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Sequence of Chloroplast Degreening in Calamondin Fruit as Influenced by Ethylene and $AgNO_3$[1]

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ABSTRACT

C_2H_4 disrupts the internal membranes of the chloroplast and induces an increase in chlorophyllase activity in degreening calamondin [× *Citrofortunella mitis* (Blanco) Ingram and Moore] fruit. Whether the loss of chlorophyll in the peel is causally related to breakdown of the chloroplast and/or chlorophyllase activity is not readily apparent. Chlorophyllase levels were inversely related to chlorophyll content, but electron micrographs also showed that internal membranes of the chloroplasts were disrupted simultaneously with the decrease in chlorophyll content. Silver, a potent inhibitor of C_2H_4-mediated effects, retarded the loss of chlorophyll in calamondin rind, reduced the C_2H_4-induced increase in chlorophyllase level, and prevented the disruption of the chloroplast membranes. The results do not permit the proposal of a mechanism of C_2H_4 metabolism in the degreening of calamondin fruit.

C_2H_4 is commonly used to destroy Chl in the rinds of citrus fruits. Nevertheless, its specific mode of action is not clearly understood. Chlorophyllase, which catalyzes the hydrolysis of Chl *in vitro*, increases dramatically when citrus fruits are exposed to C_2H_4 (3, 16). In addition, the chloroplast internal structure breaks down during both C_2H_4 degreening (15) and natural coloring on the tree (8, 19). Whether the breakdown of the chloroplast membranes and the increase in chlorophyllase activity occur simultaneously or sequentially has not been determined.

$AgNO_3$ inhibits a variety of C_2H_4-mediated effects, including Chl degradation in mature green tomato fruit (14) and green banana fruit (12, 14). $AgNO_3$ also retarded Chl degradation in citrus peel (10). However, increases in chlorophyllase levels during the first 24 h exposure to C_2H_4 were not altered by the $AgNO_3$ treatment (10). These initial results suggested that Ag may differentially alter C_2H_4 action in Chl degradation, so Ag appeared to offer a way of evaluating chlorophyllase levels and membrane breakdown during the degreening of citrus fruit. Ag has been shown to inhibit the incorporation of [^{14}C]C_2H_4 into tissue-soluble metabolites without having any effect on the rate of C_2H_4 conversion to CO_2 (5).

The experiments reported here were undertaken: (*a*) to determine the relationship between Chl destruction, chlorophyllase activity, and membrane breakdown in the rind of calamondin fruit during exposure to C_2H_4, and (*b*) to determine the effects of $AgNO_3$ on the C_2H_4-mediated Chl destruction.

MATERIALS AND METHODS

Calamondin (× *Citrofortunella mitis* (Blanco) Ingram and Moore) is a dwarf ever-bearing citrus species unusually suitable for laboratory material. Mature green fruit were harvested immediately prior to treatment and randomized into lots of 10 fruit each. Treatment consisted of a 15-min dip in solutions of $AgNO_3$ (0.1 to 10.0 mM) or distilled H_2O, each with a few drops of Triton X-100 added as a surfactant. The fruit were allowed to air-dry at room temperature before being placed in chambers maintained at 95% RH and containing either 5.3 μl/l C_2H_4 or pure air at 27 C. C_2H_4 was dispensed continuously into the chambers as described by Barmore and Wheaton (4), and the concentration was monitored with a gas chromatograph.

At prescribed times, each lot of fruit was removed from the chambers, and the rinds were extracted with cold acetone by grinding with a Sorvall Omni-Mixer until no green color was visible in the resulting acetone powders. Fifty mg $MgCO_3$ was added for each g of peel during the initial grinding. The powders were air-dried under vacuum and saved for subsequent extraction of chlorophyllase. The acetone extracts were combined, volumes were recorded, and Chl contents were determined by the method of Bruinsma (7).

