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RESEARCH PAPER

The role of the embryo and ethylene in avocado fruit mesocarp discoloration

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Abstract

Chilling injury (CI) symptoms in avocado (*Persea americana* Mill.) fruit, expressed as mesocarp discoloration, were found to be associated with embryo growth and ethylene production during cold storage. In cvs Ettinger and Arad most mesocarp discoloration was located close to the base of the seed and was induced by ethylene treatment in seeded avocado fruit. However, ethylene did not increase mesocarp discoloration in seedless fruit stored at 5 °C. Application of ethylene to whole fruit induced embryo development inside the seed. It also induced seedling elongation when seeds were imbibed separately. *Persea americana* ethylene receptor (*PaETR*) gene expression and polyphenol oxidase activity were highest close to the base of the seed and decreased gradually toward the blossom end. By contrast, expressions of *PaETR* transcript and polyphenol oxidase activity in seedless avocado fruit were similar throughout the pulp at the base of the fruit. Application of the ethylene inhibitor, 1-methylcyclopropene, decreased mesocarp browning, embryo development, seedling growth, and ion leakage, and down-regulated polyphenol oxidase activity. The results demonstrate that ethylene-mediated embryo growth in whole fruit is involved in the mesocarp response to ethylene perception and the development of CI disorders.

Key words: Chilling injury, ethylene receptor, 1-methylcyclopropene, *Persea americana*, polyphenol oxidase, seed germination, seedless avocado.

Introduction

Ripening and senescence of climacteric plant tissues, such as avocado fruit, are characterized by a burst of ethylene production and an increase in ethylene sensitivity (Abeles *et al.*, 1992). Aside from ethylene's stimulatory effect on ripening and senescence, it has also been found to be involved in the stimulation of seed germination (Kepczynski and Kepczynska, 1997; Kucera *et al.*, 2005) and precocious embryo growth (Fountain and Outred, 1990) in dry-seeded crops. Endogenous ethylene was shown to be required for optimal seed germination in several of these studies by utilizing the ethylene-action inhibitors 2,5-norbornadiene or 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997). However, the effect of ethylene on seed germination in fleshy-fruited species has barely been investigated. Moreover, as far as is known, there has been no study on the effect of ethylene on precocious germination while the seed is still inside the fruit.

The avocado (*Persea americana* Mill.) is a typical climacteric fruit that exhibits a sharp rise in ethylene production during ripening, which occurs after harvest; mature avocado fruits will not ripen while attached to the tree (Blumenfeld and Gazit, 1974).

Seedless fruits ('cukes') are a rare occurrence in avocado trees from various cultivars (Tomer *et al.*, 1980). 'Cukes' represent a type of stenospermocarpy in avocado, whereby fertilization occurs but the seed aborts and fails to develop (Lovatt, 1990). The phenomenon of stenospermocarpy also characterizes seedless grape (Hanania *et al.*, 2007), mango (Sukhvibul *et al.*, 2005), and pistachio (Polito, 1999). In the avocado cv. Arad, there is a relatively large number of seedless fruits alongside the seeded ones, which makes it an excellent experimental model for studying the involvement of seeds in avocado quality.

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Low-temperature storage is utilized to extend avocado storage life, but it also induces symptoms of chilling injury (CI) (Chaplin *et al.*, 1982), the most common one being grey–brown discoloration of the mesocarp. These symptoms do not appear at ambient temperature. It has been suggested that electrolyte leakage and disrupted ion balance resulting from ultrastructural changes in the membranes lead to the development of CI symptoms (Lyons, 1973). Mesocarp discoloration after cold storage in avocado correlates with increases in electrical conductivity (EC) values and polyphenol oxidase (PPO) activity (Hershkovitz *et al.*, 2005). Some tissues accelerate their ethylene production during chilling stress. A reduction in avocado ethylene production level during cold storage by 1-MCP or low-oxygen pretreatment has been shown to lead to a reduction in CI symptoms (Pesis *et al.*, 1994; Hershkovitz *et al.*, 2005).

This study was carried out to investigate the role of seed germination and the involvement of ethylene in CI. The effects of exogenous ethylene and the ethylene-action inhibitor 1-MCP were examined on precocious embryo growth in whole fruit, and on CI-induced mesocarp discoloration.

