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Hypoxic acclimation prevents avocado mesocarp injury caused by subsequent exposure to extreme low oxygen atmospheres

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Abstract

Avocado fruit (*Persea americana* Mill., cv. Hass), preclimacteric and ripening-initiated, were either hypoxically pretreated (HPT, 3% O_2 for 24 h) or exposed directly to 1 and 0.25% O_2 for 1–3 days (NHPT) at 20 °C. Low O_2 treatments resulted in fruit maintaining higher flesh firmness. Hypoxic acclimation of preclimacteric and ripening-initiated avocado fruit increased their tolerance to subsequent 0.25 and 1% O_2 levels, as assessed by visual quality attributes when the fruit returned to air. Hypoxic pre-treatment also produced a stronger expression of the anaerobically induced ADH isoenzymes than in NHPT avocado fruit that were subjected directly to 1 and 0.25% O_2 for 1–3 days at 20 °C. Thus, acclimation of avocado fruit to hypoxia resulted in a beneficial increase in tolerance to subsequent ultra-low O_2 treatments.

Keywords: Hypoxia; Insecticidal treatments; Flesh firmness; Respiration; Mesocarp injury; ADH; LDH

1. Introduction

Short-term exposure to ultra-low O_2 concentrations as a potential quarantine treatment for the control of insects in fruit and vegetables has recently been examined as an alternative to chemical fumigation (Carpenter and Potter, 1994; Mitcham et al., 1997). Most fresh fruit and vegetables do not tolerate these low O_2 atmospheres

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for prolonged periods, though some can tolerate them for short periods (Delate and Bretch, 1989; Ke and Kader, 1992). For example, 'Hass' avocado fruit are very sensitive to insecticidal low O_2 atmospheres and cannot tolerate 0.25% O_2 for longer than 24 h at 20 °C (Yahia and Kader, 1991; Yahia and Carrillo-Lopez, 1993); mesocarp injury observed after exposure to low O_2 for 2 days, usually started as discoloration of the vascular tissue and then extended to the rest of the tissue (Yahia and Carrillo-Lopez, 1993). In contrast, fruit such as 'Valencia' oranges and mango can tolerate 0.25% O_2 for more than 5 days at 20 °C (Ke and Kader, 1992; Yahia and Hernandez, 1993).

It is known that some plant tissues which are normally very sensitive to anoxia or severe hypoxia become more tolerant after a period of acclimation under hypoxia: for example, maize roots which die in ≈ 10 h when rapidly transferred from air to anoxia, survive more than 3 days if pretreated for 18 h in 2–4% O_2 at 20 °C (Saglio et al., 1988; Johnson et al., 1989). These acclimated tissues possess the ability to maintain a high glycolytic rate during long periods of anoxia, as well as higher ATP levels and energy charge (Johnson et al., 1989; Hole et al., 1992; Xia and Saglio, 1992). In addition, hypoxic pretreatment of cut carnation flowers enhanced their survival under anoxia (Chen and Solomos, 1996).

The specific physiological, biochemical and molecular mechanisms underlying the observed extension of the postharvest life of fruit and vegetables kept in low O_2 are largely unknown (Solomos and Kanellis, 1997). Other mechanisms involved in the short-term use of CA/MA which need elucidation are the tolerance of extreme low O_2 levels implemented in quarantine treatments, the acquired adaptation to subsequent cold storage upon transfer of the produce to air and the retarding residual effects on the ripening process when fruit are transferred from hypoxia to air.

These events have lead us to evaluate the effect of an acclimation treatment (3% O_2 for 24 h at 20 °C) on the ability of mature but preclimacteric and ripening-initiated avocado fruit to withstand a subsequent exposure to 0.25 and 1% O_2 for 1 and 3 days at 20 °C.

2. Materials and methods

2.1. Plant material

Mature (preclimacteric) avocado (*Persea americana* Mill., cv. Hass) fruit were harvested from an orchard of the Institute of Subtropical Plants and Olive Trees at Chania, Greece and immediately dipped in 600 μl l^{-1} methyl-*N*-(1-butyl carbamoyl) 2-benzimidazol carbamate (benomyl, for decay control), dried and sorted by colour and size. Uniform fruit of weight 200–220 g and free of defects were placed in 4-l jars (three fruit per jar) and kept at 20 °C under a steady flow of 50–60 ml min^{-1} humidified C_2H_4- and CO_2-free air. Selected preclimacteric fruit exhibited respiration rates < 3 mmol CO_2 kg^{-1} h^{-1}, nil ethylene production and firmness higher than 130 N. Ethylene was measured in an HP 5890 series II gas chromatograph equipped with a flame ionisation detector and fitted with an activated alumina column. Carbon dioxide concentrations were determined by means of a gas chromatograph (Carlo Erba series 2000) equipped with a thermal conductivity detector and a porapack column.

In experiment A, preclimacteric fruit were placed in glass jars (three fruit per jar) and were either exposed to different low O_2 atmospheres by subjecting the fruit to a continuous humidified gas flow (60–70 ml min^{-1}) of 0.25 and 1% O_2 (balance N_2) for 3 days (NHPT) or they were hypoxically pretreated in 3% O_2 for 24 h (HPT) before subsequent storage in 0.25 and 1% O_2 for 3 days at 20 °C. Additionally, control treatments consisted of fruit exposed to 21% and 3% O_2 over the pretreatment, acclimation and low O_2 treatment periods. In experiment B, the same treatment as that of experiment A was performed except that ripening had already been initiated in the fruit used for the experiment (see later). The fruit held continuously in air served as the control. Following low O_2 applications, fruit of both experiments were transferred to air and held for 7 days at 20 °C. During each sampling, the mesocarps of a set of three fruit were sliced in liquid nitrogen and stored at -20 °C. Other sets of fruit were used daily for firmness determination and quality evaluation. Each treatment was replicated three times.

