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Stress Ethylene does not Originate Directly from Lipid Peroxidation during Chilling-Enhanced Photooxidation[1]

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Summary

Stress ethylene synthesis was monitored in detached cucumber (*Cucumis sativus*) leaves under conditions (5 °C and 1000 μEinstein $m^{-2} s^{-1}$) that promote lipid peroxidation. Previous work has shown that such a chilling and light stress in detached cucumber leaves causes superoxide anion radical and singlet oxygen production and is accompanied by pigment bleaching, loss of endogenous antioxidants, ultrastructural damage and ethane generation due to lipid peroxidation [Wise, R. R. and A. W. Naylor: Plant Physiol. *83*, 272–278 and 278–282 (1987a, b)]. In spite of the considerable light- and chilling-enhanced lipid peroxidation, as evidenced by ethane generation, all of our observations of ethylene production in the experiments described here indicate that the S-adenosylmethionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4 enzymatic pathway was the chief, if not the sole, source of ethylene. When ethane generation was inhibited in leaves by atrazine (which blocked superoxide radical-dependent lipid peroxidation), ethylene production was unaffected. The complete photobleaching at room temperature of isolated cucumber thylakoids resulted in substantial amounts of ethane, but no detectable ethylene. The Q_{10} temperature coefficient for ethylene generation was 1.6, within the range which is typical for enzymatic reactions. Ethylene generation was significantly, but not completely, inhibited by $CoCl_2$ or aminoethoxyvinyl glycine, known inhibitors of enzymatic ethylene biosynthesis. The well-established SAM → ACC → C_2H_4 biosynthetic pathway was apparently fully engaged in the detached cucumber leaves while lipid peroxidation contributed insignificantly to stress ethylene production.

Key words: Cucumis sativus L., chilling injury, ethylene, lipid peroxidation, photooxidation, singlet oxygen, superoxide.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinyl glycine; ·OH, hydroxyl radical; LOOH, linolenic acid hydroperoxide; MET, methionine; SAM, S-adenosylmethionine; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide anion radical.

Introduction

Ethylene-forming activity and lipid peroxidation have been associated in the literature for some 25 years (Meigh, 1962; Lieberman and Mapson, 1962; 1964) but only recently has the nature of this relationship become clear (Bousquet and Thimann, 1984; Pirrung, 1986; Gardner and Newton, 1987). The original idea was that lipoxygenase (E.C. 1.13.11.12) enzymatically peroxidated linolenic acid to linolenic acid hydroperoxide (LOOH) which then non-enzymatically fragmented to yield ethylene (Meigh, 1962; Lieberman and Mapson, 1962; 1964; Galliard et al., 1968a, b; Meigh et al., 1967). Such a system fell into disfavor with the discovery of 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979) and the proposal of the methionine (MET) → S-adenosylmethionine (SAM) → ACC → C_2H_4

pathway (Lürssen et al., 1979). In fact, Yang and Hoffman (1984) flatly stated that «methionine [not linolenic acid, etc.] serves as an effective precursor of ethylene in higher plants.»

Recently however, a system using linoleic acid, lipoxygenase, ACC, and several cofactors has been shown to have many features in common with the *in vivo*, ACC-dependent ethylene forming pathway (Bousquet and Thimann, 1984). Gardner and Newton (1987) postulate that a linoleic acid hydroperoxide-Mn^{+n}-PP complex oxidizes ACC to C_2H_4, thereby successfully wedding lipoxygenase and ACC to ethylene production. Although questions have been raised concerning whether or not this is the actual *in vivo* mechanism (Wang and Yang, 1987), it may well impact on validation of the putative ethylene-forming enzyme.

Lipid peroxidation can be achieved non-enzymatically through the action of naturally-produced activated oxygen species such $O_2^{\cdot-}$, H_2O_2, $^1O_2$ or $^{\cdot}OH$ (Halliwell, 1984) and yield a variety of low molecular weight fragmentation products including ethane, propane, butane and malondialdehyde (Cohen, 1979). The suggestion that one of the lipid fragmentation products in plants is ethylene has been made by Mattoo et al. (1986) and is the hypothesis we have tested in the present work. The experimental system used to study chilling-enhanced photooxidation in detached cucumber leaves (Wise and Naylor, 1987 a, b) provides an excellent method with which to test the above hypothesis. Chilled and irradiated cucumber leaves exhibit lipid peroxidation (ethane production), loss of both lipid- and water-soluble antioxidants, pigment bleaching, and ultrastructural damage. We provided evidence for the production of both $^1O_2$ and $O_2^{\cdot-}$ and showed that all of the ethane produced was attributable to the action of these toxic oxygen species on chloroplast lipids (Wise and Naylor, 1987 a, b). In the present paper, we demonstrate that in contrast to ethane production, the ethylene generated during chilling-enhanced photooxidation in detached cucumber leaves originates from enzymatic ACC oxidation, not from lipid peroxidation.

