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ETHYLENE PRODUCTION BY HEALTHY AND *SCLEROTINIA FRUCTIGENA*-INFECTED APPLE PEEL

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Abstract—Production of ethylene by infected preclimacteric apple peel is much more sensitive to inhibition by acetate and potassium nitrate than is that from healthy climacteric peel. The opposite is true of the effect of rhizobitoxine. It is suggested that the ethylene produced from the infected tissues originates from the host's metabolism rather than from that of the fungus, but by a pathway different from that operative in healthy climacteric peel.

INTRODUCTION

IT IS A common observation that plant tissues infected by fungi, bacteria and viruses produce considerably more ethylene than related healthy tissues.[1] In studies with pre-climacteric apple fruit tissue infected with the brown-rot fungus *Sclerotinia fructigena* Aderh. & Ruhl. it was shown that a large proportion of the ethylene evolved originated from the margin of the rot, while heavily-infected tissue from the body of the rot produced very little of the gas. *S. fructigena* grown in culture did not produce ethylene, and infected heat-treated apple tissue also failed to produce the hydrocarbon, suggesting that it may be produced by the living host cells in response to infection.[1]

The enzymic conversion of methionine to ethylene in healthy apple tissue is well-documented,[2–5] although this substrate is apparently not involved in the production of ethylene by *Penicillium digitatum*, the only fungus studied in detail.[6,7] Since ethylene production by infected plant tissues has received very little study, the object of the present work was to compare factors affecting evolution of the gas by healthy and *S. fructigena*-infected apple fruit peel.

RESULTS AND DISCUSSION

In view of the osmotic instability of apple tissue[8] and the need to keep the tissue in a satisfactory physiological state, preliminary experiments were carried out with healthy and with infected fruit peel disks incubated in different aqueous sucrose concentrations. In all

[1] HISLOP, E. C., HOAD, G. V. and ARCHER, S. A. (1973) in *Fungal Pathogenicity and the Plant's Response* (BYRDE, R. J. W. and CUTTING, C. V., eds.), p. 87, Academic Press, London.
[2] LIEBERMAN, M., KUNISHI, A. T., MAPSON, L. W. and WARDALE, D. A. (1966) *Plant Physiol.* **41**, 376.
[3] BAUR, A. H., YANG, S. F. PRATT, H. K. and BIALE, J. B. (1971) *Plant Physiol.* **47**, 696.
[4] OWENS, L. D., LIEBERMAN, M. and KUNISHI, A. T. (1971) *Plant Physiol.* **48**, 1.
[5] BAUR, A. H. and YANG, S. F. (1972) *Phytochemistry* **11**, 3207.
[6] JACOBSEN, D. W. and WANG, C. H. (1968) *Plant Physiol.* **43**, 1959.
[7] KETRING, D. L., YOUNG, R. E. and BIALE, J. B. (1968) *Plant Cell Physiol. (Tokyo)* **9**, 617.
[8] BURG, S. P. and THIMANN, K. V. (1960) *Plant Physiol.* **35**, 24.

experiments, maximum ethylene evolution occurred in 0·4 M sucrose with less in 0·2 M and 0·6 M sucrose. In most cases, the amount of ethylene produced by disks incubated in distilled water was only 50% of that produced in 0·4 M sucrose.

TABLE 1. EFFECT OF SODIUM ACETATE AND SORENSEN'S CITRATE BUFFERS (0·1M) IN 0·4 M SUCROSE ON ETHYLENE PRODUCTION*

pH	Healthy peel		Infected peel	
	acetate	citrate	acetate	citrate
6·5	33 ± 3	128	4 ± 3	95
6·0	26 ± 3	122	2 ± 2	72
5·5	19 ± 1	106	2 ± 2	86
4·5	2 ± 1	89	0	94

* Values are the means of 3 replicates in an experiment, or where the s.d. is given, the means of replicate experiments (each with 3 replicates) expressed as a percentage of the mean amount of ethylene produced by disks in the sucrose sodium phosphate medium (pH 7·0).