Materials and methods

Fruits and treatments

Avocado (*Persea americana* Mill. cvs Arad and Ettinger, both local green Israeli cultivars) fruit were obtained from the orchard of Kibutz Maabarot situated centrally on the coastal side of Israel. Fruit were harvested from six to eight trees in the same orchard over two sequential seasons, three harvests in each season. The data are from representative experiments per cultivar. However, only cv. Arad produces a lot of seedless fruit along with seeded fruit on the same tree (Fig. 1). Fruit were treated on the day of harvest with ethylene (10 μl l^{-1} for 18 h at 20±1 °C) or 1-MCP (150 nl l^{-1} for 18 h at 20±1 °C) in a sealed 300-l plastic chamber. 1-MCP was generated from SmartFresh™ (Rohm & Haas, Philadelphia, PA, USA; 0.14% active ingredient by weight). Untreated, control fruit were left in air for 18 h at 20±1 °C. After treatment, the fruit were separated into two groups: one was held at 20±1 °C, simulating shelf-life, and the other was transferred to a refrigerated chamber for cold storage at 5±1 °C for 3 or 4 weeks. Fruit were sampled several times during storage: on the day of harvest; immediately after treatments on day 1 at 20 °C; days 6 and 10 at 20 °C; during cold storage for 7, 15, 21, and 28 d at 5 °C; and after removal to shelf-life for 4, 7, and 10 d at 20 °C following 28 d at 5 °C.

Ethylene production measurement

Individual fruit were sealed in 2 l glass jars and held at 20 °C for 1 h. Headspace gas samples were then withdrawn by 10 ml syringe from each jar. Fruit were ventilated for 2 h and gas samples from the seed cavity were taken under water as described previously (Pesis and Marinansky, 1992). Ethylene content in the gas sample was determined by gas chromatograph equipped with an activated alumina column and a flame ionization detector (Pesis *et al.*, 1994).

Fig. 1. Seeded and seedless avocado fruit cv. Arad.

Ethylene production measurements in various avocado parts: fruit discs (1 g) from the inner pulp close to the seed base or from the outer pulp close to the blossom end, embryo, seed coat, and seed endocarp were prepared and sealed in a 50 ml Erlenmeyer flask with 1 ml water on Whatman no. 1 filter paper. After 1 h incubation at 20 °C, ethylene measurements were determined by gas chromatograph as described above.

Fruit firmness and CI index

Fruit firmness (N) was determined on whole, unpeeled fruit using an electronic penetrometer (LTCM, Chatillon, New York, NY, USA) with a 6.5 mm conical probe. Penetration

for 12 mm was done at two equidistant points on the equatorial regions of each fruit with a speed of 3 mm s^{-1}. The fruit were considered completely soft or 'ready to eat' when the average value decreased to 5–10 N.

The CI of the pulp, expressed as mesocarp discoloration, was assessed on completely soft with firmness 5–10 N (ready to eat) fruit during shelf-life at 20 °C. Fifteen fruit per treatment were cut longitudinally into two halves for examination of the pulp appearance, and CI was assessed according to a five-point scale (0, no injury; 1, slight injury; 3, moderate damage; 5, severe injury) according to Pesis *et al.* (2002).

Embryo development measurement

Following the post-harvest treatments, seeded fruit were stored for 6 d at 20 °C or for 3 weeks at 5 °C before examination of embryo development. Eight fruit per treatment were cut in half and the seeds were removed. Then seeds were cut in half and embryo length was measured.

Seedling length measurement

Seedling elongation was examined in seeds taken from the same fruit which were used for firmness and ethylene production studies. The control, ethylene-treated, and 1-MCP-treated fruit were peeled and the seed coat removed from the seed. A 10-mm-thick cross-sectional slice was taken from the top of the seed (Berg, 1988). Healthy and uniformly sized seeds (15 seeds per treatment) were placed in plastic boxes containing moist vermiculite and kept in a germinating room maintained at 25±2 °C and 90±3% RH (ISTA, 1999). Seedling length without roots was measured 21 d after sowing (Bender and Whiley, 2002).