Ripening of fruit was initiated in experiment B by introducing 100 μl l^{-1} ethylene into the air stream and the respiration rates were monitored. When the rate of CO_2 evolution reached 5 mmol kg^{-1} h^{-1}, values that were two-thirds of those of the climacteric peak, the exogenous supply of ethylene was discontinued and the fruit were transferred to the low O_2 conditions described above.

2.2. Ripening parameters

After the daily measurements of respiration rates and ethylene production, three fruit from each treatment were removed from air and low O_2 atmospheres and flesh firmness was determined (two measurements per fruit on opposite sides at the equator) by a Chatillion pressure tester using a conical tip 6.5 mm in diameter.

A set of six fruit was taken daily from each of the low O_2 and air treatments for evaluation of exocarp and mesocarp injury. Injury was visually assessed after the fruit were held in low O_2 and/or air at 20 °C for a total holding time per treatment of 10 days (≤ 3 days low O_2 and > 7 days in air). Mesocarp injury was seen as tissue browning and breakdown and was estimated using a numerical scale where 0 = no injury, 1 = very slight, 2 = moderate, 3 = severe, and 4 = very severe. Exocarp injury (browning) was estimated as percentage of affected surface area where 0 = no injury, 1 = very slight, up to 15% affected area, 2 = moderate, 16–50%, 3 = severe, 51–85%, and 4 = very severe, 86–100% of affected area.

2.3. Extraction, PAGE and activity staining of alcohol dehydrogenase

Frozen mesocarp tissue (1 g tissue per 5 ml) was homogenized in an extraction buffer containing 100 mM Tris–HCl, pH 8.0, 10 mM DTT, 10 μM leupeptin, 0.5 mM PMSF, 10% (v/v) glycerol and 5% (g/FW) PVPP (Kanellis et al., 1991). Samples were kept on ice for 15 min with occasional vortexing and were then centrifuged at $18{,}000 \times g$ for 20 min at 4 °C. The supernatant was filtered through Miracloth, divided into 1 ml aliquots and kept at −20 °C.

Native protein gel electrophoresis and enzyme activity staining was performed according to the method of Kanellis et al. (1991). Total protein was measured according to Bradford (1976), using BSA as a standard.

2.4. Alcohol dehydrogenase activity assay

Crude extracts (40 μl) were assayed in a 1 ml assay mixture containing 333.33 mM Tris–HCl (pH 9.0), 33.33 mM ethanol and 1.0 mM NAD^+ at 30 °C. The reaction was carried out in the direction of NADH production and measured at 340 nm in a HP 89075 Multicell Transport Spectrophotometer coupled to a PC. Each sample was replicated three times. The results were expressed in enzyme units per milligram of total protein; 1 U of the enzyme catalyzes the reduction of 1 μmol NAD^+ per minute.

2.5. Lactate dehydrogenase activity assay

Lactate dehydrogenase activity in pyruvate-dependent NADH oxidation was measured by following the method of Davies et al. (1974). The oxidation of NADH was monitored at 340 nm in alkaline pH using an HP 89075 Multicell Transport Spectrophotometer coupled to a PC and in the presence of an ADH inhibitor (pyrazol). The assay mixture (final volume 1 ml) contained 166.66 mM Tris–HCl (pH 8.0), 0.188 mM NADH, 10 mM sodium pyruvate, 10 mM pyrazol and 133.33 μl enzyme extract. Pyrazol (10 mM) dissolved in acetone (10 μl) was added before starting the reaction with pyruvate (Hanson and Jacobsen, 1984). Each sample was replicated three times. The results were expressed in enzyme units per milligram of total protein; 1 U of the enzyme catalyzes the reduction of 1 μmol NAD^+ per minute.

3. Results

3.1. Effect of low oxygen on respiration

The effect of hypoxic pretreatment on the respiration rates of preclimacteric and ripening-ini-

tiated fruit are presented in Fig. 1(A,B), respectively. Preclimacteric fruit held in air showed a continued increase in respiration (Fig. 1A), while ripening-initiated fruit exhibited a typical climacteric rise in respiration followed by a decline (Fig. 1B).

Low O_2 atmospheres decreased the rate of CO_2 evolution in both preclimacteric and ripening-initiated fruit (Fig. 1A,B). Preclimacteric HPT and NHPT avocado fruit held in 1 and 0.25% O_2 for 1–3 days showed a marked decline in CO_2 production (Fig. 1A). Upon transfer to air, preclimacteric NHPT avocado fruit held in 1 and 0.25% O_2 for 2 or 3 days produced less CO_2 than the HPT ones, the values of the latter being comparable to those of air samples (data not shown). Preclimacteric avocado fruit held in 3% O_2 for 1–3 days showed a decline in CO_2 production on the first day; however, they sustained a constant rate of CO_2 production during the rest of the holding period (Fig. 1A). When these fruit were returned to air following each day's treatment in 3% O_2, the rate of respiration increased (data not shown).

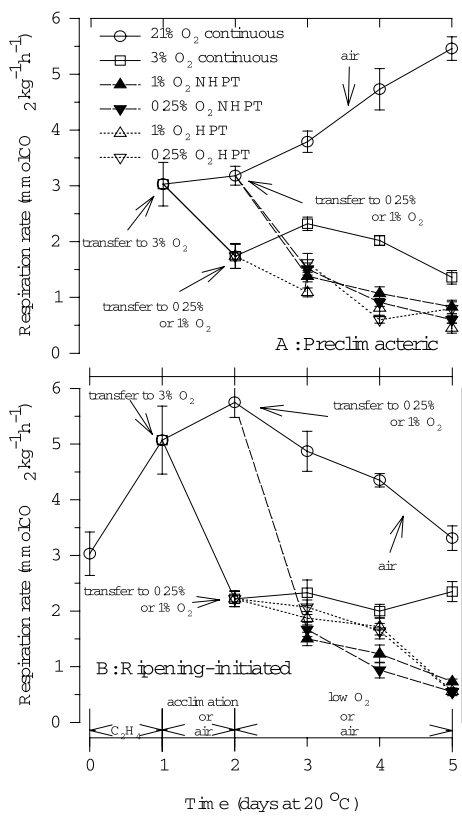

Fig. 1. Respiration rate of preclimacteric avocado fruit (A) and those treated with 100 ml l^{-1} ethylene for 24 h (B), that were either hypoxically pretreated in 3% O_2 for 24 h prior to exposure to 1 and 25% O_2 for 3 days (HPT) or subjected directly (on day 2) to 3, 1 and 0.25% O_2 for 3 days (NHPT). Vertical bars represent standard errors.