Since homogenized apple tissue has an acid pH, further incubations were carried out in 0·4 M sucrose containing 0·1 M acetate, citrate or phosphate buffers. Table 1 compares the effects of acetate and citrate buffers on ethylene production by peel disks from the cultivar Lord Lambourne as percentages of that produced in 0·4 M sucrose maintained at pH 7·0 with 0·1 M sodium phosphate buffer. The mean amounts of ethylene produced by healthy and diseased peel in the sucrose/phosphate medium were 1·90 ± 0·71 and 0·70 ± 0·18 μl/g dry wt/hr respectively, while uninfected pre-climacteric peel produced less than 20 nl/g/hr. Clearly, acetate buffer greatly reduced the production of ethylene from both healthy and diseased tissues at all pH values tested, and the inhibitory effect was greater for infected than for healthy peel. In both, inhibition became more marked with increasing acidity. The inhibitory effect of sodium acetate was confirmed by adding this compound (0·1 M) to disks of peel incubated in the sucrose/phosphate buffer medium (pH 7·0). The reason why acetate is such a potent inhibitor of ethylene production is not known, but in healthy tissues it may be related to the reported effects of acetate in the development of low-temperature breakdown in apples,[9] and in infected tissue it may be related to our own observations which show that acetate is toxic to the fungus (unpublished data). Citrate buffer was not used in further experiments, since it seemed desirable to avoid the use of organic buffers which might be readily metabolized to ethylene.[6] Therefore, most subsequent incubations were carried out in 0·4 M sucrose in 0·1 M phosphate buffer, pH 7·0.

Table 2 shows the effect of a variety of supplements, added to the sucrose/phosphate incubation medium, on ethylene production by peel disks. There was no consistent evidence that any of the potential substrates, enzymes or co-factors (e.g. phenols) greatly stimulated ethylene production from either infected or healthy tissues under these test conditions.

Since we had previously demonstrated proteolytic activity in infected apple tissue,[1] the effect of added Pronase was examined, as it was thought that this enzyme preparation

[9] WILLS, R. B. H., SCOTT, K. J. and MCGLASSON, W. B. (1970) *J. Sci. Food Agric.* **21**, 42.

might produce additional peptides of methionine[10,11] which could act as substrates for the enzymic production of ethylene. However, the failure of this treatment applied to both healthy and diseased tissue, together with the lack of a marked stimulation by methionine or its derivatives, would appear to indicate that either this factor is not limiting or that it is not involved in biogenesis of the hydrocarbon. Although methionine is a direct precursor

TABLE 2. EFFECT OF POTENTIAL SUPPLEMENTS ON ETHYLENE PRODUCTION*

Supplement	Concentration	Healthy peel	Infected peel
N-Acetyl-DL-methionine	10^{-4} M	112 ± 6	120 ± 24
N-*t*-Butoxycarbonyl-D-methionine	10^{-4} M	118 ± 12	133 ± 11
DL-Methionine	10^{-4} M	128 ± 4	115 ± 0
Pronase	9 units†/ml	94	92 ± 2
Horseradish peroxidase	18 units†/ml	96 ± 6	120 ± 18
Hydrogen peroxide	30 ppm	111 ± 14	109
Indole-3-acetic acid	10^{-5} M	119 ± 17	119 ± 22
p-Hydroxybenzoic acid	10^{-3} M	90	102
Chlorogenic acid	10^{-3} M	75	76
Caffeic acid	10^{-3} M	68	88
o-Coumaric acid	10^{-3} M	99	90
p-Coumaric acid	10^{-3} M	79	122
(+)-Catechin	10^{-3} M	94	104

* Values calculated as described for Table 1 and expressed as a percentage of the ethylene produced in the absence of supplements.
† See Experimental.

of ethylene in higher plants, for healthy peel at least, its failure to cause an increased evolution of ethylene has been previously reported,[12] and this amino acid is known to be continuously regenerated in apple tissue.[5] Thus, some other factor would appear to be limiting, although peroxidase[13,14] and hydrogen peroxide[15] did not appear to be so in the present tests.

TABLE 3. EFFECT OF POTASSIUM NITRATE ON ETHYLENE PRODUCTION*

KNO_3 concentration (M)	Healthy peel	Infected peel	KNO_3 concentration (M)	Healthy peel	Infected peel
0·2	110 ± 20	45 ± 5	0·6	99 ± 3	12 ± 3
0·4	105 ± 3	16 ± 4	0·8	47 ± 2	7 ± 3

* Values are the means and ranges of duplicate experiments (each with 3 replicates) as a percentage of the mean amount of ethylene produced by disks in distilled water.

