Pergamon

PII: S0031-9422(98)00147-2

Phytochemistry Vol. 49, No. 8, pp. 2291–2298, 1998

Printed in Great Britain
0031-9422/98/$ - see front matter

ETHYLENE ENHANCES THE ANTIFUNGAL LIPID CONTENT IN IDIOBLASTS FROM AVOCADO MESOCARP

ALICIA LEIKIN-FRENKEL and DOV PRUSKY*

Department of Postharvest Science of Fresh Produce, The Volcani Center, Bet Dagan 50250 Israel

(*Received in revised form* 2 *February* 1998)

Key Word Index—*Persea americana*; Lauraceae; *Colletotrichum gloeosporioides*; preformed compounds; fruit resistance to postharvest diseases; oil cells.

Abstract—It has previously been demonstrated that exposure of whole avocado fruits cv. Fuerte to 40 μl/l ethylene for 3 hours enhances the level of antifungal 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene (diene) in the fruit pericarp. Exposure of 1–2 mm slices of fruit pericarp and mesocarp to ethylene enhanced the level of the antifungal diene in the mesocarp only. Since most of the antifungal diene in the mesocarp is compartmentalized in idioblasts, the effect of ethylene was tested on isolated idioblasts. Exposure of idioblasts to ethylene increased the level of antifungal diene twofold within 3 hours. This effect was temperature dependent. Three hours exposure of idioblasts to ethylene at 35° doubled the diene content compared to less than 50% increase after three hours at 20°. Incubation of idioblasts with [2-^{14}C]malonyl-CoA or [1-^{14}C]acetate in the presence of ethylene, showed the incorporation of the label into a compound that co-chromatographed with the antifungal diene. The compound induced by ethylene and released from the cells was identified by NMR as the antifungal diene. The present report suggests that ethylene can induce the synthesis of the antifungal diene in idioblasts and the export of this compound to the pericarp of the fruits.

INTRODUCTION

Colletotrichum gloeosporioides attacks avocado fruits during growth in the orchard. The pathogen germinates and penetrates unripe fruit but remains quiescent until harvesting [1]. After harvest, fruit ripening is accompanied by activation of fungal development and appearance of decay symptoms. Resistance of unripe fruit to postharvest pathogens is related to the presence of preformed antifungal compounds in fruit pericarp (peel). The most active of them was found to be an antifungal diene [2]. The level of this compound decreases during ripening thus enabling the activation of the quiescent infections and symptom expression [1]. Metabolism of the antifungal diene and increased susceptibility after harvest is mediated by lipoxygenase. The activity of this enzyme is, in turn, regulated by the levels of a non-specific inhibitor in the pericarp identified as epicatechin whose levels decrease markedly in ripe fruits [1].

Enhanced levels of the antifungal diene caused by biotic and abiotic elicitors may depend on the balance between rates of synthesis and breakdown of the compound [1]. Two possibilities exist for maintaining fungitoxic levels of antifungal compounds in the tissue of ripening fruits: (i) prevention of catabolism, (ii) induction of synthesis. Several biotic and abiotic elicitors that increased the levels of the antifungal diene also led to higher levels of epicatechin [3]. These results seem to imply that abiotic elicitors may enhance the levels of antifungal diene by inhibiting its catabolism. On the other hand, increase of antifungal diene may result from the induced synthesis of the compound. The synthesis of the antifungal diene may originate from acetate and malonate, like fatty acids, or be a secondary product of lipid metabolism [4].

The location of the sites of biosynthesis of the antifungal compounds in avocado fruits has never been described. A previous report by Kobiler *et al.* [5] described the presence of antifungal compounds compartmentalized in specific oil cells in the mesocarp of avocado. Interestingly, about 85% of all antifungal compounds within the mesocarp were located in these cells which suggested the possibility that the synthesis of antifungal compounds may occur in specific idioblasts. In the present work we

*Author to whom correspondence should be addressed.

have shown that ethylene treatment that increases the level of the antifungal diene in whole fruits, has a similar effect on isolated idioblasts. Ethylene enhanced both the biosynthesis of the diene in the idioblast and the release of antifungal lipids out of the idioblast. Results suggest that idioblast are important as the source of compounds involved in the postharvest defense process of avocado fruits.