Similarly, low O_2 atmospheres resulted in a marked suppression of respiration in both HPT and NHPT ripening-initiated fruit held in 1 and 0.25% O_2 for 1–3 days (Fig. 1B). However, ripening-initiated avocado HPT fruit showed slightly higher CO_2 production in the first 2 days upon transfer to 1 and 0.25% O_2 atmospheres than ripening-initiated NHPT fruit held under the same conditions for 1–3 days. Upon transfer to air, NHPT ripening-initiated avocado fruit that were held in 1 and 0.25% O_2 for 2 or 3 days were unable to resume normal respiration, while HPT fruit showed an increased rate of CO_2 production (data not shown).

3.2. Effect of low oxygen on fruit softening

The effects of low O_2 on softening of preclimacteric and ripening-initiated fruit are presented in Fig. 2(A,B). Preclimacteric fruit held in air reduced initial firmness by 55% in 5 days (Fig. 2A), while fruit ripened with 100 μl l^{-1} ethylene for 24 h in humidified air stream, reduced initial firmness by 85% in 5 days (Fig. 2B).

Low O_2 atmospheres significantly arrested fruit softening in both preclimacteric and ripening-initiated fruit (Fig. 2A,B). Hypoxically pretreated and NHPT preclimacteric fruit held in 1 and 0.25% O_2 for 1–3 days showed a small reduction in firmness compared to those in 21% O_2 treatments (Fig. 2A). However, preclimacteric NHPT fruit showed less softening (a difference of 10 N) than HPT fruit held in 1 and 0.25% O_2 for 1–3 days (Fig. 2A). Preclimacteric fruit held in 3% O_2 for 1–3 days lost only $\approx$20% of the initial firmness.

Similarly, atmospheres of 1 and 0.25% O_2 for 1–3 days greatly reduced fruit softening in HPT and NHPT ripening-initiated fruit (Fig. 2B). Non-

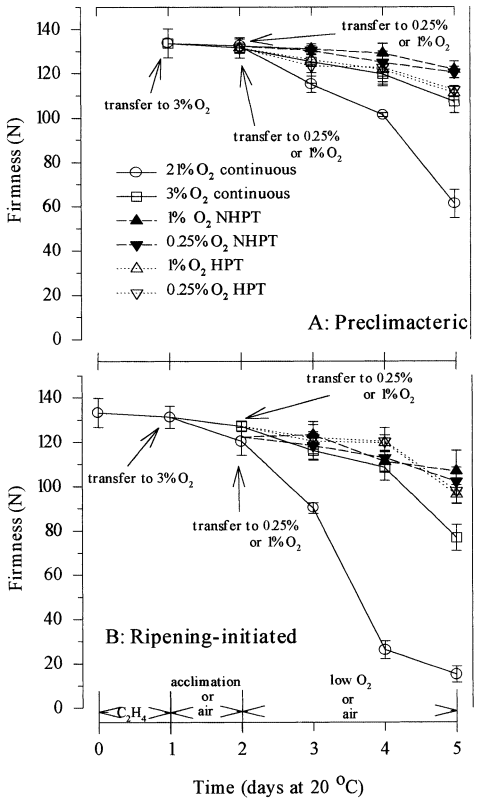

Fig. 2. Firmness of preclimacteric avocado fruit (A) and those treated with 100 ml l^{-1} ethylene for 24 h (B) that were either hypoxically pretreated in 3% O_2 for 24 h prior to exposure to 1 and 0.25% O_2 for 3 days (HPT) or subjected directly (on day 2) to 3, 1 and 0.25% O_2 for 3 days (NHPT). Vertical bars represent standard errors.

hypoxically pretreated ripening-initiated fruit showed a smaller reduction in flesh firmness (a difference of 10 N) in 1 and 0.25% O_2 atmospheres than HPT fruit.

3.3. Effect of low oxygen atmosphere on the visual quality of avocado fruit

For the evaluation of visual quality, six fruit were withdrawn daily from each low O_2 treatment and then ripened in air at 20 °C for a total holding time of 10 days.

Preclimacteric and ripening-initiated fruit held in 3% O_2 for 1, 2 and 3 days and then ripened in air for 9, 8 and 7 days, respectively, did not show any sign of injury and these fruit ripened as did fruit kept in air (Fig. 3A,B).

Non-hypoxically pretreated preclimacteric fruit held in 1% O_2 for 1–3 days and then ripened in air for a total holding time of 10 days showed no indication of injury (Fig. 3A). This was true irrespective of whether the fruit were hypoxically pretreated or exposed directly to 1% O_2 for 1–3 days (Fig. 3A). This suggested that preclimacteric fruit can tolerate an atmosphere of 1% O_2 for up to 3 days with no detrimental effect. In contrast, NHPT ripening-initiated fruit held in 1% O_2 for 2 or 3 days and then ripened in air for 8 and 7 days, respectively, developed mesocarp injury (Fig. 3B). Thus, ripening-initiated fruit are more sensitive to very low O_2 concentrations than preclimacteric ones. Non-hypoxically pre-treated preclimacteric fruit held in 0.25% O_2 for 2 days and then ripened

