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Effect of low temperature storage and ethylene removal on ripening and gene expression changes in avocado fruit

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Abstract

Changes in physiological parameters and in the RNA population from avocado (*Persea americana*, Mill.) fruit stored under different conditions were analyzed. Storage at low temperatures drastically reduced the respiration and ethylene production rates. Fruit softening was delayed at 7°C, while fruits at 3°C did not soften during long-term storage. Carbon dioxide levels in the atmosphere surrounding fruit treated with a commercial ethylene absorbent, were slightly depressed at 7°C and slightly higher at 3°C, as compared to storage without the absorbent. Ethylene production rates were drastically reduced by low temperatures and appeared not to be affected by ethylene absorption. Changes in the expression of several mRNAs, detected by *in vitro* translation of RNA during ripening at 20°C (increases in Mr 95, 72, 65, 55, 50, 41, and 40 kD and decreases in 54 and 42 kD polypeptides) were delayed or blocked at low temperatures. Under these conditions, changes in mRNAs encoding two polypeptides (Mr 80 and 42 kD) were found to be opposite those observed during normal ripening. mRNAs for other polypeptides (Mr 90, 69, 47 and 45 kD) showed variations in level depending on storage conditions. A few mRNAs were observed only during cold storage (Mr 62, 60, 58, 57, 56, and 47 kD) and could be related to cold stress or acclimation of the fruits. The results obtained suggest that the main effect of low temperatures in avocado

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is a delay or blockage in the expression of specific genes related to ripening. The effect of the ethylene absorbent was mainly noted in the polypeptide pattern for translated mRNAs, in which most of the changes observed for non-treated fruit were delayed.

Keywords: Low temperature; Avocado; *In vitro* translation; Ethylene; Respiration; Softening

1. Introduction

Low temperature storage is a widespread method for increasing post-harvest life of fruits and vegetables. However, most tropical fruits, such as avocados, undergo physiological disorders and chilling injury (CI) when exposed to low temperatures. Recommended storage temperature for the cv. Hass is between 5° and 7°C, but depending on the physiological stage and the duration of storage, fruits can develop CI symptoms (grey-brown discolouration of the vascular system, scalding, surface pitting and failure to soften properly) when removed from storage (Paull,1990).

Ethylene has been reported to increase the threshold temperature of CI in avocados, and removal of ethylene from the surrounding atmosphere was suggested to reduce or delay physiological disorders in avocado during cold storage (Zauberman and Fuchs, 1973; Lee and Young, 1984). However, ethylene removal has been mainly applied as a supplementary technique in modified or controlled atmosphere storage (Knee et al., 1985), rendering evaluation of its direct effects on the post-harvest life of fruit difficult.

The climacteric rise in ethylene production during avocado fruit ripening is normally associated with an increase in respiration rate and flesh softening. Hence, many studies on avocado deal with cell wall-degrading enzymes (Awad and Young, 1979; Christoffersen et al., 1984) and some of the changes in these enzyme activities observed during storage conditions retarding ripening appear to be a consequence of altered gene expression (Kanellis et al., 1991; Dopico et al., 1993).

Although the response of ethylene production to chilling temperatures is not the same in all species, it is suggested that the cold induction mechanism for ethylene synthesis observed upon rewarming probably involves an accumulation of ACC synthase mRNA (Field, 1990). Furthermore, Watkins et al. (1990) showed that low temperatures induce an mRNA in tomato which encodes the ethylene forming enzyme (Hamilton et al., 1991). There is also increasing evidence that exposure of fruit to low temperatures induces altered expression in other genes. The low temperature storage of Conference pears is accompanied by altered mRNA levels (Wilson et al., 1990) and Schaffer and Fischer (1990) found transcriptional activation by cold of a thiol protease gene.

To date, no applicable causal relationship between physiological changes and chilling temperature effects on avocado gene expression has been established (Dopico et al., 1993). Moreover, few studies have focused on the direct effects of ethylene absorbent technologies during cold storage of fruit. This study addresses the possible relationships between some physiological processes (respiration, ethylene production and softening) and changes in mRNA expression in avocado

fruit under low temperature storage, with and without the use of an ethylene absorbent.