RESULTS AND DISCUSSION

Response of avocado fruit tissues to exogenous C_2H_4 exposure

Exposure of freshly harvested unripe avocado fruits to ethylene enhanced the levels of the antifungal diene in the pericarp and the mesocarp of the fruit 24 h after treatment [6]. The increase in the level of the antifungal diene in the pericarp was described as a direct effect of ethylene on the regulation of the breakdown of the antifungal diene in the tissue. Ardi *et al.* [6] found that ethylene activated phenylpropanoid metabolism, especially flavanone-3-hydroxylase, at a transcriptional level and resulted in an increase of epicatechin. Present results show that exposure of avocado fruits to 40 μl/l C_2H_4 for 6 h almost doubled the level of the antifungal diene content in the pericarp in cv. Fuerte (Fig. 1). Concentration of the antifungal diene in the mesocarp of fruits exposed to 40 μl/l C_2H_4 for 3 h also increased by 80% (Fig. 2). However, when slices of either pericarp or mesocarp were sampled from freshly harvested fruits and then exposed to C_2H_4, only the mesocarp showed a 40% increase in the diene content (Fig. 2). Since Kobiler and co-workers [5] indicated that 85% of the antifungal diene in the mesocarp is located in idioblast cells, a similar ethylene treatment was applied to isolated idioblast cells.

Idioblast isolated from avocado fruits cv. Fuerte and exposed to 40 μl/l C_2H_4 showed an increase in diene from 380 to 800 $\mu g/10^6$ cells, 6 h after the treatment (Fig. 3). A similar response occurred in idioblasts obtained from mesocarp of avocado cv. Reed where the level of diene in C_2H_4 treated idioblast was twice the amount found in untreated cells (Fig. 3). The minimal time required for the cells to respond to C_2H_4 stimulus and show an increase in diene level was 60 min (Fig. 4). Maximal increase of antifungal compounds was obtained at 35° (Fig. 5). At this temperature the antifungal diene production increased 2 fold compared to untreated cells (Fig. 6). This indicates that an abiotic elicitor like ethylene is affecting the synthesis in the preformed compound and it is reasonable to suggest that idioblast are responsive for the modulation of the antifungal diene in the mesocarp.

Compartmentation of preformed antifungal compounds in oil cells has been reported also for other plants [7]. The polyacetylenic compound falcarindiol, is localized in extracetracellular oil droplets in carrot root periderm and pericycle [12]. The preformed antifungal compound gossypol is associated with pigment glands in cotton [13]. The preformed furanocoumarins from parsley are present in oil ducts and also free in the host epidermal cells [14]. In all these cases resistance to pathogen by the antifungal compounds was conferred by high amounts of free and not compartmentalized compounds.

Incorporation of [^{14}C] labeled precursors to idioblasts compounds

The hypothesis that the antifungal diene is synthesized in idioblast was supported by the incorpor-

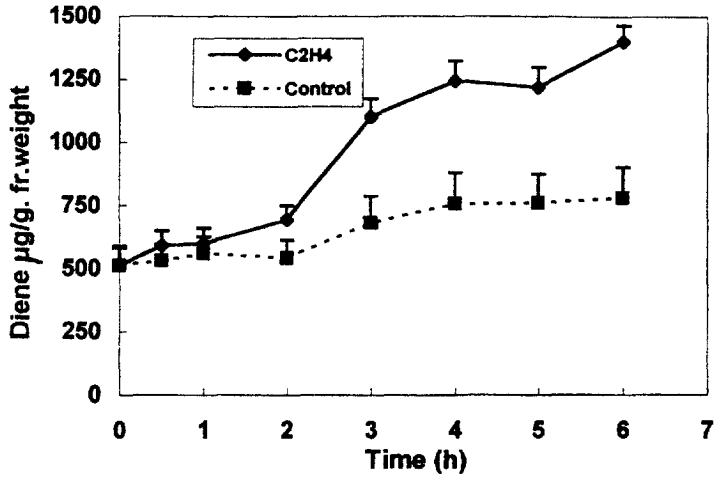

Fig. 1. Antifungal diene content in the avocado fruit pericarp after 40 μl/l ethylene treatment of freshly harvested fruits cv. Fuerte at 20°. After each period of treatment, the pericarp was sampled and the diene extracted as described in Materials and Methods. Bars indicate the standard error for each sampling time.