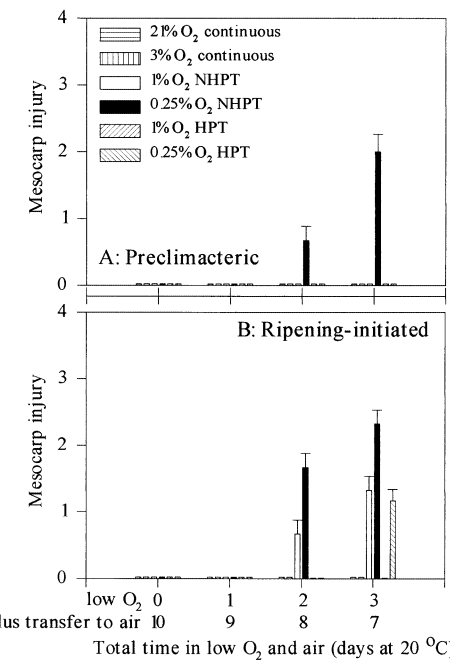

Fig. 3. Mesocarp injury in preclimacteric hypoxically (A) and ripening-initiated avocado fruit (B) that were either hypoxically pretreated (HPT) or subjected directly (NHPT) to 3, 1 and 0.25% O_2 for 0, 1, 2 and 3 days and then ripened in air for 10, 9, 8 and 7 days, respectively. Vertical bars represent standard errors.

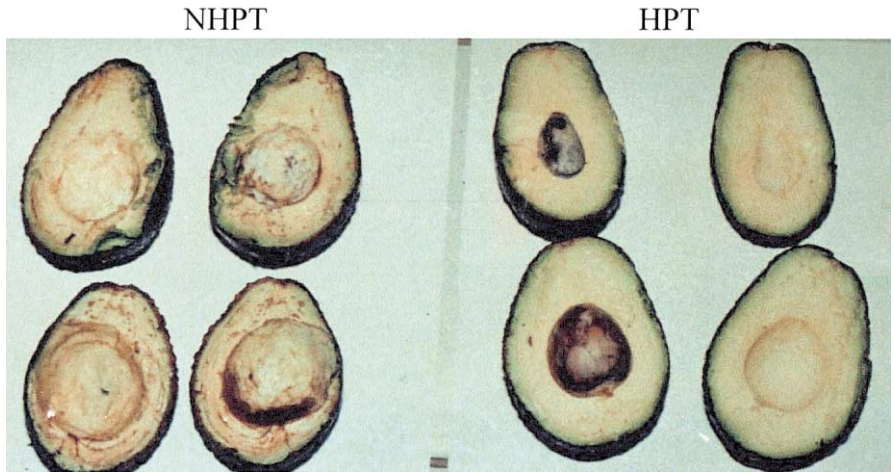

Fig. 4. Effect of acclimation to low oxygen on the visual quality of both HPT and NHPT preclimacteric avocado fruit held in 0.25% O_2 for 3 days and then transferred to air for 7 days.

in air for 8 days showed mesocarp injury (Fig. 3A). Similarly, fruit held in 0.25% O_2 for 3 days and then returned to air for 7 days expressed increased mesocarp injury (Fig. 3A). Mesocarp injury was also observed in NHPT ripening-initiated fruit held in 0.25% O_2 for 2 or 3 days and then transferred to air for 8 and 7 days, respectively (Fig. 3B).

Ripening-initiated fruit that were hypoxically pretreated prior to exposure to 1% O_2 for 1–3 days did not show any sign of mesocarp injury (Fig. 3B). Evaluation of the visual quality of these fruit in air following the 1% O_2 treatment showed clearly that these fruit ripened normally as did fruit in air (Fig. 3B). Similarly, evaluation of HPT preclimacteric fruit exposed to 0.25% O_2 for 1–3 days and then evaluated in air for a total holding time of 10 days showed no sign of mesocarp injury (Fig. 3A, Fig. 4). Hypoxically pretreated ripening-initiated fruit held in 0.25% O_2 for up to 2 days, followed by a holding period in air for 8 days, ripened normally without showing any sign of mesocarp injury (Fig. 3B, Fig. 4). However, a slight decrease in the visual quality score was evident in HPT ripening-initiated fruit exposed to 0.25% O_2 for 3 days (Fig. 3B). This supports our earlier observation that ripening-initiated fruit are more subjected to injury in low O_2 atmospheres than preclimacteric fruit.

3.4. Effect of acclimation to low oxygen on the activity of alcohol dehydrogenase and lactate dehydrogenase

Preclimacteric fruit held in 3% O_2 continuously exhibited slight increases in ADH activity in the first 2 days compared to the fruit held in air (control), but not after 3 days (Table 1). The ADH activity of both NHPT and HPT preclimacteric fruit held in 1 and 0.25% O_2 for 1–3 days was similar to the control fruit kept in 21% O_2. Ripening-initiated fruit held in 3% O_2 for 1–3 days showed slightly more ADH activity than fruit held in air (Table 1). Both HPT and NHPT ripening-initiated fruit that were held in 1 and 0.25% O_2 for 2 days exhibited slight decreases in ADH activity compared to that in fruit held in air, but, it was slightly higher after 3 days.

The activity of LDH in both HPT and NHPT preclimacteric and ripening-initiated fruit held in 3, 1 and 0.25% O_2 for 1–3 days was similar or slightly lower than that of the fruit kept in air (Table 2).

3.5. Effect of acclimation to low oxygen on the induction of alcohol dehydrogenase isoenzymes

Preclimacteric or ripening-initiated fruit held in 21% O_2 for 1–3 days contained a single dominant

ADH isoenzyme (Fig. 5). Native PAGE revealed that two additional ADH isoenzymes were induced in HPT or NHPT fruit held in 3, 1 and 0.25% O_2 for 1–3 days compared to those held in air, regardless of the stage of ripeness of the fruit (Fig. 5). However, storage for 1–3 days resulted in a progressively stronger induction of ADH isoenzymes compared to those in fruit held in similar conditions for 1 day. Additionally, the induction of ADH isoenzymes in HPT preclimacteric or ripening-initiated fruit held in 1 and 0.25% O_2 was stronger than in NHPT fruit. Similarly, isoenzyme analysis revealed stronger ADH isoenzyme staining activity for preclimacteric or ripening-initiated fruit held in 3% O_2 than for NHPT fruit held in 1 and 0.25% O_2 for 1 day. It should be noted that the intensity of the new isoenzyme staining was always higher at 1% O_2 than at 0.25% O_2.