Materials and Methods

Cucumis sativus L. (cv. Ashley) plants were raised from seed in a 1:1:1 mixture of sand:vermiculite:turface (Jaeger et al., 1981) in a temperature-controlled glasshouse (26/20 °C, D/N) in the Duke University Phytotron. Plants were watered twice daily. For some experiments, plants were grown in a soil mixture in a controlled environment chamber set at 26/20 °C (D/N) with a 14 hr photoperiod at approx. 600 $\mu E\,m^{-2}s^{-1}$. Fully-expanded leaves from 14–20 day-old plants were used as the experimental material.

Three cm by five cm rectangles of tissue were excised from the leaves and put in 22.5 ml flat-sided glass tissue culture flasks (T-15, Bellco Glass Co., Vineland, NJ) with 1 ml water, sealed, and submerged to a depth of one cm in a constant-temperature water bath set for 25° or 5 °C. Light (1000 $\mu E\,m^{-2}s^{-1}$) was provided by a 220 V, 400 W high intensity discharge lamp (Hi Tek Lighting, Crawfordsville, IN).

At various time intervals (see Fig. 1), one ml of the gas within the flasks was removed and sampled for ethylene and ethane using a Varian model 3700 gas chromatograph equipped with an Al_2O_3 column (1 m × 2 mm) held at 70 °C. Some samples were analyzed on a Hewlett Packard model 5890 gas chromatograph with a similar column at 50 °C. Ethylene and ethane identification and quantification were confirmed by co-chromatography using authentic standards from Supelco Inc. (Bellefonte, PA). The sampling techniques and the calculations for the amount of ethylene and ethane produced are detailed in Wise (1986).

For the preincubation with $CoCl_2$ (10 mM) and AVG (5 mM), 3 × 5 cm leaf segments were floated, abaxial side down, on the appropriate solution in 40 mM KH_2PO_4 (pH 7.2) at room temperature under low light (60–65 $\mu E\,m^{-2}s^{-1}$) for 30 min, blotted dry, and treated as described above for the measurement of ethylene and ethane. The herbicide atrazine (87 μM) was applied in a similar manner as an aqueous solution (with 0.05 % Tween 80) for 30 min in the dark at room temperature. Chlorophyll fluorescence was monitored with a Brancker model SF20 fluorimeter (Brancker Inc., Ottawa, Canada) to ensure that the herbicide had reached its site of action prior to inserting and sealing the leaf segments into the 25 ml tissue culture flasks described above.

Cucumber thylakoid membranes were isolated and incubated using two different protocols. The first method used a NaCl (350 mM)/Tris (100 mM, pH 7.2) grinding buffer (Packer et al., 1965) and a NaCl (175 mM)/Tris (50 mM, pH 8.0) incubation buffer (Heath and Packer, 1968). The second technique (Whitmarsh and Ort, 1984) employed a grinding medium containing 300 mM NaCl, 30 mM tricine-NaOH (pH 7.8), 3 mM $MgCl_2$ and 0.5 mM Na_2 EDTA. The membranes were washed twice and resuspended in a buffer of 200 mM sorbitol, 5 mM Hepes-NaOH (pH 7.5), and 2 mM $MgCl_2$. Chlorophyll content was determined spectrophotometrically in an 80 % acetone extract according to Ziegler and Egle (1965).

The thylakoids were incubated in 3 ml of the incubation medium in 25 ml tissue culture flasks at 25 °C and 1000 $\mu E\,m^{-2}s^{-1}$. Thylakoids were diluted to a chlorophyll concentration of 10–35 μM and allowed to photobleach in the absence of any added electron acceptors for 8 h. One ml samples of the head space gas were then collected for ethylene and ethane determination using gas chromatography (see above).

Results and Discussion

Ethylene production (Fig. 1) by illuminated cucumber leaf segments at 25 or 5 °C was observed for up to 12 hours but it was over twice as high at the warm temperature than in the cold. The 5 °C and 1000 $\mu E\,m^{-2}s^{-1}$ treatment yielded the maximum lipid peroxidation (measured as ethane generation – Wise and Naylor, 1987 a) but minimum ethylene (Fig. 1). Conversely, 25 °C and light produced the least ethane (Wise and Naylor, 1987 a) and the most ethylene (Fig. 1). We therefore conclude that the two gases originated from different reactions or processes; ethane from lipid peroxidation and ethylene from a reaction investigated below.