Chlorophyllase was extracted from triplicate samples of the acetone powders and assayed as described by Barmore (3), except that the Chl substrate was prepared from spinach instead of bean leaves. For soluble-protein determinations, 1 ml acetone powder extracts was precipitated with an equal volume of cold 10% trichloroacetic acid. The resulting precipitate was collected by centrifugation and washed with 5% trichloroacetic acid before being dissolved in 0.1 N NaOH. Soluble protein content was determined on neutralized fractions using the Bio-Rad procedure (6). The entire experiment then was repeated and the data were subjected to an analysis of variance. LSDs are shown in the figures.

At prescribed times, indicated in figure legends, sections of the epicarp were removed from the fruit and prepared for study by electron microscopy. The sections were cut in 1- × 2-mm strips and fixed in 3% glutaraldehyde in 0.2 M K-phosphate (pH 7.0) for 5 h at 25 C. The sections then were washed for 15 min in the phosphate buffer and postfixed overnight in 2% OsO_4 in 0.2 M K-phosphate at 3 C. The tissue was thoroughly rinsed in deionized H_2O and *en bloc*-stained in 2% uranyl acetate for 1 h at 25 C, followed by dehydration in an acetone series and then by embedding in Spurr's epoxy mixture (17). Ag to Au sections were made with a diamond knife on a LKB Huxley ultramicrotome and stained with 15% methanolic uranyl acetate for 15 min (18), followed by a 5-min poststaining with 0.5% lead citrate (11). The sections were viewed with a Phillips 201 electron microscope.

RESULTS

C_2H_4 induced the degradation of Chl in calamondin peel (Fig. 1). The rate of Chl degradation was maximal between 24 and 48 h. $AgNO_3$ retarded C_2H_4-induced Chl degradation, but the concentrations tested did not completely inhibit Chl destruction. A 1

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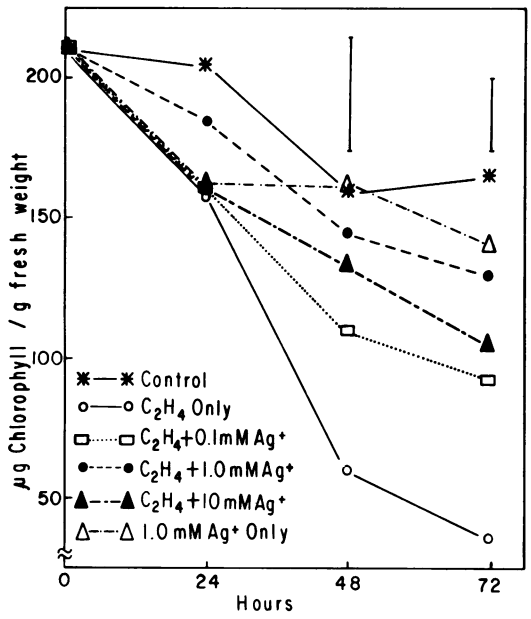


FIG. 1. Change in total Chl content of calamondin rind during exposure to 5.3 µl/l C_2H_4. Vertical bars are the LSDs among treatments at the 0.05 level of probability.

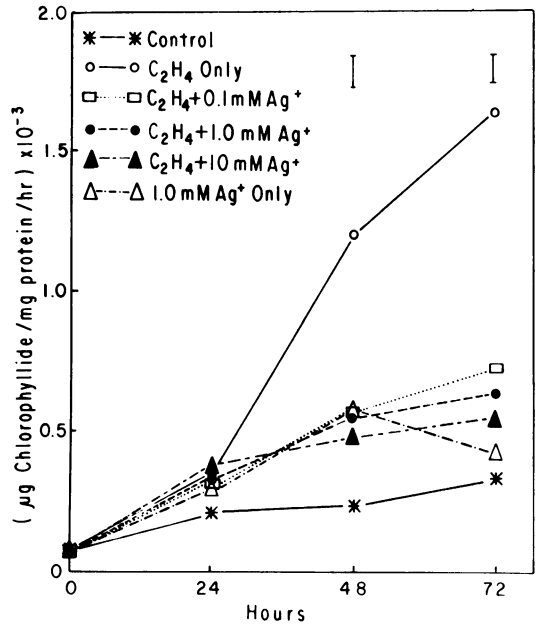


FIG. 2. Change in chlorophyllase activity in acetone powder extracts of calamondin rind during exposure to 5.3 µl/l C_2H_4. Vertical bars are the LSDs among treatments at the 0.01 level of probability.