4. Discussion

4.1. Effect of hypoxic pretreatment on respiration and firmness

Hypoxic pretreatment increases the survival and longevity of certain plant tissues during a subsequent period of anoxia or severe hypoxia (Xia and Saglio, 1992; Johnson et al., 1994; Chen and Solomos, 1996; Drew, 1997; Ellis et al., 1999; Chang et al., 2000). This acclimation treatment produces an array of physiological and biochemical changes which are expected to contribute to the increased tolerance of avocados for extremely low O_2 levels. Low O_2 atmospheres resulted in suppression of respiration in both preclimacteric and ripening-initiated fruit. It is now well-established that lowering the O_2 level in the atmosphere around fresh fruit reduces their respiration rate in proportion to the O_2 concentrations (Kanellis et al., 1989, 1991). Moreover, this effect of low O_2 occurs in tissues where ethylene is not at issue, such as potato tubers (Mapson and Burton, 1962). Interestingly, HPT ripening-initiated avocado fruit showed higher CO_2 production in the first 2 days after transfer to 1 and 0.25% O_2 than NHPT ripening-initiated fruit (Fig. 1B). Hole et al. (1992) reported that the rate of $^{14}CO_2$ evolution from HPT root tips of *Zea mays* was five times higher than that from NHPT roots under anoxia in the presence of U-^{14}C glucose. The fact that the rate of CO_2 evolution was lower in NHPT preclimacteric and ripening-initiated fruit when they were returned to air after 2 days at either 1 or 0.25% O_2 than it was in HPT fruit subjected to the same treatments, indicates that storage directly under very low O_2 is injurious to

Table 1
Alcohol dehydrogenase (ADH) activity in crude extracts from preclimacteric and ripening-initiated avocado fruit held in 21, 3, 1 and 0.25% O_2 for 1–3 days

Treatments		ADH activity (U mg^{-1} $prot^{-1}$ 10^{-3})					
O_2 level (%)	Pretreatment	Time in treatment (days)					
		Preclimacteric			Ripening-initiated		
		1	2	3	1	2	3
21	Continuous	330 ± 32	470 ± 69	690 ± 37	390 ± 9	1850 ± 112	1780 ± 13
3	Continuous	410 ± 55	530 ± 71	640 ± 91	480 ± 61	2110 ± 200	1820 ± 134
1	NHPT	350 ± 33	450 ± 1	690 ± 60	340 ± 26	1010 ± 48	1920 ± 69
1	HPT	310 ± 42	360 ± 51	520 ± 49	330 ± 29	1230 ± 30	2360 ± 24
0.25	NHPT	340 ± 8	450 ± 12	420 ± 33	290 ± 9	1060 ± 40	1870 ± 51
0.25	HPT	430 ± 66	350 ± 37	490 ± 38	320 ± 34	1190 ± 15	2220 ± 55

HPT stands for the fruits that were hypoxically pre-treated in 3% O_2 for 24 h prior to exposure to 1 and 0.25% O_2 for 1–3 days while NHPT were those subjected directly to 3, 1 and 0.25% O_2 for 1–3 days.

Table 2
Lactate dehydrogenase (LDH) activity in crude extracts from preclimacteric and ripening-initiated avocado fruit held in 21, 3, 1 and 0.25% O_2 for 1–3 days

Treatments		LDH activity (U mg^{-1} $prot^{-1}$ 10^{-3})					
O_2 level (%)	Pretreatment	Time in treatment (days)					
		Preclimacteric			Ripening-initiated		
		1	2	3	1	2	3
21	Continuous	47 ± 4	70 ± 7	64 ± 8	78 ± 1	70 ± 2	67 ± 2
3	Continuous	45 ± 5	52 ± 5	53 ± 7	58 ± 2	65 ± 5	70 ± 9
1	NHPT	40 ± 2	50 ± 1	46 ± 4	60 ± 13	76 ± 8	77 ± 11
1	HPT	47 ± 7	45 ± 5	42 ± 5	54 ± 11	77 ± 10	71 ± 1
0.25	NHPT	44 ± 1	42 ± 4	50 ± 1	59 ± 9	63 ± 4	56 ± 6
0.25	HPT	46 ± 4	48 ± 5	51 ± 5	55 ± 5	71 ± 2	64 ± 5

HPT stands for the fruit that were hypoxically pre-treated in 3% O_2 for 24 h prior to exposure to 1 and 0.25% O_2 for 1–3 days while NHPT were those subjected directly to 3, 1 and 0.25% O_2 for 1–3 days.

fruit and prevents the resumption of normal aerobic respiration.

A low O_2 environment for 1–3 days greatly reduced the loss of flesh firmness in preclimacteric and ripening-initiated fruit (Fig. 2). This was true for both HPT and NHPT fruit held in 1 and 0.25% O_2 for 1–3 days, which showed little reduction in the initial firmness. However, hypoxic pretreatment resulted in the same degree of fruit firmness as that of the NHPT fruit. Reduction of fruit softening after low O_2 pretreatment was shown also in avocado by Dori et al. (1995) and was related to a reduction in polygalacturonase and cellulase activity.