2. Material and methods

Plant material

Mature avocado fruit (*Persea americana*, Mill. cv. Hass) was harvested in Málaga in May (late season) and shipped by truck to Madrid (Spain). Uniform, mature-green avocados weighing from 210 to 250 g were selected and randomly divided into three groups and stored at 20, 7 and 3°C, 85% RH.

Seventy-five avocados — twenty-five at each temperature — were enclosed in 22-liter glass jars, and continuously flushed with ethylene- and CO_2-free air, by passing the inflowing streams through a $KMnO_4$-impregnated carrier and then bubbling them through a gas-dispersion tube holding 10% KOH aqueous solution. The flow rate was about 6 l/h.

Two other sets of fruit were placed in similar respiratory chambers to which six Green Keeper sachets (Promarket, S.A., Madrid, Spain) containing 8 g of $KMnO_4$-impregnated product each were added; one set was stored at 3° and the other at 7°C.

Respiration and ethylene determinations

Respiration chambers and open neoprene cabinets, which are more effective in maintaining temperature, were used for fruit stored without ethylene removal, as respiration chambers were used only where ethylene removal was required. Eaks (1976) reported no significant differences between "Hass" avocados stored in respiratory chambers and those kept in open containers.

CO_2 and ethylene production were determined twice daily on 1 ml samples taken from the outlet of each respiratory chamber by a syringe. A Varian 3700 gas chromatograph, equipped with a six-way switching valve and two columns (Porapak Q column and molecular sieve), in series, were used. A thermal conductivity detector was used for CO_2 measurement and a flame ionization detector for ethylene determination. Helium was used as a carrier at a rate of 30 ml/min. Quantification was performed using an external standard.

Fruit firmness

Avocados were allowed to reach room temperature before determining firmness. Flesh firmness was measured in three 1-cm peeled areas of the equator using an Instron Testing Machine, model 1140, fitted with a double plate probe (De La Plaza et al., 1977). Force–distance curves were recorded and results expressed as the maximum penetration force at the point the tissue broke, in Newtons. Data are the average of three replicates.

RNA isolation and in vitro translation

Three representative avocados from each treatment were peeled, cut into pieces and immediately frozen in liquid nitrogen. Randomized samples were stored at

−70°C until used. This sampling procedure has been reported more satisfactory than coring repeatedly from the same fruit, because of the adverse effects on ripening behaviour of coring fruit (Starret and Laties, 1991). For the *in vitro* translation experiments frozen avocado fruit samples were air-shipped from the Instituto del Frio (Madrid) to Nottingham University. Total RNA extraction was performed as described in Dopico et al. (1993).

In vitro translation of the total RNA was performed using a nuclease treated rabbit reticulocyte lysate from Promega (Promega Corporation, USA) according to the manufacturer's protocol. 4, 7 and 10 μg of total RNA were used to determine the best concentration for *in vitro* translation, and finally 10 μg in 2 μl of water were used for all the experiments in the presence of 1 μl ^{35}S-methionine (Amersham, UK; 370 MBq/ml), 1 μl of 1 mM amino acid mixture (except methionine) plus 9 μl lysate in a total volume of 13 μl. Brome Mosaic Virus (BMV) RNA provided with the lysate was used as the positive control reaction. Measurement of the incorporation of radioactivity was performed by trichloroacetic acid precipitation of protein in an aliquot of the products obtained after *in vitro* translation, followed by scintillation counting. The labelled products obtained were then separated in a 10% SDS-PAGE gel, loading similar cpm per lane. ^{14}C-labelled proteins (Rainbow Coloured Markers, range 14.3–200 kD, Amershan, UK) were used as molecular weight markers. Following electrophoresis the gels were fixed overnight in 30% (v/v) methanol and 14% (v/v) acetic acid, dried at 60°C, exposed to X-ray film and photographed.

3. Results

Respiration rate, C_2H_4 production and firmness

The respiration rate during normal ripening showed a typical climacteric pattern peaking on day 7 (263 mg CO_2/kg h) and decreasing thereafter (Fig. 1). Low temperatures (3°C and 7°C) clearly lowered rates of CO_2 production (Fig. 1) with respect to normal ripening. This was followed by a slow increase, beginning after 25 days, which was greater at 7°C than at 3°C. The effect of ethylene absorption was small relative to the effects of low temperature, but differences were observed between the effects of ethylene absorption at 3°C and 7°C. At 7°C the respiration rate was slightly depressed when ethylene was removed as compared to storage at 7°C without ethylene removal, while at 3°C the respiration rate rose slightly when ethylene absorbents were used (Fig. 1).