RNA extraction and isolation of cDNA clone

The samples for RNA extraction were taken from tissue located at various distances from the seed (or seed cavity in seedless fruit): (i) inner pulp close to the seed base; (ii) mid-section pulp 0.5 cm from seed base; (iii) mid-section pulp 1.0 cm from seed base; (iv) outer pulp close to the blossom end. Total RNA was extracted from frozen mesocarp tissue in liquid nitrogen which was stored at –80 °C until analysis, using phenol–SDS as described by Or *et al.* (2000). First-strand cDNA was synthesized using the ABgene reverse-iT 1st strand Synthesis Kit (ABgene, Epson, UK) from 2 μg of total RNA, pre-treated with 1.5 units of RQ1 (Promega, Madison, WI, USA). The *Persea americana* ethylene receptor (*PaETR*) cDNA fragment was amplified using degenerate primers designed against conserved regions of subfamily-II ethylene receptor genes: (F)5′-GCYTAYT-TYTCVATCCCBMTDGAG-3′, (R)5′-DATYGMRTGC-ATBGGVSKYCTCAT-3′. Reactions were carried out by Mastercycler gradient (Eppendorf, Hamburg, Germany) using the following thermocycling profile: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 56 °C for 1.5 min, 1 min for 72 °C, and a final extension for 7 min at 72 °C. The PCR product of around 994 bp was subcloned into pGEM-T Easy vector System I (Promega) and five plasmids from recombinant colonies were sequenced using both SP6 and T7 primers by Hy-labs Laboratory (Rehovot, Israel). Sequence data were deposited in the GenBank database for *PaETR* cDNA under accession number EU370699. Deduced amino acid sequence comparison was performed using BlastP and BlastN algorithms (National Centre for Biotechnology Information).

Expression analysis by quantitative real-time PCR (qRT-PCR)

Accumulation of *PaETR* transcript was evaluated by qRT-PCR using the Absolute QPCR SYBR Green ROX Mix (ABgene, Epson, UK). Reactions were carried out using 5 μl of SYBR Green PCR Master mix according to the manufacturer's instructions and 2 pmol of specific primers, determined to be the most efficient concentration. The primers were as follows: *PaETR* (F) 5′-TGATCGGCATACCATC-TTGTACA-3′, (R)5′-TCACCGTACGCTCCTCGTTT-3′; *r18S* (F)5′-GTTACTTTAGGACTCCGCC-3′, (R)5′-TTC-CTTTAAGTTTCAGCCTTG-3′. The analysis was performed in a Rotor GENE 6000 instrument (Corbett Life Science, Sydney, Australia), with *r18S* serving as the standard. PCR conditions were as follows: incubation for 15 min at 95 °C to activate the enzyme. Then the next cycle was repeated 40 times: 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. The amount of specific transcript was calculated using the comparative CT method (Livak and Schmittgen, 2001).

Extraction and assay of PPO activity

Samples for PPO were taken from avocado cv. Arad tissue located at various distances from the seed (or seed cavity in seedless fruit): (a) inner pulp close to the seed base; (b) mid-section pulp; (c) outer pulp close to the blossom end (Table 5). Extraction was performed as previously described (Hershkovitz *et al.*, 2005). PPO activity was determined according to Esterbauer *et al.* (1977). The spectrophotometric method measures the decrease in absorbance (λ=412 nm) of the intensely yellow-coloured 2-nitro-5-thiobenzoic acid due to the coupled reaction with quinones generated by enzymatic oxidation of 4-methyl-1,2-catechol. Changes in absorbance were recorded every 10 s for 2 min with a spectrophotometer (GBC 911; Victoria, Australia).

The activity of the enzyme was calculated and expressed as units per milligram of protein. The protein content of each sample was determined with the Bradford Dye-Binding Assay (BioRad, Hercules, CA, USA) in triplicate according to the method of Bradford (1976), using bovine serum albumin as the standard.

Electrical conductivity

The EC of the internal tissue located in the mid-section pulp between the seed (or seed cavity in the seedless fruit) and the blossom end was measured in avocado cv. Arad as described previously (Hershkovitz *et al.*, 2005).

Statistical analysis

The ethylene production measurements presented are means of four fruit per treatment, firmness values are means of eight

fruit, embryo lengths are means of eight seeds, and seedling lengths are means of 15 seeds. The CI index is calculated using the means of 15 fruit per treatment. The data were statistically analysed with JMP 5.0 software (SAS Institute, Cary, NC, USA) and expressed as mean ±standard deviation.