4.2. Visual quality of avocado fruit is positively affected by acclimation to low oxygen

The results obtained from evaluation of visual quality when the NHPT O_2-stressed avocado fruit were returned to air suggest that preclimacteric and ripening-initiated fruit are very sensitive to extremely low O_2 conditions and that the injury increases with the length of exposure to these atmospheres. Further, NHPT ripening-initiated fruit subjected directly to 1 and 0.25% O_2 showed more mesocarp injury than NHPT preclimacteric fruit. Yahia and Carrillo-Lopez (1993) observed mesocarp injury in 'Hass' avocado fruit exposed to 0.1–0.4% O_2 + 50% to 75% CO_2 atmospheres for longer than 1 day. Yahia and Kader (1991) found that 'Hass' avocado tolerated atmospheres of 0.25% O_2 or 0.25% O_2 + 80% CO_2 for only 1 day; mesocarp injury developed after 2 days and was more severe due to high CO_2 than to low O_2 alone. In our experiment, no exocarp injury was observed in any of the treatments. Yahia and Carrillo-Lopez (1993) reported that exocarp injury became apparent after 4 days in MA plus 6 days in air and increased thereafter. It should be noted that because the visual quality was assessed after the recovery period in air, it is feasible that the observed injuries were either due to the residual effects induced during the hypoxic treatment or to post-hypoxic or post-anoxic injury. Oxygen toxicity has been proposed as the principal mechanism of post-anoxic, post-hypoxic injury (Monk et al., 1987a,b; VanToai and Bolles, 1991; Crawford and Vollenweber-Ratzer, 1992).

4.3. Effect of acclimation to low oxygen on ADH and LDH enzymatic activities

The extractable ADH activity during low O_2 treatments (3, 1 and 0.25% O_2) in both HPT and NHPT fruit was similar to that of the air control fruit (Table 1), irrespective of both duration of the treatment and stage of ripeness of the fruit

(preclimacteric or ripening-initiated). Ke et al. (1995) reported that subjecting 'Hass' avocado to 0.25% O_2, 20% O_2 + 80% CO_2 or 0.25% O_2 + 80% CO_2 for up to 3 days did not influence the extractable activity of ADH, although there was a change in the accumulation of ethanol and an induction of ADH hypoxic isoenzymes. The authors explained that the high ADH activity in control avocado fruit made it difficult to detect any small changes in the total enzyme level due to the induction of the hypoxia-induced isoenzymes. They added that the molecular induction of the expression of this fermentation enzyme did not play a major role in regulating fermentation in avocado fruit (Ke et al., 1995).

The data presented in Table 2 show that hypoxic treatments (3, 1 and 0.25% O_2) for 1–3 days do not change the extractable LDH activity in either NHPT or HPT fruit compared to that of the control. Ke et al. (1995) also reported that exposure of avocado fruit to 0.25% O_2 for 3 days had only a marginal effect on the changes in LDH activity. When, after 3 days in hypoxia, the fruit were transferred back to air, there was an increase in LDH activity.

4.4. Effect of acclimation to low oxygen on the induction of alcohol dehydrogenase isoenzymes

From the results presented in this section, five observations warrant discussion. Firstly, low O_2 environments of 0.25, 1 and 3% O_2 for 1–3 days caused an induction of two new isoenzymes in addition to the constitutive isoenzyme. The results reported here are in agreement with those of Kanellis et al. (1991, 1993), who showed that two anaerobic ADH isoenzymes appeared in avocado fruit kept at 2.5–5.5% O_2 for 3 days. It is well-established that ADH in avocado fruit is a dimer with two isoenzymes: ADH1 and ADH2. Torres et al. (1978) reported that ADH1 isoenzyme is the slower-moving one in gel separations, a product of the *Adh1* gene, while ADH2 is the faster-migrating one, a product of the *Adh2* gene. Inter-

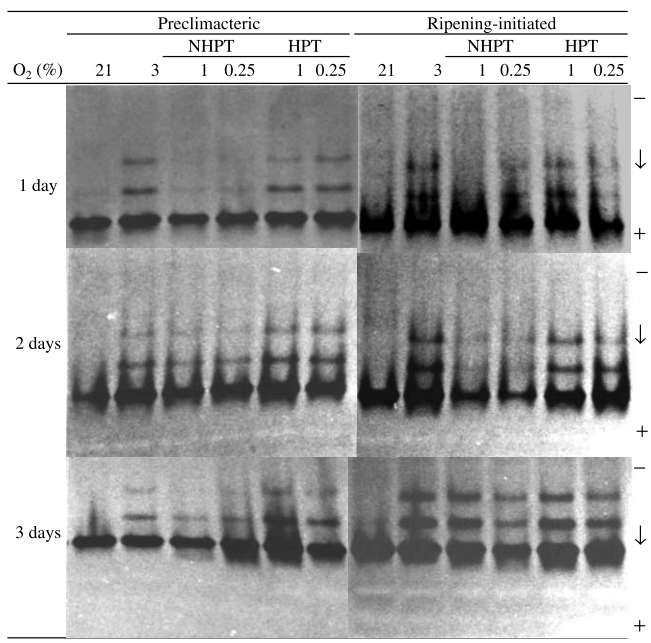

Fig. 5. Effect of acclimation to low oxygen on the induction of alcohol dehydrogrenase isoenzymes. Protein extracts (30 μg/lane) from preclimacteric and ripening-initiated avocado fruit were separated by native-PAGE and stained for ADH activity. Fruits were either not hypoxically pretreated (NHPT) and subjected directly to 3, 1 and 0.25% O_2 or they were hypoxially pretreated (HPT) prior to exposure to 1 and 0.25% O_2 for 1, 2 and 3 days.

genic isoenzymes are dimerization products of two subunits specified by the two separate *Adh* genes. Accordingly, the slower-migrating band is a homodimer for ADH1, while the faster-migrating band is a homodimer for ADH2 and the intermediate band is the dimerization product of the two alleles.

Secondly, ripening-initiated avocado fruit held in low O_2 always had stronger activity staining for the anaerobically induced ADH isoenzymes than did preclimacteric fruit. These data suggest that ripe avocados may have a greater potential for expressing ADH isoenzymes than unripe ones. Kanellis et al. (1993) reported that the intensity of ADH isoenzymes in air or low O_2 was stronger in ripening-initiated than in preclimacteric fruit.