Ethylene production at 20°C also showed a climacteric pattern, peaking at about 7 days. Ethylene production during low temperature storage declined substantially, and levels below 1.2 μl/kg h were observed throughout the storage period at 3°C and 7°C irrespective of whether or not ethylene was removed (data not shown).

During normal ripening at 20°C, fruit softening increased dramatically after 7 days (Fig. 2) coinciding with the peaks in CO_2 and ethylene production. Cold storage treatment had different effects on the softening process, depending on the temperature. At 7°C softening was delayed in relation to normal ripening and firmness began to decline after 25 days, coinciding with the increase observed

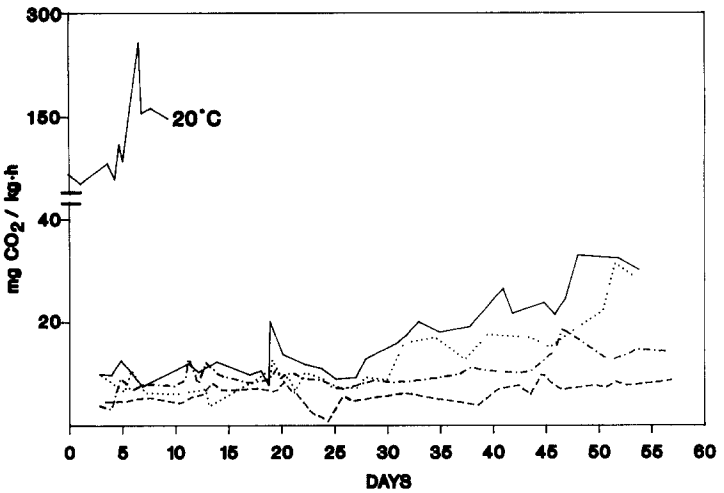

Fig. 1. Changes in the respiration of avocado fruit under different storage conditions. – – – –, 3°C; – · – · –, 3°C treated with ethylene absorbent; ——, 7°C; · · · · · ·, 7°C treated with ethylene absorbent, and 20°C.

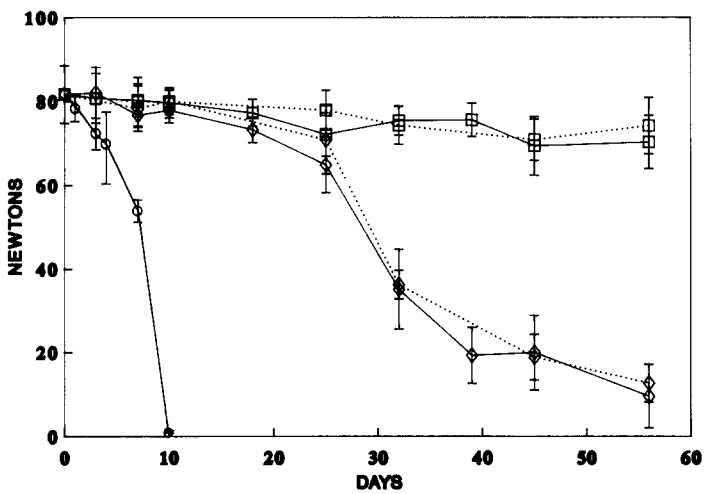

Fig. 2. Changes in firmness of avocados under different storage conditions. (□), 3°C; (◇), 7°C; (○), 20°C. Dotted lines indicate treatment with ethylene absorbent at 3°C (□) and 7°C (◇). Values are means of three replicates ± SD.

in the respiration rate (Figs. 1 and 2). After 39 days of storage at 7°C fruit reached a flesh firmness value similar to that of fruit stored at 20°C for 9 days (postclimacteric). In contrast, 3°C storage of avocado fruit completely inhibited the softening process even after 56 days under such conditions. Analysis of variance (ANOVA) at $P \leq 0.05$ showed that the effect of ethylene removal from the

surrounding atmosphere on softening was not significant in low temperature storage, at either 3°C or 7°C storage temperature (data not shown).