Results and discussion

Effect of applied exogenous ethylene or 1-MCP on mesocarp discoloration

Control or ethylene-treated seeded and seedless avocado fruit stored at ambient temperature for 10 d did not develop any CI symptoms expressed as browning of the mesocarp (data not shown). Severe CI symptoms developed during cold storage at 5 °C, especially in seeded ethylene-treated fruit, exhibited as mesocarp discoloration located close to the seed base in both cvs Ettinger and Arad (Table 1, Fig. 2). However, ethylene did not increase mesocarp browning in seedless avocado stored at 5 °C (Fig. 2). Both control and ethylene-treated seedless avocado exhibited a similar CI index (Table 1). Moreover, in the seedless fruit, the mesocarp discoloration appeared close to the stem end and not close to the base of the seed cavity.1-MCP treatment effectively prevented mesocarp discoloration during cold storage and subsequent shelf-life in both seeded and seedless fruit (Fig. 2). CI development in control avocado fruit has been shown previously to be increased by ethylene exposure prior to cold storage (Pesis *et al.*, 2002). Delay of internal discoloration in avocado fruit by 1-MCP treatment has also been reported (Pesis *et al.*, 2002; Hershkovitz *et al.*, 2005; Woolf *et al.*, 2005).

Effect of ethylene or 1-MCP on embryo germination, seedling growth, firmness, and ethylene production

Control, ethylene-treated, or 1-MCP-treated seeded avocado fruit cvs Ettinger and Arad were stored for 6 d at 20 °C or 3 weeks at 5 °C, then the fruit were cut and the embryo length examined. Storage at 20 °C hastened embryo growth as compared with storage at 5 °C (Table 2). Ethylene treatment prior to storage of whole avocado fruit consistently accelerated embryo growth in seeds of both cultivars under both temperature regimes (Table 2, Fig. 3). By contrast, 1-MCP treatment inhibited embryo development in avocado fruit stored under both regimes, with embryo length remaining the same in both cases (Table 2). Thus ethylene can induce seed germination, even at low storage temperatures. Ethylene is associated with the acceleration of seed germination in many species (Kepczynski and Kepczynska, 1997; Kucera *et al.*, 2005). Here it is demonstrated that ethylene treatment of whole avocado fruit prior to storage consistently accelerated embryo growth in seeds of both cultivars under both temperature regimes (Table 2, Fig. 3).

Fruit firmness was checked in the various fruit stored for 1 d or 6 d at 20 °C or 28 d at 5 °C or 28 d at 5 °C plus 7 d at 20 °C (Table 3). Control and ethylene-treated fruit had already begun to soften during 28 d in cold storage at 5 °C, and they completed their ripening after 7 d of shelf-life at 20 °C. The softening of 1-MCP-treated fruit was significantly delayed, and after the cold storage and 7 d at 20 °C their firmness value was 32 N (Table 3); these fruit reached similar degrees of softness as their control counterparts after an additional 3 d (data not shown). Similar effects of 1-MCP in delaying avocado fruit softening have been observed before (Hershkovitz *et al.*, 2005; Woolf *et al.*, 2005).

Ethylene production was checked in mesocarp discs of avocado fruit which were examined for firmness. No

Table 1. Effect of ethylene or 1-MCP treatments on CI expressed as mesocarp discoloration (index) in avocado cvs Ettinger and Arad

Control and ethylene-treated avocados were measured after 4 weeks at 5 °C followed by 4 d at 20 °C and 1-MCP-treated fruit after 4 weeks at 5 °C followed by 10 d at 20 °C when fruit are ready to eat. 1-MCP (150 nl l^{-1}) and ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage.

	CI: mesocarp discoloration (index, 0–5)		
	Ettinger	Arad	
Treatments	Seeded	Seeded	Seedless
Control	0.15±0.1 b*	0.5±0.3 b	1.1±0.2 a
Ethylene	0.47±0.1 a	2.4±0.6 a	0.9±0.3 a
1-MCP	0 c	0 c	0 c

* Values for each cultivar within a column followed by a same letter are not significantly different according to Tukey's test (P <0.05, n=15).