Thirdly, the results obtained from ADH isoenzymes contradicted those from ADH enzymatic activities. The ADH zymogram proved that low O_2 exhibited an apparent induction, as evidenced by the increase in the intensity of ADH isoenzymes, whereas the ADH enzymatic activities showed no increase. Hassan (1993) reported that although low O_2 treatments (0–5%) caused an induction of the two additional anoxic ADH isoenzymes in both preclimacteric and ripening-initiated fruit, they did not result in increased ADH activities in these tissues. Ke et al. (1995) reported that subjecting 'Hass' avocado to 0.25% O_2, 20% O_2 + 80% CO_2 or 0.25% O_2 + 80% CO_2 for up to 3 days did not influence extractable activities of ADH. However, crude protein extracts analysed by native gel electrophoresis from these stressed avocado fruit revealed the induction of new ADH isoenzymes compared to the ADH isoenzyme profile of the air control fruit. Therefore, electrophoresis might have removed the presumed inhibitor of ADH and permitted the maximum expression of its enzymatic activity. However, further experiments are still needed to elucidate the discrepancy between induction of ADH anoxic isoenzymes and the lack of increase in extractable ADH activity.

Fourthly, the characterization of the induction of ADH isoenzymes in relation to the O_2 tensions has led us to question whether 3% O_2 or lower O_2 levels (1 and 0.25% O_2) induced stronger ADH activity. Our results showed that preclimacteric and ripening-initiated avocado fruit held in 3% O_2 for 1–3 days had higher intensity staining of the low O_2-induced ADH isoenzymes compared to those that were NHPT and held in 1 and 0.25% O_2 for the same period. In maize root tips, hypoxia rather than anoxia induced elevated ADH activity (Saglio et al., 1988; Johnson et al., 1989).

Hassan (1993) reported that fruit held in 1 and 2% O_2 for 48 h exhibited a stronger staining activity of ADH compared to those held in 3 and 5% O_2. This was more clearly shown in fruit held without O_2, which had the strongest staining for the anaerobic isoenzymes. However, the study of this phenomenon is complicated by the imprecise definition of hypoxia because when respiration is rapid, steep gradients in O_2 concentrations can result in an anaerobic core of cells surrounded by fully aerobic ones (Thomson and Greenway, 1991). In avocado fruit, the issue of whether 3% O_2 or more extreme low O_2 levels result in a stronger induction of ADH isoenzymes merits further investigation.

Fifthly, the most interesting aspect of all our observations is the strong induction of the anaerobically induced ADH isoenzymes in HPT preclimacteric and ripening-initiated avocado fruit held in 1 and 0.25% O_2 for 1–3 days. These HPT fruit showed much greater increase in intensity of the two newly induced ADH isoenzymes compared to those that were NHPT and held in 1 and 0.25% O_2 for up to 3 days.

The pioneering work of Sachs et al. (1996) showed that in maize roots, anoxia induced and/or enhanced the synthesis of a number of polypeptides (anoxic proteins) that were later identified as the enzymes that are expected to enhance production of ATP under subsequent anoxia. It was later shown that hypoxic pretreatments increased the survivability of maize seedlings in subsequent anoxia (Saglio et al., 1988). It is well known that the potentiating effects of hypoxic conditioning are attended by an increase in the activity of a number of glycolytic enzymes and, in the case of corn roots, the formation of aerenchyma (He et al., 1996; Drew, 1997; Chang et al., 2000). Our previous work demonstrated that the induction of the anoxic ADH isoenzymes is saturable with respect to O_2 concen-

tration in that for the induction to occur, the level of O_2 must drop below 6% (Kanellis et al., 1991). Thus, the induction of anoxic proteins is initiated at levels of O_2 where the rate of ATP synthesis is expected to be higher than under partial or total anoxia. Since protein synthesis is considered a strong sink for ATP utilization, it is reasonable to assume that the rate of anoxic protein synthesis is faster under O_2 concentrations, which do not create partial anaerobiosis, than it is under partial or total anoxia.

It is concluded that extremely low O_2 atmospheres can be used as a potential strategy for postharvest avocado fruit disinfection, provided that it is preceded by a period of low O_2 (3%) treatment which is necessary to permit acclimation of the avocado to extremely low O_2, resulting in a greater tolerance.

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References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carpenter, A., Potter, M., 1994. Controlled atmospheres. In: Sharp, J.L., Hallman, G.J. (Eds.), Quarantine Treatments for Pests of Food Plants. Westview Press, Boulder, pp. 171–198.

Chang, W.W.P., Huang, L., Shen, M., Webster, C., Burlingame, A.L., Roberts, J.K.M., 2000. Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. Plant Physiol. 122, 295–317.

Chen, X., Solomos, T., 1996. Effects of hypoxia on cut carnation flowers (*Dianthus caryophyllus* L.): Longevity, ability to survive under anoxia, and activities of alcohol dehydrogenase and pyruvate kinase. Postharvest Biol. Technol. 7, 317–329.

Crawford, R.M.M., Vollenweber-Ratzer, B., 1992. Influence of L-ascorbic acid on post-anoxic growth and survival of chickpea seedlings (*Cicer arietinum* L.). J. Exp. Bot. 43, 703–708.

Davies, D.D., Greco, S., Kenworthy, P., 1974. The control of production of lactate and ethanol by higher plants. Planta 118, 297–310.

Delate, K.M., Bretch, J.K., 1989. Quality of tropical sweet potatoes exposed to controlled atmosphere treatments for postharvest insect control. J. Am. Soc. Hort. Sci. 114, 963–968.

Dori, S., Butron, J.N., Lomaniec, E., Pesis, E., 1995. Effect of anaerobiosis on aspects of avocado fruit ripening. Acta Hort. 379, 129–136.