When a variety of salts were added to peel disks in the sucrose/buffer medium to give a 1·0 M concentration of salt there was a very large reduction of ethylene evolution from

[10] KU, H. S. and LEOPOLD, A. C. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1155.
[11] DEMOREST, D. M. and STAHMANN, M. A. (1971) *Plant Physiol.* **47**, 450.
[12] GALLIARD, T., RHODES, M. J. C., WOOLTORTON, L. S. C. and HULME, A. C. (1968) *Phytochemistry* **7**, 1465.
[13] YANG, S. F. (1969) *J. Biol. Chem.* **244**, 4360.
[14] TAKEO, T. and LIEBERMAN, M. (1969) *Biochim. Biophys. Acta.* **178**, 235.
[15] LUND, B. A. and MAPSON, L. W. (1970) *Biochem. J.* **119**, 251.

both healthy and diseased peel which could not be attributed to a specific cation (Ca^{2+}, K^+, Na^+) or anion (NO_3^-, Cl^-). This effect was examined further by incubating peel disks in different concentrations of aqueous potassium nitrate. The results (Table 3) show that whilst ethylene production from healthy peel was reduced only by concentrations greater than 0·6 M, production of the gas by infected peel was greatly reduced by 0·2 M and almost eliminated by 0·8 M salt.

TABLE 4. EFFECT OF POTENTIAL INHIBITORS ON ETHYLENE PRODUCTION*

Inhibitor	Concentration	Healthy peel	Infected peel
Rhizobitoxine preparation†	—	9 ± 4	87 ± 7
Catalase	2000 units†/ml	97 ± 6	92 ± 19
Sodium azide	10^{-3} M	17 ± 3	44 ± 8
Sodium fluoride	10^{-3} M	61 ± 9	40 ± 10
Potassium cyanide	10^{-3} M	66 ± 21	39 ± 15
Cycloheximide	10^{-3} M	57 ± 2	70
Na *cis*-3-chloroacrylate	10^{-3} M	103 ± 12	77 ± 22
Na *cis*-3-chloroacrylate	5×10^{-3} M	116	120

* Values calculated as for Table 1 and expressed as a percentage of the ethylene produced in the absence of inhibitors.
† See Experimental.

A selection of potential inhibitors of ethylene production was also examined for their effect on peel disks in the sucrose/phosphate buffer medium (Table 4). The classical enzyme inhibitors together with cycloheximide all reduced ethylene production; the differential effect of cyanide snd azide on the two tissues could reflect different metabolic pathways. Rhizobitoxine[16] was outstanding in almost eliminating ethylene production by healthy tissues (even when added with methionine) while not greatly affecting production by infected peel. These observations suggest that methionine is not involved as a substrate for ethylene production in infected pre-climacteric peel since rhizobitoxine inhibits a step in the conversion of methionine to ethylene,[17] but do not provide information as to whether the ethylene is generated by the host, the pathogen or by the host and pathogen. Rhizobitoxine does not inhibit ethylene production by *P. digitatum*.[17]

Catalase, which antagonized the stimulatory effects of pectic enzymes on ethylene production by cauliflower floret tissue,[15] had no such effect in our tests. This is consistent with our inability to stimulate the production of ethylene from healthy peel disks incubated in extracellular pectolytic enzyme preparations from *S. fructigena*.[1] Similar tests repeated during the present study with polygalacturonase fractions purified by isoelectric focusing and which caused permeability changes and death of healthy peel cells,[18] had no effect on ethylene production. Despite the fact that sodium *cis*-3-chloroacrylate caused a reduction of ethylene production from infected tissue in one test, this was not confirmed in other experiments with both tissues. An acrylic acid pathway for the production of ethylene has

[16] OWENS, L. D. (1969) *Science* **165**, 18.
[17] LIEBERMAN, M. and KUNISHI, A. T. (1971) *Hort. Science* **6**, 355.
[18] BYRDE, R. J. W., FIELDING, A. H., ARCHER, S. A. and DAVIES, E. (1973) in *Fungal Pathogenicity and the Plant's Response* (BYRDE, R. J. W. and CUTTING, C. V., eds.), p. 39, Academic Press, London.

been proposed for higher plants,[19–21] but if it occurs at all is apparently very inefficient. In contrast, *P. digitatum* metabolizes acrylic acid[6] rather more efficiently, and evolution of the gas is inhibited by sodium *cis*-3-chloroacrylate.[6] If this observation applies to fungi generally, then our failure to inhibit ethylene production from infected peel with chloroacrylate could be interpreted as supporting our earlier suggestion[1] that the hydrocarbon originates from the injured host tissues, but in view of the effect of rhizobitoxine, probably not via methionine. During the present investigation production of ethylene by the fungus alone was also re-examined. The gas was not detected above cultures growing on a variety of synthetic or plant-derived media even when supplemented with likely precursors, despite the fact that cultures were incubated for 20 hr (compared with 3 hr for disks) and contained many more growing hyphal tips than the infected peel disks.

