (7, 14, 20, 23, 24). The observation that irradiated (11), stressed (19), wounded (9, 12), or infected plants (9, 18, 21) produce more ethylene suggests that ethylene may play a role in increased PAL activity. This idea is also supported by reports that ethylene can regulate protein synthesis (1) and PAL activity in sweet potato roots (10).

This paper presents data which show that ethylene plays a role in increased PAL activity in citrus flavedo.

Materials and Methods

Mature grapefruits (*Citrus paradisi* Macf., cv. Marsh Seedless) were used in these experiments.

Disc Incubation. In most cases discs 18 mm in diameter were cut from the peel and further sectioned with a sharp razor blade so as to leave only flavedo tissue (about 1 mm thick). Petri dishes (11.5 cm in diameter) were padded with 2 layers of filter paper Whatman No. 1, wetted with 5.5 ml H_2O (or the solution required); 16 discs laid flat in each dish were incubated at 25° under light (600 ft-c of fluorescent and incandescent light). At the end of incubation time PAL was assayed as described below.

Ethylene and Carbon Dioxide Supply to Tissues. Ethylene at different concentrations was supplied to intact fruits in 13 liter jars with a continuous air stream at the rate of 200 ml/min. Carbon dioxide and ethylene were supplied to flavedo discs incubated in Petri dishes placed in 2 to 4 liter jars, with an air stream of 100 ml/min. Air and gas were supplied through a system of flowmeters permitting adjustment but the fine calibration was performed by gas chromatography (C_2H_4) and Orsat gas analyzer (CO_2). Experiments were carried out at temperatures of 25°.

Enzyme Assay. PAL was extracted from acetone powders of peel tissues. The enzyme extract was partially purified with ammonium sulfate and assayed by following the increase in absorbance at 290 mμ as previously described (16). Activity is expressed as μmoles of cinnamic acid produced per gram fresh weight per hr.

Ethylene Measurements. For the determination of ethylene production 25 discs, 14 mm in diameter, incubated as described above, were transferred to sealed Erlenmeyer flasks for 2 hr before sampling. Ethylene was measured by gas chromatography in 1 ml air samples taken from the flasks with an air-tight syringe. We used a Packard gas chromatograph with a hydrogen flame ionizing detector fitted with a 192 × 0.38 cm column of activated alumina containing 4% H_2O (v/w). The column temperature was 24°. Ethylene was expressed as mμ liter per gram fresh weight per hr.

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All data reported throughout this paper are averages of 4 to 6 replicates.

Results

Effects of Light on PAL Activity. The PAL activity of many plants has been shown to be influenced by light (4, 5, 6, 17, 23). However as shown in table I, 22 hr of light had no effect on the induction of PAL in citrus flavedo discs. Other workers (20) have also reported PAL induction systems unaffected by light.

PAL Activity and Ethylene Production of Discs as Functions of the Period of Incubation. Fig. 1 shows the time curves for PAL activity as found in 2 different experiments. The curve of activity seems to proceed at an increasing rate, and there is no indication of slowing down after 48 hr.

Fig. 2 presents data which show that flavedo discs produce increasing amounts of ethylene with time. A comparison between Fig. 1 and 2 indicates that there is a similarity between the rise in PAL activity and ethylene production of isolated flavedo discs. The rate of ethylene production of flavedo discs is high compared to intact citrus fruits (2).

Table I. *PAL Activity of Grapefruit Flavedo Discs at Zero Time and After 22 Hr of Incubation at 25° Under Light or Darkness*

Date of picking	Zero time	22 Hr Light 600 ft-c	22 Hr Darkness
	μmoles cinnamic acid per g fresh wt per hr		
Feb. 11	0.024	0.490	0.495
May 7	0.024	0.510	0.540