Drew, M.C., 1997. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 223–250.

Ellis, M.H., Dennis, E.S., Peacock, W.J., 1999. Arabidopsis roots and shoots have different mechanisms for hypoxic stress tolerance. Plant Physiol. 119, 57–64.

Hanson, A.D., Jacobsen, V.J., 1984. Control of lactate dehydrogenase, lactate glycolysis, and a amylase by O_2 deficit in barely aleuron layers. Plant Physiol. 75, 566–572.

Hassan, M.M., 1993. Some biochemical and molecular aspects of low oxygen action on fruit ripening. M.Sc. Thesis, Mediterranean Agronomic Institute of Chania, Greece, 86p.

He, C., Finglayson, S.A., Drew, M.C., Jordan, W.R., Morgan, P.W., 1996. Ethylene biosynthesis during aerenchyma formation in roots of maize subjected to mechanical impedance and hypoxia. Plant Physiol. 112, 1679–1685.

Hole, D.J., Cobb, B.G., Hobe, P.S., Drew, M.C., 1992. Enhancement of anaerobic respiration in root tips of *Zea mays* following low-oxygen (hypoxic) acclimation. Plant Physiol. 99, 213–218.

Johnson, J.R., Cobb, B.G., Drew, M.C., 1989. Hypoxic induction of anoxia tolerance in roots tips of *Zea mays*. Plant Physiol. 91, 837–841.

Johnson, J.R., Cobb, B.G., Drew, M.C., 1994. Hypoxic induction of anoxia tolerance in roots of *Adh1* null *Zea mays* L. Plant Physiol. 105, 61–67.

Kanellis, A.K., Solomos, T., Mattoo, A.K., 1989. Changes in sugars, enzymic activities and acid phosphatase isoenzyme profiles of bananas ripening in air or stored in 2.5% O_2 with and without ethylene. Plant Physiol. 90, 251–258.

Kanellis, A.K., Solomos, T., Roubelakis-Angelakis, K.A., 1991. Suppression of cellulase and polygalacturonase and induction of alcohol dehydrogenase isoenzmyes in avocado fruit mesocarp subjected to low oxygen stress. Plant Physiol. 96, 269–274.

Kanellis, A.K., Loulakakis, K.A., Hassan, M., Roubelakis-Angelakis, K.A., 1993. Biochemical and molecular aspects of low oxygen action on fruit ripening. In: Pech, C.J., Latche, A., Balague, C. (Eds.), Cellular and Molecular Aspect of the Plant Hormone Ethylene. Kluwer Academic, Dordrecht, pp. 117–122.

Ke, D., Kader, A.A., 1992. External and internal factors influence fruit tolerance to low-oxygen atmospheres. J. Am. Soc. Hort. Sci. 117, 913–918.

Ke, D., Yahia, E.M., Hess, B., Zhou, L., Kader, A.A., 1995. Regulation of fermentation metabolism in avocado fruit under oxygen and carbon dioxide stresses. J. Am. Soc. Hort. Sci. 120, 481–490.

Mapson, L.W., Burton, W.G., 1962. The terminal oxidases of potato tuber. Biochem. J. 82, 19–25.

Mitcham, E.J., Zhou, S., Kader, A.A., 1997. Potential of CA for postharvest insect control in fresh horticultural perishables: an update of summary tables compiled by Ke and Kader, 1992, vol. 1. Proceedings of the Seventh International Controlled Atmosphere Research Conference, July 13–18, University of California, Davis, CA, pp. 78–90.

Monk, K.S., Braendle, R., Crawford, R.M.M., 1987a. Catalase activity and post-anoxic injury in monocotyledonous species. J. Exp. Bot. 38, 233–246.

Monk, K.S., Fagerstadt, K.V., Crawford, R.M.M., 1987b. Superoxide dismutase as an anaerobic polypeptide. Plant Physiol. 85, 1016–1020.

Sachs, M.M., Subbaiah, C.C., Saab, I.W., 1996. Anaerobic gene expression and flooding tolerance in maize. J. Exp. Bot. 47, 1–15.

Saglio, P., Drew, M.C., Pradet, A., 1988. Metabolic acclimation to anoxia induced by low (2–4 kPa) partial pressure oxygen pretreatment (hypoxia) in root tips of *Zea mays*. Plant Physiol. 86, 61–66.

Solomos, T., Kanellis, A.K., 1997. Hypoxia and fruit ripening. In: Kanellis, A.K., Chang, C., Kende, H., Grierson, D. (Eds.), Biology and Biotechnology of the Plant Hormone Ethylene. Kluwer Academic, Dordrecht, pp. 239–252.

Thomson, C.J., Greenway, H., 1991. Metabolic evidence for stelar anoxia in maize roots exposed to low O_2 concentrations. Plant Physiol. 96, 1294–1301.

Torres, A.M., Diedenhofen, U., Bergh, B.O., Knight, R.J., 1978. Enzyme polymorphisms as genetic markers in the avocado. Am. J. Bot. 65, 134–139.

VanToai, T.T., Bolles, C.S., 1991. Postanoxic injury in soybean (*Glycine max*) seedlings. Plant Physiol. 97, 588–592.

Xia, J.H., Saglio, P.H., 1992. Lactic acid efflux as a mechanism of hypoxic acclimation of maize root tips to anoxia. Plant Physiol. 100, 40–46.

Yahia, E.M., Kader, A.A., 1991. Physiological and biochemical responses of avocado fruits to O_2 and CO_2 stress. Plant Physiol. 96, S96.

Yahia, E.M., Carrillo-Lopez, A., 1993. Responses of avocado fruit to insecticidal O_2 and CO_2 atmospheres. Food Sci. Technol. (Lebenn-Smittel-Wissensch. Technol.) 26, 307–311.

Yahia, E.M., Hernandez, M.T., 1993. Tolerance and responses of harvested mango to insecticidal low-oxygen atmospheres. HortScience 28, 1031–1033.