Our previous work (Wise and Naylor, 1987 a, b) has indicated that superoxide anion radicals were generated during photobleaching in the cold via the Mehler reaction (Mehler, 1951). The $O_2^{\cdot-}$ then caused the peroxidation of chloroplast thylakoid lipids which fragmented to yield ethane. Atrazine, an inhibitor of photosynthetic electron transport (Pfister and Arntzen, 1979), effectively halted ethane production (Wise and Naylor, 1987 a) because $O_2^{\cdot-}$ was not generated and therefore the lipids were not peroxidated. Even though ethane generation from leaf segments was almost completely eliminated by an atrazine pretreatment, ethylene synthesis was unaffected (Fig. 2). Hence, $O_2^{\cdot-}$-mediated lipid peroxidation does not appear to be associated with ethylene production in intact leaf segments.

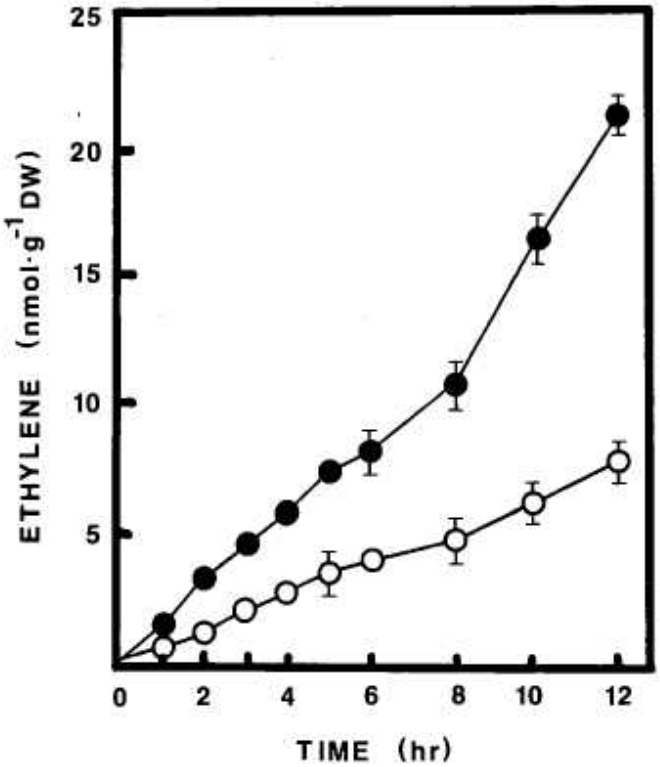

Fig. 1: Time course of ethylene production by detached, 3 cm × 5 cm cucumber leaf segments irradiated with 1000 μE m^{-2}s^{-1} at either 25 °C (●) or 5 °C (○). Mean ± S.E., n = 8.

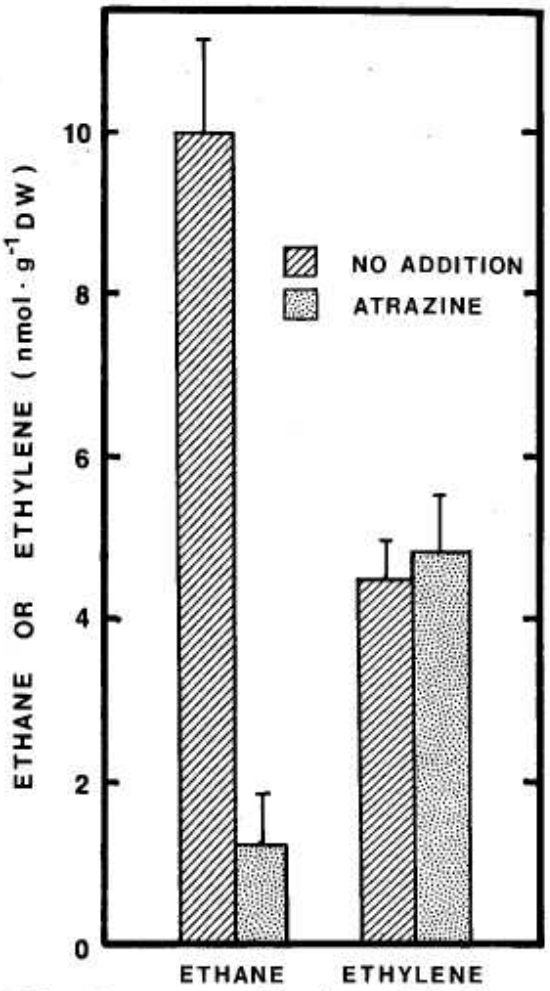

Fig. 2: Effect of pretreatment with a photosynthetic electron transport inhibitor (atrazine, 87 μM) on ethane and ethylene production by chilled (5 °C) and irradiated (1000 μE m^{-2}s^{-1}) cucumber leaf segments. Leaf tissue was floated on a buffered solution (40 mM KH_2PO_4, pH 7.2) of the inhibitor for 30 min at room temperature prior to treatment. Cumulative ethane and ethylene were measured after 8 hours. Mean ± S.E., n = 8.