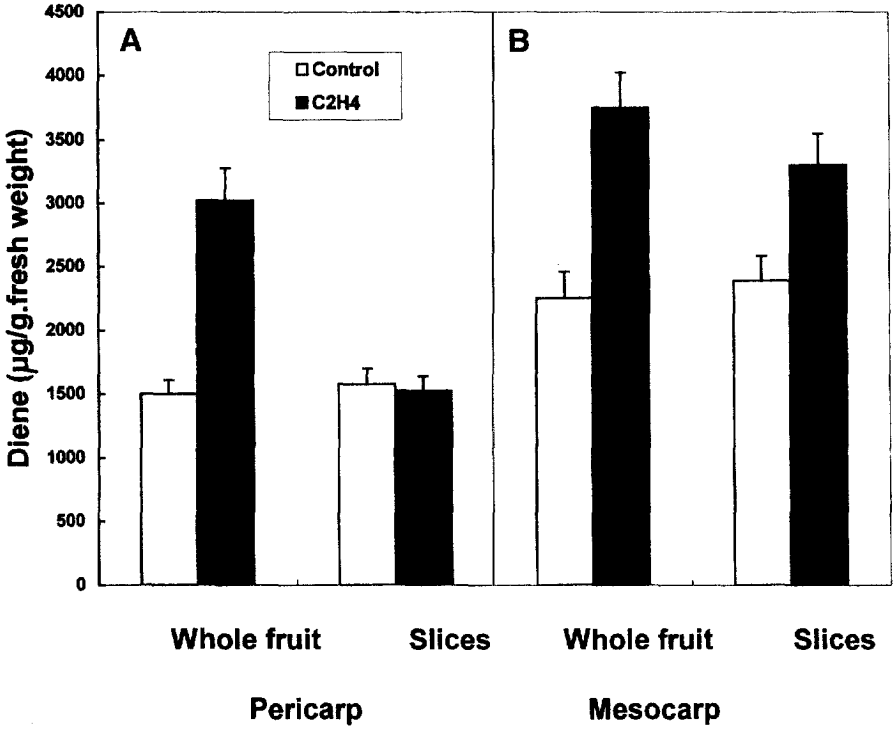

Fig. 2. Antifungal diene content in the pericarp and mesocarp of avocado fruits cv. Fuerte exposed to ethylene before and after sampling. **A**. Antifungal diene in the pericarp of freshly harvested fruits exposed to 40 μl/l C_2H_4 for 3 h and on pericarp slices exposed to the same conditions after sampling. **B**. Antifungal diene in the mesocarp of freshly harvested fruits exposed to 40 μl/l C_2H_4 for 3 h and on mesocarp slices exposed to the same conditions after sampling from the fruit. Bars indicate the standard error for each sampled treatment.

ation of [2-^{14}C]malonyl-CoA and [1-^{14}C]acetate into a compound that initially co-chromatographed with the antifungal diene in TLC, HPLC and was finally characterized as the antifungal diene. Ethylene treatment of idioblast cells incubated in the presence of [1-^{14}C] acetate and [2-^{14}C] malonyl CoA enhanced the incorporation of both labeled materials (Fig. 6). Not only the precursors were incorporated by the cells but they were also transformed into a compound that co-chromatographed with the diene by TLC and HPLC (Fig. 6). Boiled cells did not show any transformation of labeled precursors in the presence or absence of ethylene (results not shown), indicating that a metabolically active system is involved. When pericarp and mesocarp slices were exposed to ethylene in the presence of [1-^{14}C] acetate they did incorporate and transform this precursor into a compound that co-chromatographed with the antifungal diene by TLC. Mesocarp slices transformed 5% of the incorporated material into