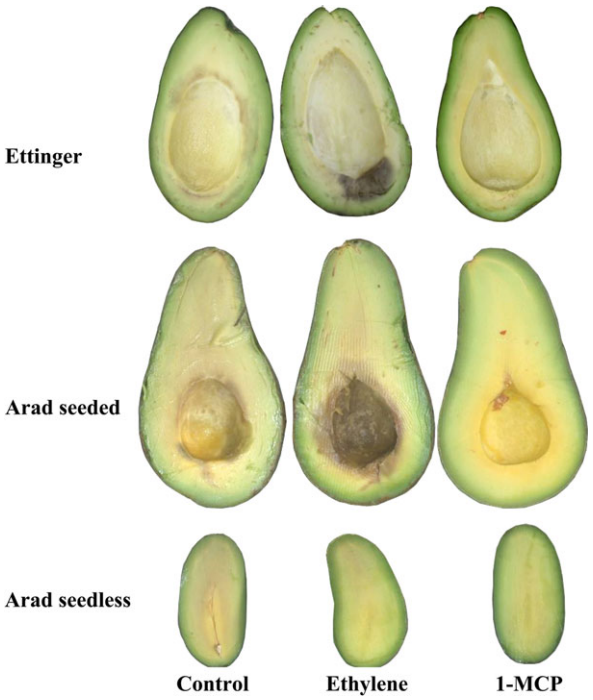

Fig. 2. Appearance of avocado mesocarp in control, ethylene-treated, and 1-MCP-treated seeded avocado fruit cvs Ettinger and Arad and seedless fruit cv. Arad stored for 4 weeks at 5 °C followed by 4 d at 20 °C. 1-MCP (150 nl l^{-1}) or ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage.

Table 2. Effect of ethylene or 1-MCP treatments on embryo length (mm) inside seeded avocado fruit cvs Ettinger and Arad stored for 6 d at 20 °C or for 21 d at 5 °C

1-MCP (150 nl l^{-1}) and ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage

		Embryo length (mm)	
Cultivars	**Treatments**	**6 d at 20 °C**	**21 d at 5 °C**
	Control	11.8±4.2 b*	7.0±2.7 b
Ettinger	Ethylene	21.4±1.6 a	13.6±2.3 a
	1-MCP	8.6±3.4 b	7.5±1.9 b
	Control	3.3±0.3 b	0.5±0.5 b
Arad	Ethylene	8.0±2.3 a	2.1±0.4 a
	1-MCP	0.2±0.2 c	0.3±0.2 b

* Values for each cultivar within a column followed by the same letter are not significantly different according to Tukey's test (P <0.05, n=8).

ethylene production was detected on days 1 and 6 in the control and 1-MCP-treated seeded fruit, whereas in ethylene-treated fruit ethylene production was detected on day 6 (Table 3). During cold storage (day 28), the level of ethylene production in ethylene-treated fruit was ~3-fold that in control or 1-MCP-treated fruit (Table 3).

Ethylene treatment effectively induced seedling growth immediately after removal from treatment (day 1) (Table 3). The length of the seedlings from ethylene-treated fruit stored for 6 d at 20°C or 28 d at 5 °C plus 7 d of shelf-life did not change significantly, whereas seedlings from control fruits lengthened gradually, reaching the length of the ethylene-treated group only after 28 d at 5 °C plus 7 d at 20 °C (Table 3). Seedling length derived from imbibed seeds of 1-MCP-treated fruit stored for 1, 6, and 28 d was significantly shorter than those of the control (Table 3). However, this growth inhibition was with time at 20 °C, because seedlings from 1-MCP-treated fruit after 28 d at 5 °C followed by 7 d at 20 °C, reached lengths similar to those from the other treatments (Table 3). The occurrence of seed germination, even in 1-MCP-treated avocado fruit, supported the notion that ethylene does not induce germination but rather increases seedling growth. The requirement of ethylene for healthy germination has been shown in papaya fruit, in which more vigorous seed germination was exhibited with seeds taken from post-climacteric fruit (Aroucha *et al.*, 2005). In *S. hermonthica* seeds, ethylene treatment has also been found to consistently induce germination (Sugimoto *et al.*, 2003).