In vitro translation of RNA samples

Total RNA from fruit at all stages of storage stimulated the reticulocyte *in vitro* translation system at least five-fold over background incorporation, usually reaching 30,000–100,000 cpm/μl reaction mix, but in some cases the stimulation was up to 20-fold. Autoradiography of the labelled polypeptides separated by SDS-PAGE revealed products with an Mr range between 10 and 150 kD, indicating that the RNA was un-degraded and translation products were representative of the *in vivo* mRNA population. The results obtained after *in vitro* translation of the total RNA extracted from avocado fruit stored under different conditions are showed in Figs. 3–6 and the differences between treatments summarized in Table 1.

A comparative analysis of the labelled polypeptides produced by *in vitro* translation of all our RNA samples showed that storage of Hass avocado fruit at both 3°C and 7°C was accompanied by altered levels of specific mRNA with respect to

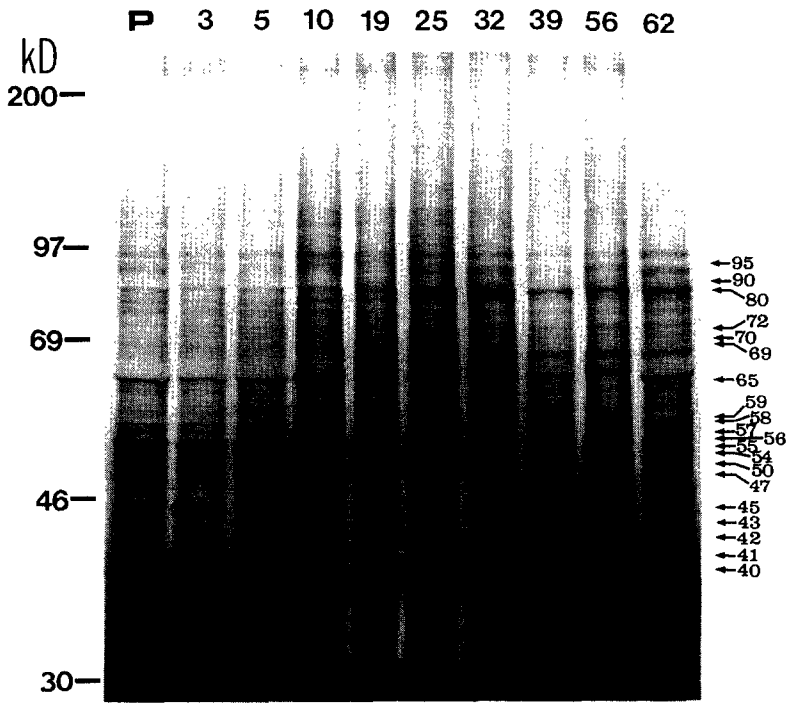

Fig. 3. Autoradiograph of SDS-PAGE gel of *in vitro* translation products from total RNA isolated from avocado fruit tissue during storage at 7°C. *P* is pre-stored tissue. Numbers at top refer to days of storage. The molecular weights of marker proteins run in the same gel are indicated on the left in kD. Polypeptides cited in Table 1 and their respective molecular weights in kD are indicated by arrows and numbers on the right.

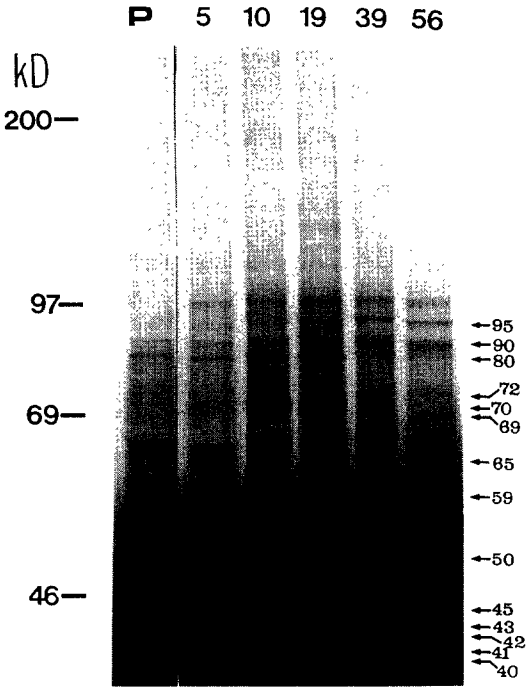

Fig. 4. Autoradiograph of SDS-PAGE gel of *in vitro* translation products from total RNA isolated from avocado fruit tissue during storage at 3°C. *P* is pre-stored tissue. Numbers at top refer to days of storage. The molecular weights of marker proteins run in the same gel are indicated on the left in kD. Polypeptides cited in Table 1 and their respective molecular weights in kD are indicated by arrows and numbers on the right.