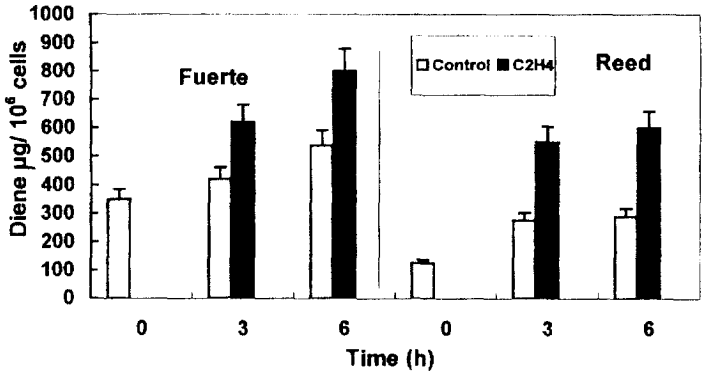

Fig. 3. Antifungal diene content in idioblast exposed to ethylene. Idioblasts isolated from mesocarp of avocado fruits cv. Fuerte and Reed were exposed for 3 and 6 h at 20° to 40 μl/l C_2H_4. Bars indicate the standard error for each sampling time.

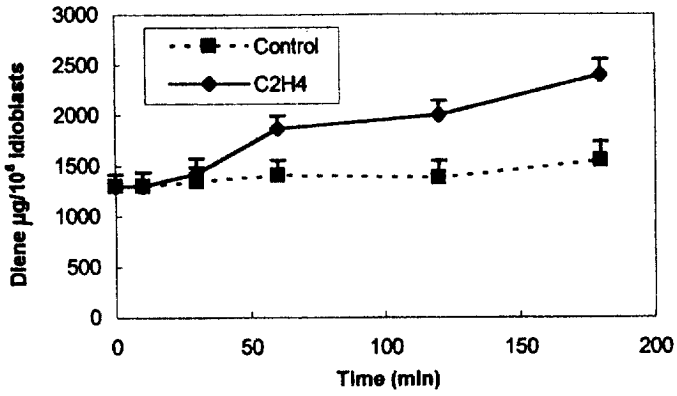

Fig. 4. Antifungal diene content in idioblast of avocado fruit mesocarp cv. Fuerte exposed for different periods to 40 µl/l C_2H_4 at 20°. Bars indicate the standard error for each sampling time.

the putative diene and pericarp slices transformed only 0.7% of it. Whereas purified idioblast cells transformed 37% of the labeled material into diene under similar conditions (Fig. 7), indicating the higher efficiency of the cells compared to other tissues, to transform precursors into the antifungal diene. The observations of large lipoidal inclusions in the plastid of similar developing oil cells has led to the conclusion that these organelles also may be involved in oil synthesis in other cases [8]. Observations in cells of *Citrus deliciosa* [9] and *Poncirus trifoliata* [10] support this conclusion. Terpene-producing trichomes and oil cavities in *Citrus* have smooth endoplasmic reticulum, which may be involved in oil synthesis [9, 11] or in the transport of oil from the plastids to the oil cavity [9].

Effect of ethylene treatment on the export of the diene from idioblasts

Present results indicate that ethylene treatment to pericarp does not induce an increase in the antifungal diene content unless the treatment is applied to whole fruits. This suggests that whereas the biosynthesis of the diene occurs in the oil cells, the compound may be relocated to other parts of the fruits. Ethylene treatment of idioblasts in the presence of floating mesocarp lipids (FL fraction) enhanced the diene content in the cells and in the lipid media (Table 1). The accumulation of the diene in the lipid media doubled after 6 h treatment compared to a 3 h treatment only. When idioblast cells and the lipid media were separated before ethylene treatment, no change in the diene level was observed suggesting that no synthesis of the diene occurs in the lipid media (not shown results). In order to determine that idioblast released the diene compound, cells were incubated in the presence of a solution of 10% bovine serum albumin as a non-specific hydrophobic acceptor. The material released in the presence of albumin and ethylene treatment was extracted and fractionated by TLC and NMR identified the component with R_f 0,47 as the described antifungal diene. The proton NMR spectrum showed δ: 0.90 (CH_3), 1.32 [$(CH_2)n$, 2.10 (CH_3COO–), 2.43 ($CH_2C{=}O$, *d*), 2.78 (bisallytic