Embryo development proceeds in fruit concomitant with fruit maturation. For example, in avocado cv. Hass, the embryo reaches the fully mature stage at 305 d after pollination, when the fruit is fully developed (Perán-Quesada *et al.*, 2005). Embryo development can lead to seed germination even inside the fruit. Nevertheless, it is possible that seed germination is induced by fruit detachment from the tree.

Ethylene evolution in various avocado organs

The distribution of ethylene evolution in mature seeded avocado cv. Ettinger was checked in tissue discs or organs.

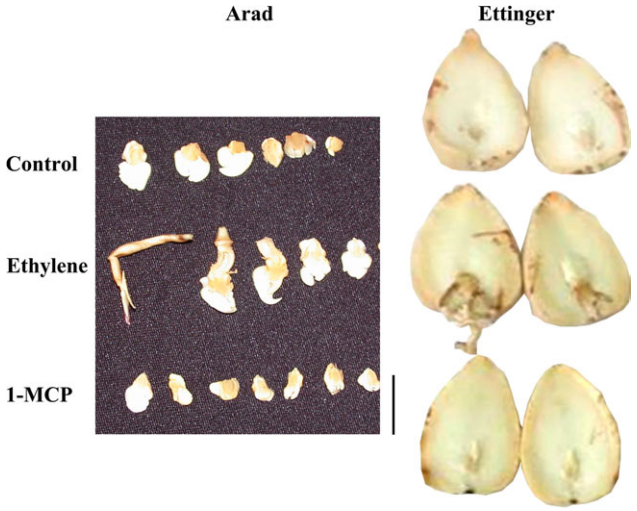

Fig. 3. Embryo development inside the seeds of control, ethylene-treated, and 1-MCP-treated avocado fruit cv. Arad, stored for 4 weeks at 5 °C and cv. Ettinger, stored for 2 weeks at 5 °C followed by 1 week at 20 °C. 1-MCP (150 nl l^{-1}) or ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage. Scale bar=1 cm.

The cv. Ettinger was chosen because it has a relatively large seed cavity that enables ethylene withdrawal. The highest ethylene production was detected in inner pulp close to the seed base while outer pulp close to the blossom end produced markedly lower amounts (Table 4). The level of ethylene evolution by embryo was 14 nl g^{-1} h^{-1} and ethylene concentration in the seed cavity was 2.6 µl l^{-1}. In mature passion fruit, the rate of ethylene production in seeds has been found to be lower than that in arils (Mita *et al.*, 1998). By contrast, the plum seed is not able to produce ethylene at the climacteric phase but does produce it in the early development stage (Fernandez-Otero *et al.*, 2006). In the present study, no ethylene evolution was detected in the seed coat or endosperm (Table 4), supporting the previous finding that the seed coat produces ethylene only in immature avocado fruit up to maturation, at which point it shrivels (Davenport and Manners, 1982). The present results demonstrate that the embryo actively participates in ethylene production of mature avocado fruit at the climacteric stage.

PPO activity

PPO activity was measured in avocado cv. Arad pulp tissue located at various distances from the seed or seed cavity close to the blossom end (sections a–c, Table 5) in seeded and seedless avocado cv. Arad after cold storage at 5 °C for 28 d. The PPO activity level was accelerated by ethylene treatment in seeded fruit. The PPO level in control and ethylene-treated seeded fruit was highest in the inner pulp close to the seed base (a), and gradually decreased toward the outer pulp close to the blossom end (c) (Table 5). By contrast, such a gradient was not found in seedless avocado; moreover, the level of PPO activity in seedless control fruit was the same as that in ethylene-treated ones (Table 5). Ethylene treatment induced PPO activity in the inner pulp

Table 3. Effect of ethylene or 1-MCP treatment on fruit firmness (N), ethylene production (nl g^{-1} h^{-1}), and seedling length (mm) in avocado fruit cv. Arad stored under different conditions

Firmness and ethylene production were measured in the same fruit. 1-MCP (150 nl l^{-1}) and ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage.