Further support for our hypothesis came from a much-simplified system of isolated cucumber thylakoids (Table 1). Two different isolation and incubation procedures were used (Heath and Packer, 1968; Whitmarsh and Ort, 1984) to reduce the possibility that our results were caused by an artifact of isolation. Thylakoids were allowed to bleach at room temperature, in the light (1000 μE m^{-2}s^{-1}), and in the absence of an artificial, terminal electron acceptor. The washing procedure used during thylakoid isolation would have removed most, if not all, of the endogenous ferredoxin and NADP and results in high rates of O_2^- production (Halliwell, 1984). Lipid peroxidation was evidenced by ethane production but no ethylene could be detected (Table 1). Again, lipid peroxidation did not produce ethylene in our system. It is significant that the rates of ethane production, when expressed on a per mg chlorophyll basis, were the same for both intact, detached leaves and isolated thylakoids given the same irradiance (Table 1).

Enzymatic reactions can often be distinguished from physical reactions on the basis of their respective temperature dependencies, i.e. temperature coefficient (Q_{10}). This value is characteristically 1.4 to 2.0 for enzymatic reactions and generally below 1.3 for physical reactions (Larcher, 1980). The Q_{10} between 5 and 25 °C for ethylene production in the light (1000 μE m^{-2}s^{-1}) was 1.6 (Fig. 1), indicating that the phenomenon is very likely an enzyme-mediated process.

Several compounds are known to inhibit enzymatic ethylene biosynthesis; among them are aminoethoxyvinyl glycine (Yu and Yang, 1979) and the cobalt ion (Lau and Yang, 1976). AVG inhibits ACC synthase activity while co-

Table 1: Cumulative ethylene and ethane production by cucumber leaf segments or isolated cucumber thylakoids during photooxidation. Leaf segments were treated at 5 °C and 1000 μE m^{-2}s^{-1} for 8 hours. Isolated thylakoids were incubated for 8 hours at 25 °C and 1000 μE m^{-2}s^{-1}. Thylakoids I were isolated and incubated according to Heath and Packer (1968); Thylakoids II according to Whitmarsh and Ort (1984). Mean ± S.D., n = 12.

	mmol gas/mol chlorophyll	
	Ethane	Ethylene
Leaves	1.0±0.1	0.5±0.1
Thylakoids I	1.3±0.2	N.D.
Thylakoids II	1.2±0.3	N.D.

* A mathematically derived figure based on 3.6 mg DW · cm^{-2} leaf area, 35 nmol Chl · cm^{-2} leaf area, and the values given in Fig. 2. N.D. = not detectable.