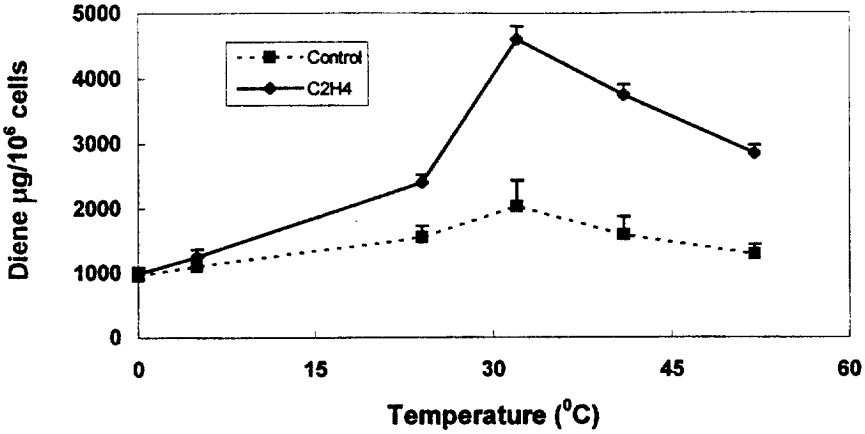

Fig. 5. Effect of temperature on the antifungal diene content of idioblast from avocado mesocarp cv. Fuerte exposed to 40 µl/l C_2H_4 for 3 h. Bars indicate the standard error for each sampling time.

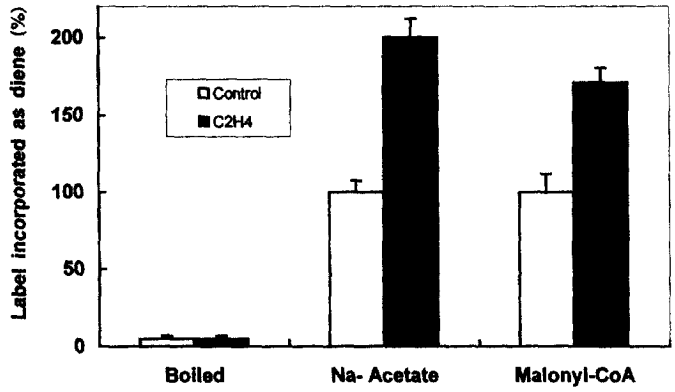

Fig. 6. Effect of ethylene on the incorporation of [^{14}C] labeled precursors by idioblasts from avocado fruit mesocarp of cv. Fuerte. Idioblast cells were exposed to 40 μl/l C_2H_4 at 35° and after 3 h filtered, washed and extracted as described in Experimental. The extracts were analyzed by TLC, the spots with a similar R_f as the diene standard were scraped off and the incorporated label measured by liquid scintillation counting. Bars indicate the standard error for each sampled treatment.

CH_2, *t*), 3.15 (OH, *s*), 4.10 (AcO–CH_2–), 4.30 (–CHOH, *m*), 5.36 (–CH=CH–, *m*).

In another set of experiments the export of labeled diene formed in idioblasts towards the incubation medium was followed. Idioblast cells exposed to ethylene under optimal time and temperature conditions in the presence of [1-^{14}C] acetate were filtered, washed and transferred to new fresh medium of varied composition and exposed again to 40 μl/l C_2H_4 for 3 h in the presence of proteins obtained by acetone powder and pure lipids obtained by Folch procedure from the mesocarp [15]. When the incubation media was composed of liposomes from pure lipids only, twenty percent of the labeled diene was released from the oil cells compared to 45% release in the present of soluble protein preparation (Fig. 8). This fact suggests that either simple solubility in the lipid fraction or factor(s) present in the acetone powder preparation of mesocarp might be involved in the diffusion or acceptance of the diene from the idioblasts towards other sites of the fruit.

Since neither epicatechin nor lipoxygenase activity could be detected in the idioblasts cells [5], the increase in the level of the diene is clearly not the result of inhibition of diene breakdown but the result of direct biosynthesis [1]. This does not preclude the possibility that other cells have the possibility to synthesize the antifungal diene, particularly because the increase of the diene at the site of fungal infection in the fruit peel would need a significant relocation of the antifungal compound. The elucidation of this process may shed light for under-