	Storage period			
Treatments	**1 d at 20 °C**	**6 d at 20 °C**	**28 d at 5 °C**	**28 d at 5 °C+7 d at 20 °C**
		Firmness (N)		
Control	127.6±6.6 a*	44.6±13.6 b	15.5±15.6 b	6.5±0.7 b
Ethylene	117.6±5.4 a	21.8±19.2 b	15.4±4.1 b	6.1±0.9 b
1-MCP	123.0 ±11.4 a	108.1±5.5 a	103.4±10.4 a	32.8±19.0 a
		Ethylene production (nl g^{-1} h^{-1})		
Control	ND†	ND	27.6±17.3 b	29.0±15.1 b
Ethylene	ND	13.5±5.5 a	72.8±3.1 a	23.1±6.2 b
1-MCP	ND	ND	24.1±2.4 b	62.3±2.4 a
		Seedling length (mm)		
Control	9.0±6.4 b	28.8±5.7 b	36.0±3.2 b	52.3±5.2 a
Ethylene	40.1±8.2 a	51.9±4.8 a	51.1±3.9 a	52.1±5.3 a
1-MCP	2.0±2.4 b	7.6±1.1 c	13.8±1.9 c	42.5±4.7 a

* Values within a column in each group followed by the same letter are not significantly different according to Tukey's test (P <0.05) (firmness, n=8; ethylene, n=4; seedling length, n=15).
† ND, Non-detectable.

Table 4. Ethylene production (nl g^{-1} h^{-1}) in whole fruit, embryo, inner pulp close to the seed base, outer pulp close to the blossom end, seed endosperm and ethylene concentration (µl l^{-1}) in the seed cavity of avocado fruit cv. Ettinger

The parameters were measured in five soft (ready to eat) fruits stored for 10 d at 20 °C. Values are presented as means of five fruit ±standard deviation.

Organs	Units	Ethylene production
Whole fruit	nl g^{-1} h^{-1}	22.8±10.8
Inner pulp close to the seed base	nl g^{-1} h^{-1}	48.4±25.4
Outer pulp close to the blossom	nl g^{-1} h^{-1}	12.1±6.20
Seed cavity	µl l^{-1}	2.6±1.26
Embryo	nl g^{-1} h^{-1}	14.0±8.98
Seed coat	nl g^{-1} h^{-1}	ND*
Endosperm	nl g^{-1} h^{-1}	ND

* ND, Non-detectable.

Table 5. Effect of ethylene and 1-MCP treatments on PPO activity (units mg^{-1} protein) in seeded and seedless avocado fruit cv. Arad after cold storage for 28 d at 5 °C

PPO activity (units mg^{-1} protein) was measured in pulp taken at various distances from the seed base: a, inner pulp; b, mid-section pulp; c, outer pulp. 1-MCP (150 nl l^{-1}) and ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage.

		Areas of the avocado pulp		
Fruit type	**Treatments**	**a**	**b**	**c**
Seeded	Control	0.79±0.03 b*	0.45±0.10 b	0.58±0.09 b
	Ethylene	1.07±0.11 a	1.01±0.08 a	0.84±0.15 a
	1-MCP	0.53±0.02 c	0.53±0.02 b	0.66±0.01 b
Seedless	Control	0.72±0.04 a	0.88±0.04 a	0.68±0.05 b
	Ethylene	0.72±0.03 a	0.77±0.17 a	0.94±0.08 a
	1-MCP	0.52±0.04 b	0.65±0.02 b	0.69±0.09 b

* Values within each column in each group followed by the same letter are not significantly different according to Tukey's test (P <0.05, n=4).

of seeded fruit, while in seedless fruit there was no induction in this section, which associates with mesocarp discoloration (Table 5 versus Table 1, Fig. 2). Application of 1-MCP was more effective at reducing PPO activity in the inner pulp tissue close to the seed base or seed cavity (section a) than in outer pulp close to the blossom end (section c) in both types of fruit (Table 5).

Pulp browning in avocado fruit, which is induced by chilling temperatures and ethylene application, has previously been attributed to the activity of PPO (Kahn, 1975; Chaplin *et al.*, 1982; Pesis *et al.*, 2002). PPO activity has been found to be induced in ethylene-treated avocado fruit which were cold-stored, whereas 1-MCP treatment reduced mesocarp discoloration and PPO activity (Hershkovitz *et al.*, 2005; Woolf *et al.*, 2005; Pesis *et al.*, 2007). The present data also show that PPO activity is induced by ethylene and inhibited by 1-MCP; hence, this activity can serve as an indicator for ethylene activity. The gradual decrease in PPO activity in seeded fruit from the seed base towards the blossom end of the fruit indicates that ethylene activity is higher near the seed: the tissue closest to the seed responded to 1-MCP more strongly than tissue located further away.