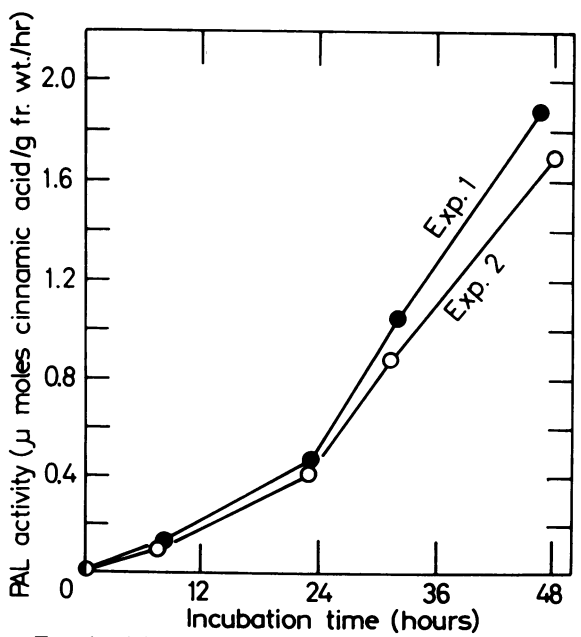

FIG. 1. Time course of PAL activity of grapefruit flavedo discs. Discs were incubated on water under 600 ft-c of light at 25°. Experiment 1, April 16; Experiment 2, May 28.

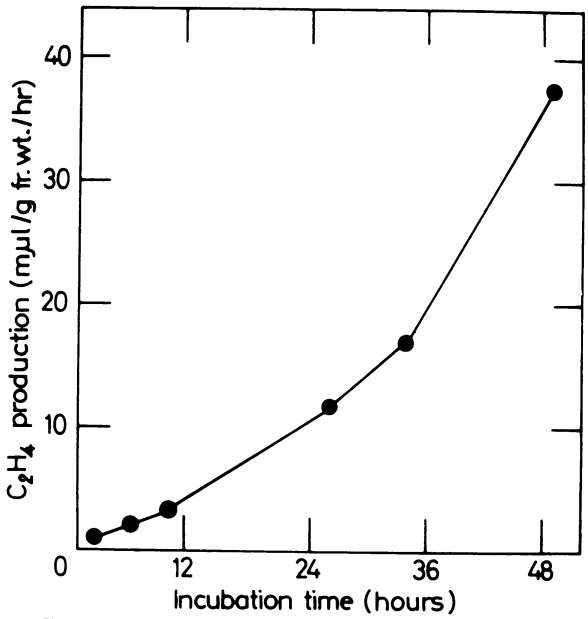

FIG. 2. Time course of ethylene production of grapefruit flavedo discs. Incubation conditions as in Fig. 1, except that discs were transferred to sealed Erlenmeyer flasks 2 hr before ethylene determination.

Effects of Exogenous Ethylene on PAL Activity of Intact Fruits. Since intact fruits have both a very low PAL activity (16, see also table I) and ethylene production (2), it seemed interesting to investigate whether added ethylene would enhance their PAL activity.

Fig. 3 shows that in the presence of 50 ppm ethylene PAL activity in flavedo of intact fruits increased in a sigmoid fashion while PAL activity for controls remained low and unchanged. When fruit was removed from the ethylene atmosphere PAL activity rapidly decreased toward control values (see broken line in Fig. 3). The data in Fig. 3 therefore show that a continuous exposure to ethylene is required to increase PAL activity and in the absence of the gas PAL activity rapidly decreases. The sigmoid shaped curve of Fig. 3 is different from the exponentially shaped curve of Fig. 1 which is probably induced by the endogenous upsurge of ethylene (Fig. 2). A concentration curve of ethylene action on PAL activity in flavedo of intact fruits during 24 hr is shown in Fig. 4. Ethylene effects are evident at 1 ppm and attain a maximum at 100 ppm. Half maximum stimulation of PAL activity occurs at about 15 ppm.