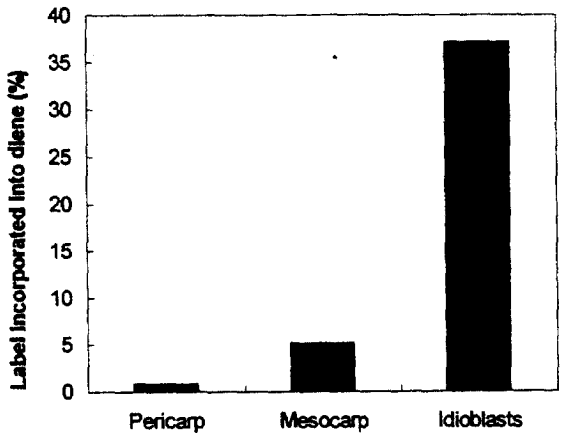

Fig. 7. [1-^{14}C] acetate transformation into diene by pericarp, mesocarp and isolated idioblasts cells from avocado cv. Fuerte exposed to 40 μl/l ethylene for 3 h at 35°. After exposure of equivalent amounts of tissue to the precursor, they were separated from the incubation medium, washed and the antifungal diene was extracted as described in Experimental. The extracts were applied to TLC plates, the spots with R_f 0.47 (like the diene standard) were scraped and the incorporated label measured by liquid scintillation. The acetate transformed into diene was calculated as the ratio between the radioactivity measured in the diene spot related to the total radioactivity applied to the TLC plate ×100.

Table 1. Effect of ethylene (40 μl/l) on the diene content of idioblasts incubated in the presence of mesocarp lipids*

Time (h)	Diene concentration†			
	Idioblasts‡		Mesocarp lipids	
	Control	C_2H_4	Control	C_2H_4
3	740 ± 45	1250 ± 100	187 ± 20	300 ± 32
6	800 ± 50	1600 ± 150	190 ± 25	640 ± 55

*Mesocarp lipids were separated from the idioblast cells by vaccum filtration of the floating layer (FL)

†Diene concentrations presented as μg per total cells ± SE.

‡$1-2 \times 10^6$ cells were incubated in the presence of 2 ml of mesocarp lipids at 35° for 3 h.

standing the basis of fruit defense and its possible regulation.

EXPERIMENTAL

Silica gel G plates, were purchased from Merck, Germany. Bovine serum albumin, sorbitol, MES buffer were obtained from Sigma Chem. Co., USA Film was obtained from Kodak, USA. Cellulozyme, macerozyme and pectolyase were from Calbiochem, USA. Organic solvents and analyticals were from BioRad, Jerusalem, Israel. 1-[^{14}C] acetate, 60 mCi/ml and 2-[^{14}C] malonyl CoA 55 mCi/ml were from Dupont de Nemours. Scintillation liquids Ultima Gold and Optifluor, for organic and aqueous samples, respectively, were from Packard, The Netherlands.

Source of material

Avocado fruits (*Persea americana*, Mill.) cultivars "Fuerte" and "Reed" were obtained from an orchard at Kibbutz Guivat Brenner. One to two mm thick slices of pericarp and mesocarp were sampled for experimental purposes. Firmness of avocado fruits, an inverse parameter of ripening, was measured as the force (kg) required to penetrate the fruit skin and flesh with a 5 mm diameter, 4 mm long conical probe [16] Mesocarp proteins were obtained by precipitation with cold acetone [6]. Mesocarp lipids were extracted according to the procedure of Folch [15]. Liposomes from mesocarp lipids were obtained as follows: the appropriate amount of mesocarp lipids extracted as described above were dried under N_2 and sonicated in the presence of buffer (as described bellow, without enzymes) in a Ultrasonic processor, W-225, Heat System ultrasonic, with a microtip, at 60% of duty capacity, for 3 min.