mM concentration of $AgNO_3$ was more effective than either 0.1 or 10.0 mM in inhibiting Chl degradation.

C_2H_4 also caused an increase in chlorophyllase activity, as has been previously reported (3, 16) (Fig. 2). The greatest increase in chlorophyllase activity occurred between 24 and 48 h. $AgNO_3$ reduced the C_2H_4-induced increase in chlorophyllase activity, but the reduction was small during the first 24 h compared to its effect after 24 h (Fig. 2).

Fruit which had been maintained in pure air also exhibited a small increase in chlorophyllase activity and a decrease in Chl content (Figs. 1 and 2). $AgNO_3$ also caused an increase in chlorophyllase activity and a decrease in Chl content in fruit in the absence of exogenous C_2H_4 (Fig. 2). However, $AgNO_3$ at both 1.0 and 10.0 mM injured the peel of calamondin fruit and the injured peel may have produced some C_2H_4. The Chl content of the peel was inversely related to the extractable chlorophyllase activity for all treatments and times ($r = -0.918$, $P < 0.01$).

Ultrastructural changes in the epicarpal cells of calamondin fruit during 72-h exposure to C_2H_4 were primarily limited to the chloroplasts and were similar to those described for chromoplasts developing naturally from chloroplasts in 'Valencia' orange peel (19). Chloroplasts in green fruit had an extensive grana-fretwork system (Fig. 3). The thylakoid membranes were of even density and uniformly tight spacing. Ultrastructural changes were not evident after 24 h exposure to C_2H_4. However, after 48 h, much of the grana had disappeared (Fig. 4). The stroma lamellae concentrated around the periphery of the chloroplast were the last Chl-containing membranes to disappear (Fig. 4). The membranes after 48 h C_2H_4 appeared to dilate and become irregularly spaced (Fig. 4).

After 72 h, only a few chloroplasts in fruit treated with C_2H_4 contained any organized membrane structures (Fig. 5). The dispersed membranes appeared to develop into vesicles (Fig. 5). Plastoglobuli with differential staining intensities also accumulated (Fig. 5) and some of these were frequently seen extruding from the chloroplasts into the cytoplasm and vacuoles (Fig. 5). In the absence of C_2H_4, chloroplasts appeared unchanged during the 72-h period. The chloroplasts of fruit, which had been dipped in