Inhibition of PAL Activity of Flavedo Discs by Carbon Dioxide. Carbon dioxide is known to act as a competitive inhibitor of ethylene (1, 3). Such an effect would be expected in our disc system, if the induction of PAL is a typical ethylene mediated

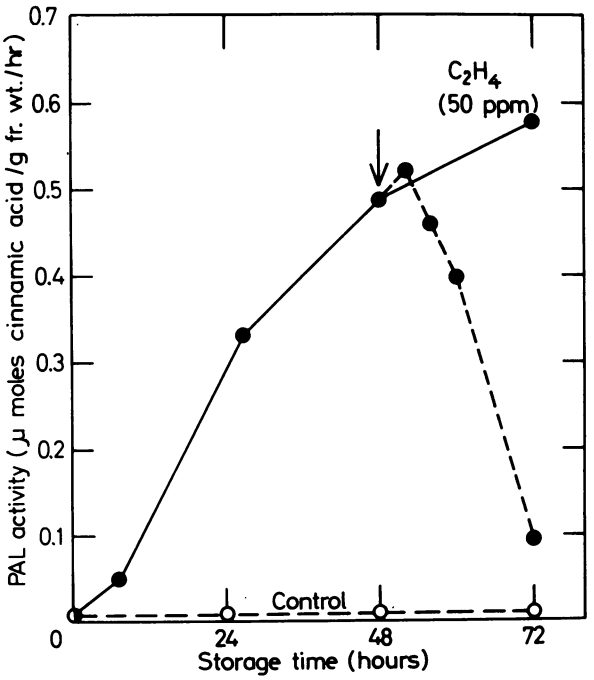

FIG. 3. Effect of ethylene on development of PAL activity in flavedo of intact grapefruits. Fruit was stored in 50 ppm ethylene at 25°. Broken line: activity after fruit removal from ethylene atmosphere, at time shown by arrow.

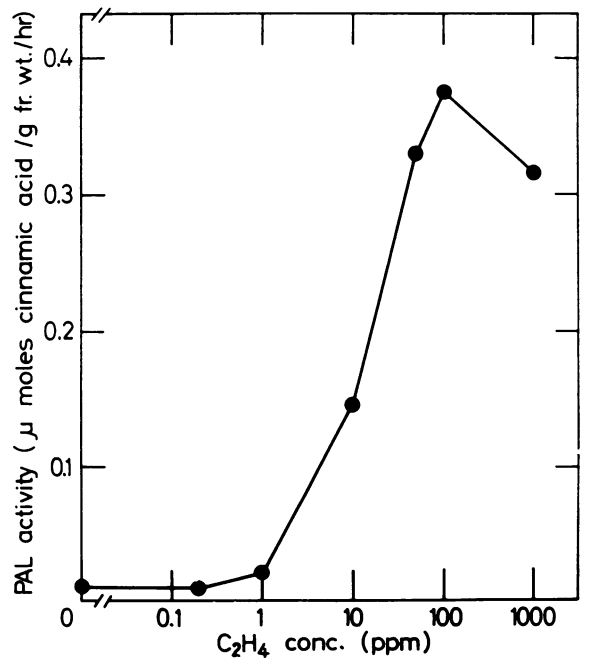

FIG. 4. Effect of ethylene concentration on PAL activity in flavedo of intact grapefruits. Fruit was stored for 24 hr in ethylene at 25°, before enzyme was extracted and assayed.

response. Flavedo discs were therefore incubated under a flow of air containing different concentrations of carbon dioxide and their PAL activity measured after 28 hr. Fig. 5 shows a progressive decrease in PAL activity with increasing carbon dioxide concentration. Upon addition of 300 ppm ethylene to the 20 % carbon dioxide concentration the inhibitory effect was partly relieved, PAL activity increasing from 39.6 % (Fig. 5) to 57.7 % of air control.

On the other hand, 20 % carbon dioxide were found to inhibit the production of ethylene by flavedo discs, resulting in only 7.5 % of air control. This observation is not in accordance with the ideas about competitive interaction between carbon dioxide and ethylene.