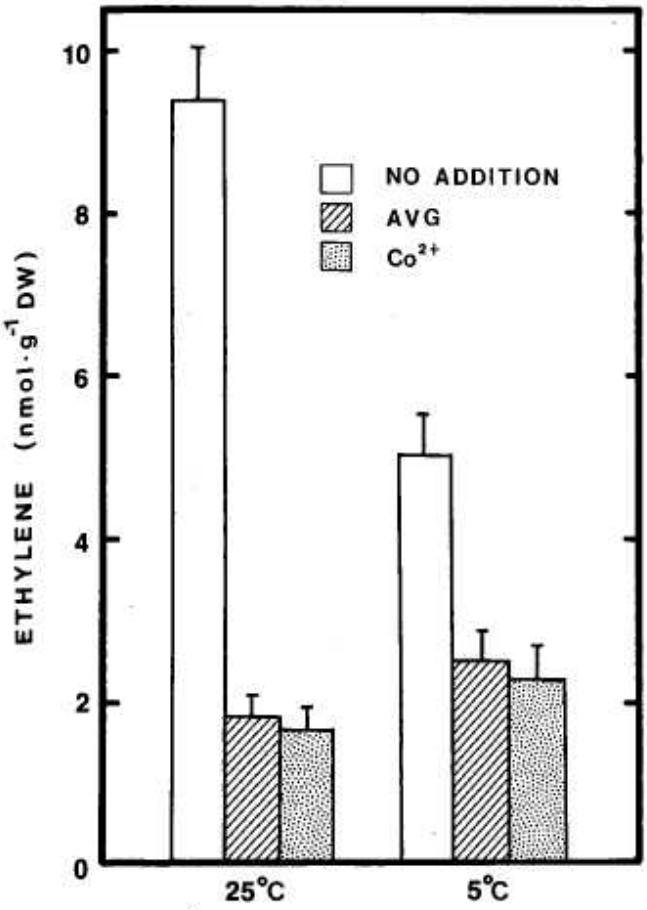

Fig. 3: Inhibition of cucumber leaf segment ethylene production by a pretreatment with either $CoCl_2$ (10 mM) or AVG (5 mM) at 5 or 25 °C. See legend to Fig. 2 for pretreatment protocol. Incubation was for 8 hours in 1000 $\mu E\, m^{-2} s^{-1}$. Mean ± S.E., n = 8.

balt inhibits the conversion of ACC to ethylene (Yu and Yang, 1979). When applied to cucumber leaf segments prior to 8 h treatments of either 5 °C or 25 °C at 1000 $\mu E\, m^{-2} s^{-1}$ both AVG and $CoCl_2$ inhibited ethylene production (Fig. 3). These data show the dependence of ethylene production on the ACC-dependent pathway during chilling-enhanced photooxidation.

AVG or cobalt both inhibited room temperature ethylene synthesis by about 82% compared to 53% in the cold (Fig. 3). Since the inhibitor pretreatment was done at room temperature (regardless of incubation temperature), it seems unlikely that uptake accounted for the difference. It is possible that some of the 5 °C, AVG- and Co^{+2}-insensitive ethylene production is caused by a direct oxidation of cytoplasmic ACC by H_2O_2. Superoxide generated in the chloroplast during the cold and light (Wise and Naylor, 1987 a) will dismutate to H_2O_2 (Halliwell, 1984) which would then diffuse across the chloroplast envelope into the cytoplasm (Robinson et al., 1980). Once in the cytoplasm, H_2O_2 can oxidize ACC to ethylene without the involvement of any of the enzymes normally inhibited by AVG or Co^{+2} (Stegink and Siedow, 1986). Fig. 2 shows C_2H_4 production to be insensitive to atrazine, an inhibitor of $O_2^{\cdot-}$ production, which argues against any $O_2^{\cdot-}/H_2O_2$ involvement in ethylene synthesis from ACC. However, under the conditions of atrazine treatment, the SAM → ACC → C_2H_4 pathway should be fully engaged consuming all the ACC produced and the absence of cytoplasmic H_2O_2 would not be noticeable. During inhibition of the ethylene biosynthetic pathway by AVG or Co^{+2} the postulated $O_2^{\cdot-}/H_2O_2$ oxidation of ACC could become a significant source of ethylene.

Copper toxicity apparently leads directly to ethylene production from linolenic acid by peroxidation (Sandmann and Böger, 1980 a, b; Lieberman and Mapson, 1964; Abeles and Abeles, 1972; Mattoo et al., 1986). Sandmann and Böger (1980 b) proposed a mechanism in which both Cu^{+1} and Cu^{+2} are directly involved at multiple sites during copper-mediated lipid peroxidation. The first two sites inhibit photosynthetic electron transport, thus enhancing both $^1O_2$ and $O_2^{\cdot-}$ production. Copper then was said to react, in two steps, to form the hydroxyl radical from $O_2^{\cdot-}$. The $^{\cdot}OH$ radical was suggested to peroxidate esterified linolenic acid and initiate a radical chain reaction generating a constant supply of LOOH. In this system the copper could have other sites of action in LOOH degradation ultimately yielding ethylene through the action of Cu^{+2} or ethane through the action of Cu^{+1}. This follows since only ethane, and not ethylene, is produced during lipid peroxidation in the absence of copper (Sandmann and Böger, 1980 b).

Photooxidation, accompanied by lipid peroxidation, can be caused by a number of environmental stresses (for review see Powles, 1984). Such conditions can also induce stress ethylene synthesis (Yang and Hoffman, 1984) and the suggestion has been made that stress ethylene is a direct product of lipid peroxidation (Mattoo et al., 1986), a process that can yield several low molecular weight hydrocarbon gases (Cohen, 1979). Our data demonstrate that even under severe photooxidative stress, accompanied by lipid peroxidation, ethylene is synthesized exclusively by the ACC-dependent pathway. Only in the special case of copper toxicity, in which Cu^{+n} plays several catalytic roles (Sandmann and Böger, 1980 b), can ethylene be shown to arise directly from lipid peroxidation (Mattoo et al., 1986).

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