Isolation of idioblasts

Idioblasts were isolated from avocado mesocarp essentially according to the method of Platt *et al.* [17] with slight modifications. One hundred g of avocado mesocarp, 1–2 mm thick slices, were sampled and placed in 200 ml of 10 mM MES buffer pH 5.5 containing 16.500 u cellulase (ICN Biomedicals Inc), 5 u pectinase (Sigma) and 1000 u macerase (Calbiochem), 100 mM sorbitol, 1 mM $CaCl_2$, 0.2% dithiothreitol and 0.2% bovine serum albumin. The tissue was incubated for 15 h, overnight, on a stirring plate at room temperature until complete maceration. The macerated tissue was filtered through 300 μm mesh nylon net and unfiltered debris discarded. The filtrate was further transferred through 45 μm mesh nylon net and the unfiltered residue collected, placed in double distilled water and centrifuged twice for 10 min. at 12,000*g*. The upper layer containing avocado lipids was discarded and the pellet containing idioblasts, was further cleaned by suspension on 2X distilled water and

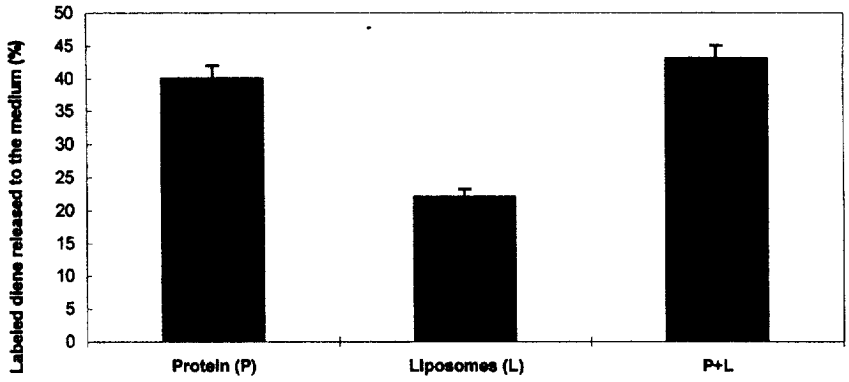

Fig. 8. Distribution of labeled [^{14}C] antifungal diene content between the incubation medium and idioblasts after exposure to 40 μl/l ethylene for 3 h at 35°. After 3 h initial incubation with [1-^{14}C] acetate in buffer in the presence of ethylene, idioblasts were filtered, washed and distributed in new incubation media containing proteins precipitated from the mesocarp after acetone extraction (2 mg/10^6 cells, P), liposomes obtained as described in Experimental (L) or a combination of proteins and liposomes. The suspension of idioblast was exposed to 40 μl/l ethylene for 3 h at 35°C and afterwards the medium was separated by filtration from idioblast and the distribution of the incorporated label was measured by liquid scintillation. Bars indicate the standard error for each sampled treatment.

centrifugation in an Eppendorf centrifuge 5–6 times until a white pellet was obtained. Cell concentration was determined by haemocytometer. The average cell concentration obtained was between 0.5×10^5 to 1×10^5 cells/g Fr. tissue.

In some cases the macerated tissue was directly centrifuged at 5000*g* for 10 min. and the floating layer (FL) containing cells and mesocarp lipids was used for experimental purposes. Both fractions were separated from lipids by vacuum filtration under a Millipore, 1225 Sampling Manifold.

Ethylene treatments of avocado fruits and idioblasts cells

Freshly harvested fruits were exposed to 40 μl/l ethylene stream in 30 l jars [6]. At different experimental periods after treatment, the jars were opened and pericarp and mesocarp slices were sampled. For the study of isolated tissues, 5 g of slices from pericarp and mesocarp (1–2 mm thick) were sampled and placed in glass containers with the bottom covered with filter paper humidified with 10 ml of a incubation solution containing 100 mM sorbitol, 1 mM $CaCl_2$ and 0.2% bovine serum albumin in 10 mM MES buffer pH 5.5. Ethylene was injected through a rubber stopper to a final concentration of 40 μl/l.

For experiments with idioblast, up to five ml of idioblasts suspension containing $1-2 \times 10^6$ cells were placed in 20 ml plastic vials and the volume was made up to 10 ml with the same buffer solution. Ethylene was injected through a rubber stopper to a final concentration of 40 μl/l. In some cases, idioblasts were isolated with mesocarp lipids after the first centrifugation and exposed together or separately to ethylene.

Isolation of antifungal diene

Extraction of antifungal diene was carried out from pericarp and mesocarp slices (1–2 mm thick) according to [2]. The tissues were homogenized in 95% ethanol and the filtrate concentrated in vacuum at 40° following by partition with dichloromethane/water. The organic phase was concentrated to dryness and solubilized in ethanol or ethyl acetate for analysis by HPLC or TLC respectively.