the pre-stored tissue (Figs. 3 and 4) as well as to normal ripening (Fig. 5). Fruit ripened at 20°C showed altered mRNA levels throughout the ripening period, with particularly prominent increases in mRNAs encoding polypeptides of Mr 50, 55, 72 and 95 kD (Fig. 5). Christoffersen et al. (1982) also observed such an increases, showing that some mRNAs are closely related to avocado fruit ripening, although the polypeptides involved had different Mr.

RNA from fruit stored at 7°C showed several differences with respect to the RNA obtained during normal ripening at 20°C. The expression of at least 4 RNAs increased markedly during storage at 7°C (those encoding polypeptides with Mr 42, 69, 80 and 90) (Fig. 3), the 69 kD polypeptide barely being expressed at 20°C (Fig. 5). At least two mRNAs not detected at 20°C were observed under 7°C storage, encoding polypeptides with Mr of 47 and 58 kD. The expression of some mRNAs was also delayed with respect to normal ripening (e.g., those encoding polypeptides of 40, 54, 55 kD, compare Figs. 3 and 5) while others decreased or remained stable (encoding polypeptides of 50, 65 and 70 kD, compare Figs. 3 and 5) whereas their production increased during normal ripening. Where ethylene was removed, these changes were observed to be lesser and to occur later (Table 1, Fig. 6).

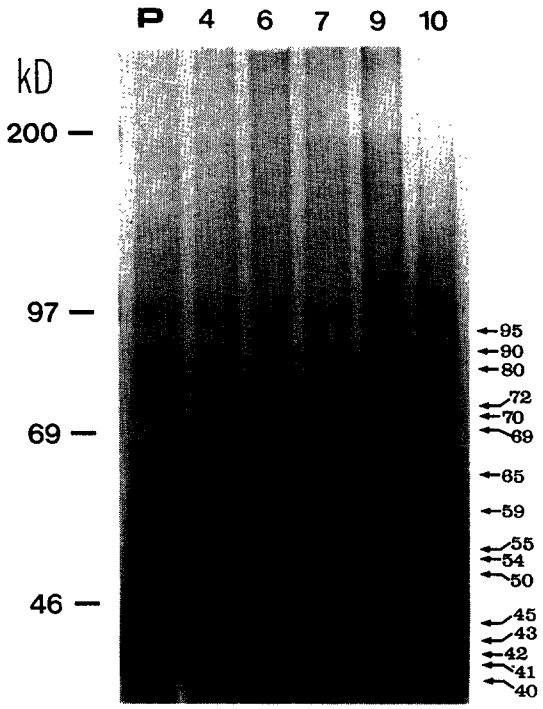

Fig. 5. Autoradiograph of SDS-PAGE gel of *in vitro* translation products from total RNA isolated from avocado fruit tissue during normal ripening at 20°C. *P* is pre-stored tissue. Numbers at top refer to days of storage. The molecular weights of marker proteins run in the same gel are indicated on the left in kD. Polypeptides cited in Table 1 and their respective molecular weights in kD are indicated by arrows and numbers on the right.

The expression of mRNAs encoding 70 and 80 kD polypeptides increased from the day 10 to day 19 in avocados stored at the chilling temperature (3°C) (Fig. 4) and decreased thereafter. The expression pattern of some mRNAs was similar at 3° and 7°C but different from that at 20°C (those encoding polypeptides of 42, 45, 50 kD), while mRNAs encoding Mr 59, 65, and 72 kD polypeptides seemed to be at least partially inhibited at 3°C. The removal of ethylene from the atmosphere led to the appearance of two RNAs (encoding 60 and 62 kD proteins) that appeared after 56 days of storage in such conditions (Fig. 6) and were not detected in any other cold storage treatment or during ripening at 20°C.