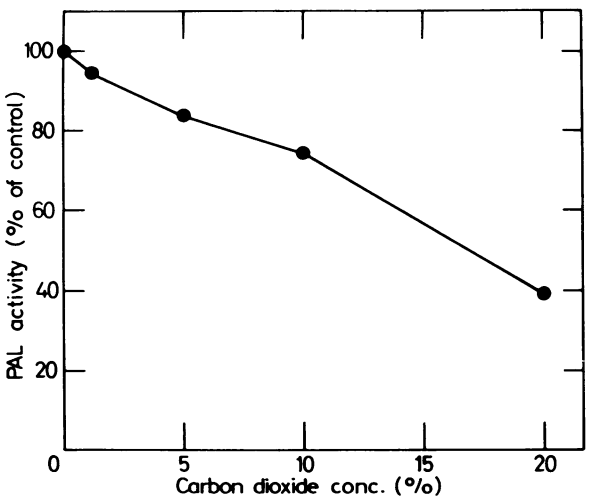

FIG. 5. Effect of carbon dioxide concentration on PAL activity of grapefruit flavedo discs. Discs were incubated for 28 hr in carbon dioxide at 25°, before enzyme was extracted and assayed. Activity of air control, 0.769 μmoles cinnamic acid per g fresh wt per hr.

Effects of Inhibitors of Nucleic Acid and Protein Synthesis on PAL Activity of Incubated Discs. Nucleic acid and protein synthesis inhibitors were used to determine whether the increase in PAL activity was a consequence of *de novo* protein synthesis.

The inhibitors were supplied in the solution wetting the padded Petri dishes and PAL activity of flavedo discs was measured after 28 hr of incubation under the usual conditions. Infiltration techniques were not used since water infiltration was found to decrease PAL activity by about 70 %. The results, expressed in percent inhibition as compared with water control values, are reported in table II.

A different degree of inhibition was obtained with different inhibitors and only cycloheximide (CH) produced almost complete PAL inhibition at low molar concentrations. Fig. 6 further shows a concentration curve of CH inhibition after 28 hr

Table II. *Percent Inhibition of PAL Activity of Grapefruit Flavedo Discs Brought About by Inhibitors of Nucleic Acid and Protein Synthesis*

Discs were incubated on solutions of inhibitors or water for 28 hrs before enzyme was extracted and assayed. Incubation conditions as described in Fig. 1. PAL activity of water controls ranging between 0.554 to 0.637 μmoles cinnamic acid per g fresh wt per hr.

Inhibitor	Concn.	Inhibition
	M	%
Puromycin	3×10^{-4}	18.6
Actinomycin D	4.0×10^{-5}	25.0
Cycloheximide	1.4×10^{-5}	97.5

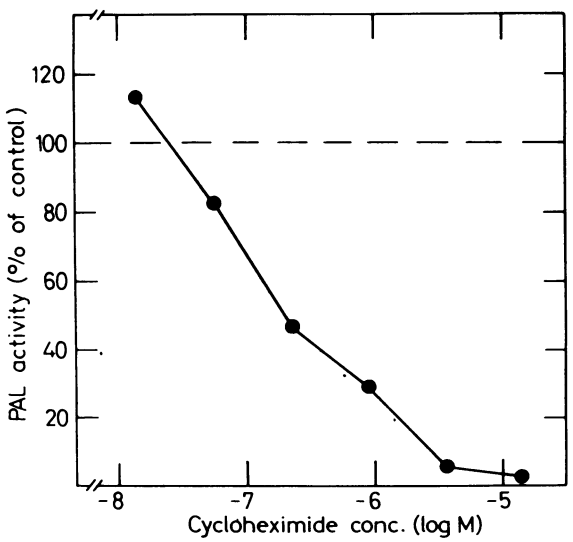

FIG. 6. Effect of cycloheximide concentration on PAL activity of grapefruit flavedo discs. Discs were incubated for 28 hr, in conditions as in Fig. 1, before enzyme was extracted and assayed. Activity of water control, 0.584 μmoles cinnamic acid per g fresh wt per hr.

of incubation. The exceedingly low concentration of 1.2×10^{-7} M caused about 50 % PAL inhibition.