For extraction of antifungal diene from idioblasts, 2 ml of a cell suspension containing approximately 2×10^6 cells were boiled for 2–3 min., and 10 ml 95% ethanol were added. After blending for 3 min, the ethanol extract was processed as described above. Diene quantification was performed in a 25×0.5 cm RP-18 Partisil column with an isocratic program employing 95% methanol at a 1 ml/min flow. The eluate was analyzed by refractive index and the retention times and areas of the peak recorded and compared to those of a known antifungal diene standard. TLC of the diene was performed in $20 \times 20 \times 0.5$ cm Silica gel G (Merck) plates with hexane–ethyl acetate–methanol (60:40:1) as solvent. The R_f was compared to that of pure diene standard. When radioactive precursors were incorporated, the radioactive spots were detected by autoradiography, scraped, eluted with ethyl acetate, filtered and the eluate concentrated for scintillation counting. Proton NMR of the material isolated from TLC (R_f 0.47) was performed in a Bruker AM X-400 instrument.

Incorporation of [^{14}C] labeled precursors into antifungal diene and its analysis

Incorporation of labeled precursors was carried out in the presence of 1 μCi of [2-^{14}C] malonyl CoA or 2.5 μCi [1-^{14}C] acetate. Precursors were added to the incubation solution containing slices of tissue or idioblast cells and exposed to ethylene while shaking. After the elicitation period, the slices of tissue and the cells were separated from the incubation media by filtration, washed thoroughly and placed in freshly added 10 mM MES buffer pH 5.5, with 100 mM sorbitol, 1 mM $CaCl_2$. In some experiments, cells were incubated in the above mentioned medium in the presence of i. 10% bovine serum albumin, ii. mesocarp lipids separated from idioblast by by centrifugation or iii. liposomes from pure mesocarp lipids [15]. Cells were separated from the media by Millipore filtration. Filtration washes were collected and added to the respective medium and the diene extracted with ethanol as described before.

Acknowledgements—This work was supported by grants funded by BARD, GIARA and CDR-AID.

REFERENCES

1. Prusky, D. and Keen, N. T., *Plant Disease*, 1993, **77,** 114.
2. Prusky, D., Keen, N. T., Sims, J. J. and Midland, S. L., *Phytopathology*, 1982, **72,** 1578.
3. Karni, L., Prusky, D., Kobiler, I., Bar-Shira, E. and Kobiler, D., *Physiological and Molecular Plant Pathology*, 1989, **35,** 367.
4. Geissman, T. A. and Crout, D. H. G., *Organic Chemistry of Secondary Plant Metabolism*, 1969.
5. Kobiler, I., Prusky, D., Midland, S., Sims, J. J. and Keen, N. T., *Physiological and Molecular Plant Pathology*, 1993, **43,** 319.
6. Ardi, R., Kobiler, I., Keen, N. T. and Prusky, D., *Physiological and Molecular Plant Pathology*, 1998, in press.
7. Amelunxen, F. and Gronan, G., *Zeitscheift fur Pflanzenphysiologic*, 1969, **60,** 156.
8. Bosabalidis, A. and Tsekos, J., *Protoplasma*, 1982, **112,** 63.

9. Henrich, G., Schultze, W. and Wegener, R., *Protoplasma*, 1980, **103,** 115.
10. Thompson, W. W., Platt-Aloia, K. A. and Endress, A. G., *Botanical Gazete*, 1976, **137,** 330.
11. Garrod, B. and Lewis, B. G., *Transactions of the British Mycological Society*, 1970, **72,** 515.
12. Bell, A. A., *Phytopathology*, 1967, **59,** 1119.
13. Jahnen, W. and Hahlbrock, K., *Planta*, 1988, **173,** 453.
14. Prusky, D., *Annual Review Phytopathology*, 1966, **34,** 413.
15. Folch, J., Lees, M. and Sloane-Stanley, G. A., *Journal of Biological Chemistry*, 1957, **226,** 497.
16. Prusky, D., Plumbley, R. and Kobiler, I., *Physiological and Molecular Plant Pathology*, 1991, **39,** 325.
17. Platt, K. A. and Thompson, W. W., *International Journal of Plant Sciences*, 1992, **153,** 301.