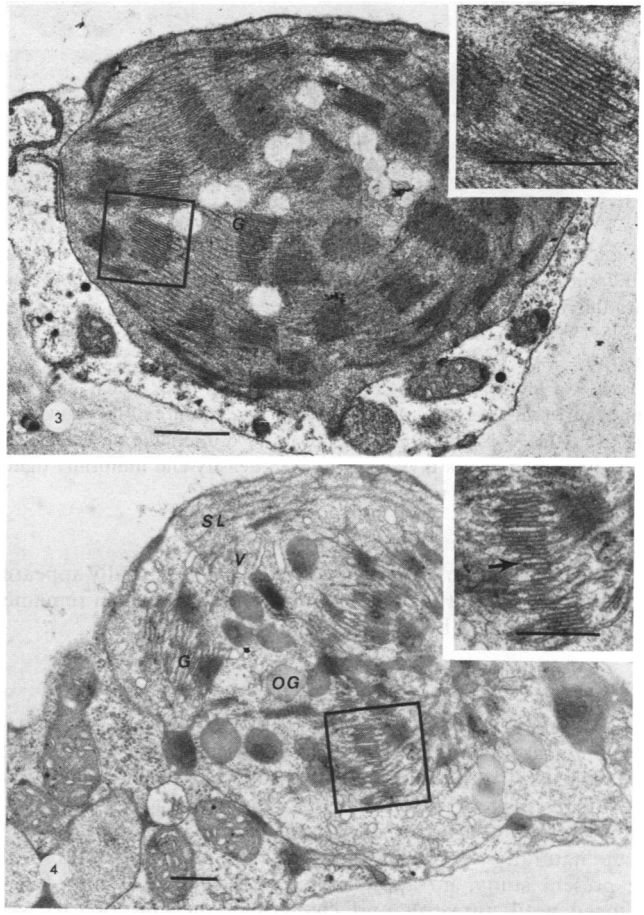
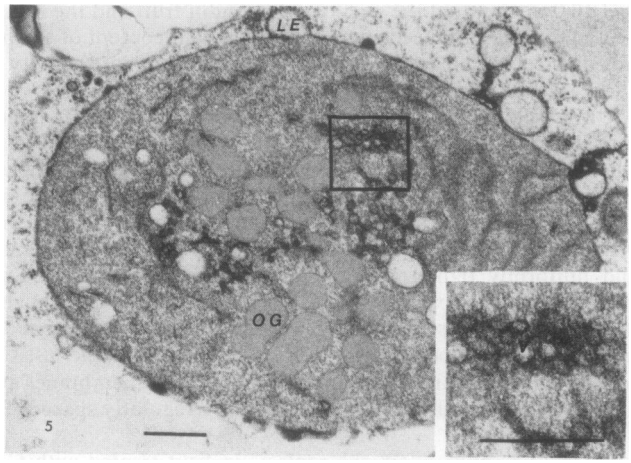


FIG. 3. Chloroplast from a green calamondin fruit peel, showing extensive grana-fretwork system (G) (× 48,000). Inset shows thylakoids of even density and uniformly tightly spaced. Bars equal 400 nm.

FIG. 4. Chloroplast from a calamondin fruit peel after 48 h exposure of the fruit to 5.3 µl/l C_2H_4, showing grana (G), vesicles (V), stroma lamellae (SL), and osmiophilic globules (OG) (× 30,100). Arrow in inset shows thylakoids are unevenly spaced. Bars equal 400 nm.



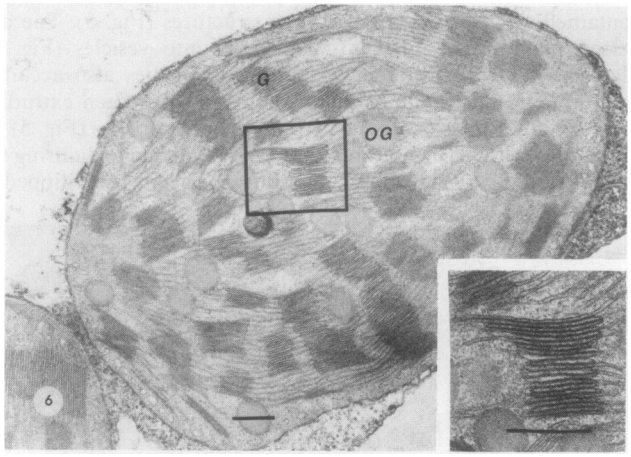


FIG. 5. Chloroplast from a calamondin fruit peel after 72 h exposure of the fruit to 5.3 μl/l C_2H_4, showing osmiophilic globules (OG) and lipids extruding through the chloroplast envelope (LE) (× 40,600). Inset shows vesicles (V) which may arise from the grana-lamellae fretwork. Bars equal 400 nm.