The greater sensitivity shown by the infected peel ethylene-generating system towards both inorganic salts and acetate could in part be a result of the damage that these tissues have already sustained as a result of infection. However, this comment does not apply to the results of the rhizobitoxine inhibition experiments. Our earlier observation that brown-rot-infected but not healthy climacteric apples evolve ethylene from both peel and pulp tissues,[1] together with these present observations, indicate a different biogenetic origin for the gas from healthy and diseased tissues. In the only other reported study of this nature, Sakai *et al.*[22] from experiments with glucose-U-^{14}C reached similar conclusions concerning the origin of ethylene in healthy and black-rot-infected sweet potatoes.

On the other hand, a recent report[23] has indicated that ethylene produced from leaves treated with toxic chemicals arose from methionine by the same biochemical pathway as involved in the evolution of the gas from uninjured tissue. Thus, the nature of the stress factor may have an important bearing upon the biogenetic origin of ethylene, and further studies with healthy tissues and with tissues exposed to various degrees of physiological and pathological stress are clearly warranted.

EXPERIMENTAL

Plant material. Pre-climacteric and climacteric apple fruits from the cultivar Lord Lambourne were used. Pre-climacteric fruit was artificially infected with *Sclerotinia fructigena* and incubated for 5 days before the peel (1·0–1·5 mm thick) was removed with a potato peeler. Disks (10 mm dia.) which were half infected and half healthy were cut from the peelings with a cork borer. Healthy peel disks were obtained from climacteric fruit in a similar manner. Four disks selected at random were placed in each glass specimen tube (74 × 23 mm) containing 2 ml of incubating medium. Supplements and inhibitors were made up in the incubating medium and aliquots added to the desired concentration. At least 3 replicate tubes were prepared for each test. Tubes containing peel were preincubated with mild agitation for 1 hr at 25°, flushed with oxygen (15 sec) and sealed with polyethylene stoppers fitted with gas septa. Sealed tubes were incubated for 3 hr (25° with agitation) before a gas sample was removed with a syringe and assayed for ethylene by GC.[24]

Reagents. *N*-Acetyl-DL-methionine, *N*-*t*-butoxycarbonyl-D-methionine and catalase were obtained from Sigma; horseradish peroxidase (salt-free, lyophylised) from Koch-Light; and Pronase (grade B) from Calbiochem. The units of enzyme activity referred to in Tables 2 and 4 are as defined by the appropriate manufacturer. Sodium *cis*-3-chloroacrylate was a gift from Dr. R. A. Herrett (Union Carbide Corpn., 270 Park Avenue, New York.)

Rhizobitoxine. The 'rhizobitoxine' used in this work was produced and concentrated from cultures of *Rhizobium japonicum* (Strain 94)[25] as modified by Dr. L. D. Owens (private communication). The prepara-

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[20] SHIMOKAWA, K. and KASAI, Z. (1970) *Agr. Biol. Chem.* **34**, 1640.
[21] SHIMOKAWA, K. and KASAI, Z. (1970) *Agr. Biol. Chem.* **34**, 1646.
[22] SAKAI, S., IMASEKI, H. and URITANI, I. (1970) *Plant Cell Physiol. (Tokyo)* **11**, 723.
[23] ABELES, A. L. and ABELES, F. B. (1972) *Plant Physiol.* **49**, (Supplement) 21.
[24] HISLOP, E. C. and STAHMANN, M. A. (1971) *Physiol. Plant Path.* **1**, 297.
[25] OWENS, L. D. and WRIGHT, D. A. (1965) *Plant Physiol.* **40**, 931.

tion yielded a yellow reaction product with ninhydrin on chromatographic paper (R_f 0·15 using 80% phenol as solvent) and caused chlorosis of 5-day-old sorghum seedlings. On the bases of these characteristics (and the effect on ethylene production by healthy peel) it was presumed that the preparation contained rhizobitoxine although the toxin was not purified and characterized further.

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