4. Discussion

These results confirm that low temperature storage delays (7°C) or blocks (3°C) the ripening of avocado fruit, as is shown by the changes in flesh firmness (Fig. 2), respiration rate (Fig. 1) and polypeptide profile during cold storage (Figs. 3, 4 and 6) in comparison with those of fruit ripened at 20°C (Fig. 5).

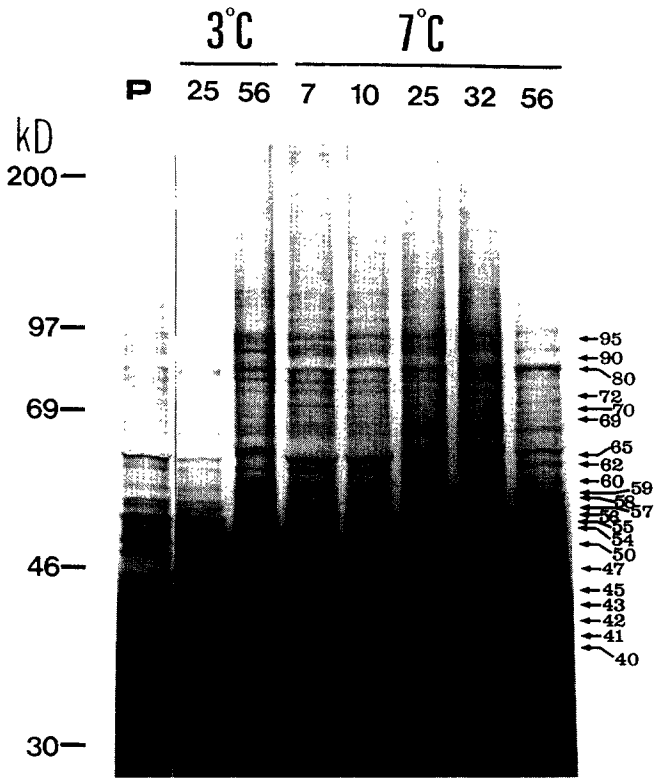

Fig. 6. Autoradiograph of SDS-PAGE gel of *in vitro* translation products from total RNA isolated from avocado fruit tissue during storage at 3°C and 7°C with ethylene removal from the surrounding atmosphere. *P* is pre-stored tissue. Numbers at top refer to days of storage. The molecular weights of marker proteins run in the same gel are indicated on the left in kD. Polypeptides cited in Table 1 and their respective molecular weights in kD are indicated by arrows and numbers on the right.

At both storage temperatures, with and without ethylene removal, ethylene production was clearly depressed. Although ethylene has been considered to play an essential role in fruit ripening, it seems that elevated levels of ethylene production are not strictly needed for some changes concurrent with avocado ripening, since fruit softened at 7°C without it. This would seem to be in line with previous reports related to the role of wound and climacteric ethylene in ripening of avocado discs (Starret and Laties, 1991).

The ethylene absorbent was observed to provoke no clear effect on ethylene production, due perhaps to very low ethylene levels at low temperatures. It is suggested that other volatiles related to enhanced oxidative metabolism may evolve in the storage atmosphere at chilling temperatures, as a general response to stress. Nonethylenic volatiles (such as acetaldehyde, ethanol and ethane) may be produced during chilling of avocados, as Lyons (1973) reported for other fruit and vegetables. Oxidation of one or several of these volatiles might lead to the higher values of external CO_2 levels observed during storage at 3°C with ethylene removal.

Table 1
Polypeptides detected by *in vitro* translation of avocado mRNAs and changes in abundance during fruit storage and ripening