FIG. 6. Chloroplast from a calamondin fruit peel which had been dipped in 1.0 mM $AgNO_3$ for 15 min prior to exposure of the fruit to 5.3 μl/l C_2H_4 for 72 h, showing grana (G) and osmiophilic globules (OG) (× 27,400). Inset shows thylakoids of even density and uniformly tightly spaced. Bars equal 400 nm.

a 1 mM silver solution prior to exposure to C_2H_4, typically appeared unaltered by C_2H_4 (Fig. 6). The grana-fretwork system remained intact during the 72-h C_2H_4 treatment (Fig. 6).

DISCUSSION

The epicarpal cells of green citrus peel have highly organized chloroplasts with an extensive grana-fretwork system. Several lines of evidence suggest that disruption of chloroplast membranes is a primary process occurring during degreening whether degreening occurs naturally (8, 19) or is induced by exogenous C_2H_4 (15). In the present study, no appreciable color change or loss of Chl occurred until ultrastructural changes in the chloroplasts were apparent. The first and most obvious ultrastructural change in the chloroplast was the complete disappearance of the grana-fretwork system. Grana are also among the first structures to breakdown during the natural conversion of chloroplasts to chromoplasts (19). When chromoplasts revert back to chloroplasts during regreening, an extensive grana-fretwork system develops again (8, 20).

Chlorophyllase apparently is also involved in the ultimate degradation of Chl during C_2H_4 degreening of citrus fruit. The proposed role of chlorophyllase in degreening is that of converting the lipid-soluble Chl into the more H_2O-soluble chlide which then is catalytically bleached by H_2O_2 (9) or enzymically bleached by peroxidase activity (1). During degreening with C_2H_4, an inverse relationship between chlorophyllase activity and Chl content of the peel was observed. However, the chlorophyllase levels in the peel of fruit degreening naturally (in the absence of exogenously applied C_2H_4) are not always correlated with Chl content and green calamondin fruit frequently have higher chlorophyllase levels than calamondin fruit which have lost approximately half of their Chl (data not shown). A high level of chlorophyllase activity alone may not necessarily cause degreening. Aljuburi *et al.* (1) reported that during the regreening of 'Valencia' oranges (a process occurring in the spring following flowering), chlorophyllase levels increased in parallel with Chl content, suggesting that the enzyme may also be involved in Chl synthesis. Alternatively, chlorophyllase and Chl may be spatially separated until the chloroplast envelope is made permeable by a C_2H_4-mediated process. Inhibition of chlorophyllase synthesis by cycloheximide during degreening of citrus fruits (3, 18) and senescence of detached leaves (13) supports the concept of spatial separation.

The mechanism by which $AgNO_3$ interfered with C_2H_4 in degreening citrus peel is not known. It is apparent that $AgNO_3$ did not simply combine with the C_2H_4 since C_2H_4 was continuously supplied to the degreening chambers (4). Alternatively, the Ag may occupy the C_2H_4 receptor sites in the tissue. Beyer (5) showed that Ag does not inhibit the oxidation of C_2H_4 to CO_2 by pea tissue but does inhibit C_2H_4 incorporation into H_2O-soluble tissue metabolites; hence, the sites of C_2H_4 conversion to CO_2 and its incorporation into tissue-soluble metabolites are apparently different. The fact that $AgNO_3$, even at concentrations which caused peel injury, did not completely inhibit Chl destruction and the C_2H_4-induced increase in chlorophyllase activity further supports the unlikelihood that all of the C_2H_4 receptor sites were simultaneously occupied by Ag. A third possibility is that Ag catalytically converts C_2H_4 to an inactive form other than CO_2 in the presence of particular cellular constituents. Until the mechanism by which C_2H_4 mediates plant processes is known, the role Ag plays in counteracting C_2H_4 action remains speculative.