Effects of Cycloheximide on Ethylene Production by Flavedo Discs. We further studied ethylene production of grapefruit flavedo discs supplied with CH. With 1.4×10^{-5} M CH concentration ethylene production was much inhibited, as compared with non inhibited controls: percentage inhibition was 80 % after 8 hr and 91.5 % after 28 hr of incubation (ethylene production of control discs was 5.1 and 12.8 mμl per g fresh weight per hr after 8 and 28 hr, respectively). It was therefore shown that ethylene production of discs is strongly inhibited by CH. A similar inhibition of ethylene production by CH has been recently reported for incubated discs of preclimacteric apple peel (8).

Effects of Cycloheximide on PAL Activity of Flavedo Discs Supplied With Exogenous Ethylene. Flavedo discs were placed on filter paper wetted with water or CH (1.4×10^{-5} M) and placed in an air stream containing 300 ppm ethylene. After 28 hr of incubation, PAL activity of inhibited discs was only 3.3 % of ethylene water controls (control values: 0.410 μmole cinnamic acid per g fresh weight per hr).

Inhibition of PAL activity comparable to that of table II is therefore obtained also in the presence of exogenous ethylene. These data show that CH will block the increase of PAL activity in ethylene as well as in air.

Discussion

Effects of wounds (9, 12) stress (19) or other physical damage on ethylene production have been shown previously, but in these cases parallel changes in PAL activity have not been studied. Conversely, effects of physical damage on PAL activity have been studied in different plants (4, 13, 23) but mostly without parallel determination of ethylene production. Because of the concurrent increases in ethylene production and PAL activity, we have shown in Fig. 1 and 2, flavedo discs seem to be a very convenient model for studying the possible relationships between these 2 phenomena.

The present results indicate that PAL activity in the flavedo tissues of intact grapefruit peel is low. However, flavedo discs cut from the peel evince upon incubation a remarkable increase in PAL activity. Increased PAL activity of flavedo is also observed when intact fruits are treated with ethylene or irradiated with gamma radiation (16). The development of PAL activity in discs is inhibited by CH and carbon dioxide.

On the other hand, endogenous ethylene production of intact citrus fruit tissues is also low (2), and increases upon cutting in incubated flavedo discs, as does PAL activity. Increased ethylene production of intact fruits is also induced by gamma radiation (11). The production of ethylene by flavedo discs is inhibited by CH and carbon dioxide.

On the basis of the data described it seems reasonable to consider the upsurge of ethylene in discs as a causal agent for increased PAL activity for several reasons. PAL activity is induced by exogenous ethylene in intact fruits, but decreases when fruit is removed from the ethylene atmosphere. Moreover, in irradiated fruit there is a close correlation between ethylene production and PAL activity in the flavedo (unpublished data). Carbon dioxide which inhibits ethylene production of discs also inhibits PAL activity. The inhibitory effect of carbon dioxide is partly relieved by exogenous ethylene. The appearance of PAL activity in discs seems to be due to *de novo* protein synthesis, which ethylene is considered to regulate (1). Increased PAL activity in sweet potato root discs has also been recently shown to be induced by ethylene (10).

The increase in ethylene production of flavedo discs is inhibited by CH, suggesting protein synthesis is necessary for the additional capacity in ethylene production.

CH inhibits PAL also in the presence of exogenous ethylene, and this is not unexpected if enhanced PAL activity is a consequence of *de novo* synthesis as previously advanced (7, 14, 20, 23, 24). The fact that other inhibitors which were found active on PAL in other cases (14, 20) do not inhibit PAL activity in flavedo discs, may be due to different reasons, as lack of penetration in the tissues or enzyme specificity.

In flavedo discs no indication is found (see Fig. 1) of the existence of an inactivating system for PAL activity as shown by Zucker (24) and others (7, 20). Some data from irradiated intact grapefruits (16), the differences in patterns of curves in Fig. 1 and 3 and the rapid decrease in PAL activity when intact fruits are removed from ethylene (Fig. 3) seem however to point to the possibility that a system inactivating PAL may exist in intact citrus fruits.

The emphasis on the role of ethylene as a factor inducing enzymatic activity under stress conditions (in this case physical damage produced by cutting) is in line with recent work in the field of plant pathology (10, 18) and stress physiology (19).

Acknowledgment

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