Mr (kD)	20°C	7°C	7°C + E.A.	3°C	3°C + E.A.
95	I from 9 d	I from 25 d	S	I from 19 d	=
90	S	I	=	S	=
80	D	D at 32 d	D at 56 d	Fluctuation	A at 56 d
72	I	I	S	H.D.	A at 56 d
70	S	D at 32 d	=	Fluctuation	A at 56 d
69	H.D.	I	=, delayed	A at 39 d	S
65	I	S	=	H.D.	S
62	N.D.	N.D.	N.D.	N.D.	A at 56 d
60	N.D.	N.D.	N.D.	N.D.	A at 56 d
59	S	D at 39 d	=, delayed	H.D.	N.D. at 56 d
58	N.D.	A at 56 d	=	N.D.	N.D.
57	N.D.	N.D. at 32 d	=	N.D.	H.D.
56	N.D.	N.D. at 32 d	=	N.D.	H.D.
55	A at 9 d	A at 32 d	A at 56 d	N.D.	N.D.
54	D at 9 d	D at 25 d	=, delayed	H.D.	S
50	I	S	=	S	I
47	N.D.	Fluctuation	=, delayed	N.D.	N.D.
45	S	D at 32 d	=, delayed	D after 39 d	I
43	D	Fluctuation	=, delayed	D	S
42	D	Fluctuation	=, delayed	Fluctuation	I
41	Fluctuation	Fluctuation	=, delayed	Fluctuation	I
40	I	I	=, delayed	I	Fluctuation

E.A., ethylene absorbent; I, increases; D, decreases; N.D., not detected; H.D., hardly detected; A, appearance; =, evolution as at the corresponding temperature without ethylene absorbent.

While in the early stages of avocado ripening, overall increases in RNA translation have been detected, it is only late in the respiratory climacteric that changes in gene expression have been noted (Tucker and Laties, 1984). Our results also show that most of the changes in gene expression during ripening at 20°C appear after the climacteric peak, when sharp rises in the expression of several mRNAs (notably those encoding polypeptides in the Mr range 50–55 kD) were observed. Similarly, most of the changes at 7°C were observed late in the storage period, when CO_2 production had already increased.

The decline in avocado fruit firmness coincides with the increase in both cellulase and polygalacturonase (PG) enzyme activities (Awad and Young, 1979) due to increases in the expression of the corresponding mRNAs during ripening (Christoffersen et al., 1984; Dopico et al., 1993). Thus, the rate of softening during storage should correspond to changes in the expression of both enzymes. The *in vitro* translation product of cellulase has been reported as 53–54 kD (Christoffersen et al., 1984, Bennett and Christoffersen, 1986) whilst PG appears as a number of polypeptides with molecular masses of 55, 52, 49, 48 and 46 kD, as shown by immunological detection (Kanellis et al., 1991). These might correspond to the 50–55 kD polypeptides in Figs. 3–6. Thus, the delay in flesh softening at 7°C storage with

respect to normal ripening may be related to the delay in the changes observed in the polypeptides with an Mr between 50–55. At 3°C, where no significant softening was observed, these polypeptides were barely detected.

Our results suggest that the main effect of low temperature storage is a delay in the accumulation of ripening-specific mRNAs rather than synthesis of specific cold induced proteins. Such a delay is more evident in fruit in which ethylene has been removed from the surrounding atmosphere. Delayed ripening may also be due to the failure of mRNAs encoding some polypeptides to decline. For example, the 80 kD polypeptide decreases during ripening at 20°C, while it increases in early storage stages at 7°C and begins to decline when softening first appears at this temperature. Furthermore, two polypeptides have been detected whose mRNAs decrease slowly and disappear late, and which are not present in normal ripening (56 and 57 kD). Some of these polypeptides may possibly be involved in the delay in ripening. The very late modifications in the pattern of mRNAs revealed by *in vitro* translation after prolonged storage could be due to a specific cold-acclimation response.

The accumulation of at least two mRNAs not affected by the presence of ethylene suggests that low temperatures caused direct induction of gene expression (90 and 58 kD, Fig. 6). Such effect has been noted in mRNA for a protease inhibitor (Schaffer and Fischer, 1988) and an EFE gene (Watkins et al., 1990), both in tomato. Further work will be necessary in order to establish whether any of the low-temperature-induced changes are related to chilling injury.

Further, our study showed that while the use of an ethylene absorbent in a flow system had no effect on ethylene production or softening of avocado fruit, some effects on the respiration rate and polypeptide pattern were observed during low temperature storage with ethylene absorption. Moreover, it has been shown that the delays in the expression of several genes (PG, cellulase, EFE, and others of unknown function) during low temperature storage of avocados were longer with ethylene absorption (Dopico et al., 1993). It would, then, be desirable to study other physiological parameters that would make it possible to assess the feasibility of these treatments during the storage period, without having to resort to rewarming. Such parameters may be related to oxidative metabolism and nonethylenic volatiles.

Acknowledgements

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