The primary role of C_2H_4 in degreening citrus fruit is still not clear. Apelbaum *et al.* (2) present evidence which indicates that endogenous C_2H_4 is not the primary inducer for the natural color change in detached 'Shamouti' oranges. Nevertheless, ultrastructural changes are similar in the peel of citrus fruit which degreen naturally (19) and those which are degreened with C_2H_4 (this study). Thus, C_2H_4 may not necessarily trigger color change in citrus fruits but simply accelerates or amplifies processes which are already in progress. For example, the level of chlorophyllase activity in calamondin fruit degreening naturally is less than one-fourth that of fruit being degreened with C_2H_4. The question remains as to whether or not very low endogenous levels of C_2H_4, which may never leave the tissue, produce a metabolic effect without any C_2H_4 being observed in the atmosphere surrounding the tissue. Since chlorophyllase was always found, albeit at low levels, the primary mechanism of exogenously applied C_2H_4 in degreening may well be disruption of chloroplast integrity.

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LITERATURE CITED

1. ALJUBURI H, A HUFF, M HSHIEH 1979 Enzymes of chlorophyll catabolism in orange flavedo. Plant Physiol 63: S-73
2. APELBAUM A, EE GOLDSCHMIDT, S BEN-YEHOSHUA 1976 Involvement of endogenous ethylene in the induction of color change in Shamouti oranges. Plant Physiol 57: 836-838
3. BARMORE CR 1975 Effect of ethylene on chlorophyllase activity and chlorophyll content in calamondin rind tissue. HortScience 10: 595-596

4. BARMORE CR, TA WHEATON 1978 Diluting and dispensing unit for maintaining trace amounts of ethylene in a continuous flow system. HortScience 13: 169–171
5. BEYER EM JR 1979 Effect of silver ion, carbon dioxide, and oxygen on ethylene action and metabolism. Plant Physiol 63: 169–173
6. BIO-RAD LABORATORIES 1977 Bio-Rad Protein Assay. Technical Bull 1051
7. BRUINSMA J 1963 The quantitative analysis of chlorophylls *a* and *b* in plant extracts. Photochem Photobiol 2: 241–249
8. EL-ZEFTAWI BM, RG GARRETT 1978 Effects of ethephon, GA, and light exclusion on rind pigments, plastid ultrastructure, and juice quality of Valencia oranges. J Hort Sci 53: 215–223
9. NOACK K 1943 Biological decomposition of chlorophyll. Biochem Z 316: 166–187
10. PURVIS AC 1979 Differential inhibition of ethylene by $AgNO_3$ and α,α'-dipyridyl in the degreening of calamondin fruit. Plant Physiol 63: S–91
11. REYNOLDS ES 1963 The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17: 208–212
12. ROUHANI I 1978 Effect of silver ion application on the ripening and physiology of stored green bananas. J Hort Sci 53: 317–321
13. SABATER B, MT RODRIQUEZ 1978 Control of chlorophyll degradation in detached leaves of barley and oat through effect of kinetin on chlorophyllase levels. Physiol Plant 43: 274–276
14. SALTVIET ME JR, KJ BRADFORD, DR DILLEY 1978 Silver ions inhibits ethylene synthesis and action in ripening fruits. J Am Soc Hort Sci 103: 472–475
15. SHIMOKAWA K, A SAKANOSHITA, K HORIBA 1978 Ethylene-induced changes of chloroplast structure in Satsuma mandarin (*Citrus unshiu* Marc) Plant Cell Physiol 19: 229–236
16. SHIMOKAWA K, S SHIMADA, K YAEO 1978 Ethylene-enhanced chlorophyllase activity during degreening of *Citrus unshiu* Marc. Sci Hort 8: 129–135
17. SPURR AR 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruc Res 26: 31–43
18. STEMPAK JC, RT WARD 1964 An improved staining method for electron microscopy. J Cell Biol 22: 697–701
19. THOMSON WW 1966 Ultrastructural development of chromoplasts in Valencia oranges. Bot Gaz 127: 133–139
20. THOMSON WW, LN LEWIS, CW COGGINS 1967 The reversion of chromoplasts to chloroplasts in Valencia oranges. Cytologia 32: 117–124