Electrical conductivity

Electrical conductivity (EC) levels in avocado cv. Arad, which was measured in the mid-section pulp located between the seed base and the blossom end, increased gradually during storage at 5 °C in seeded fruit in both control and ethylene-treated fruit (Fig. 4A). Ethylene treatment induced significantly the EC levels compared with the EC levels of control and 1-MCP-treated fruits during

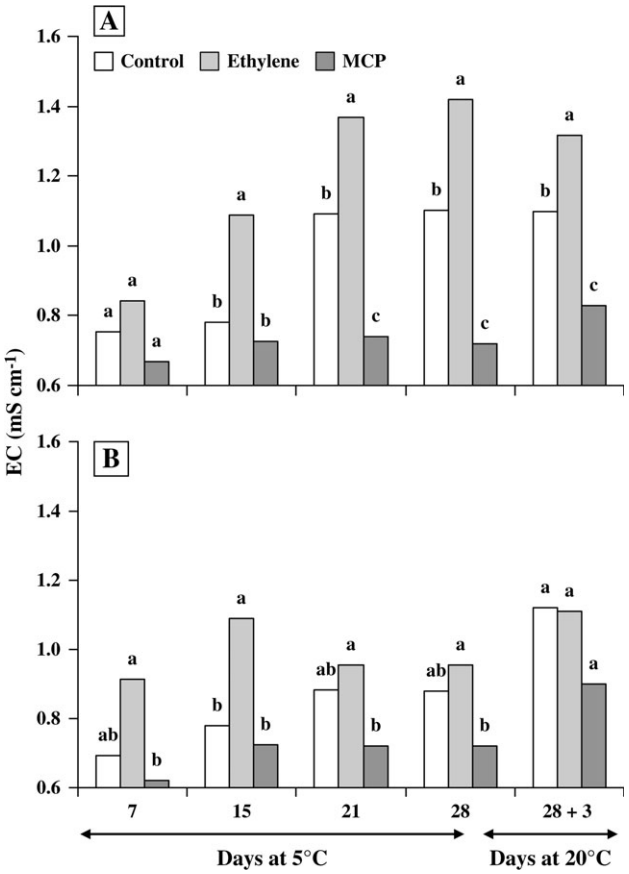

Fig. 4. Change in electrical conductivity (EC) (mS cm^{-1}) in control, ethylene-treated, and MCP-treated seeded (A) and seedless (B) avocado fruit cv. Arad. EC was measured for 28 d at 5 °C followed by 3 d at 20 °C. 1-MCP (150 nl l^{-1}) and ethylene (10 µl l^{-1}) treatments were given for 18 h at 20 °C prior to storage. Columns with a different letter within each treatment are significantly different (P <0.05, n=10).

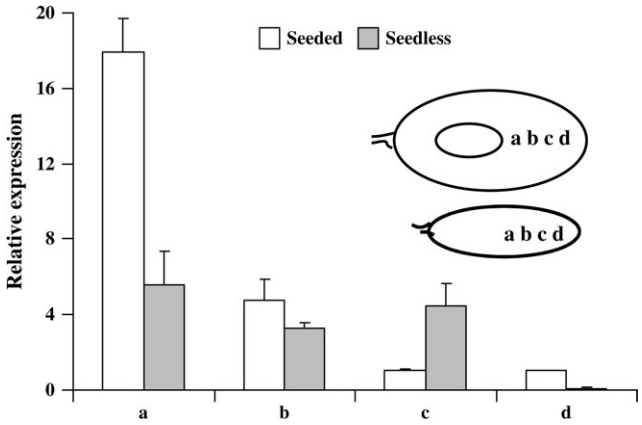

Fig. 5. Relative expression profile (transcript accumulation) determined by qRT-PCR of avocado ethylene receptor *PaETR* in seeded and seedless control fruit stored for 4 weeks at 5 °C followed by 4 d at 20 °C. Samples were taken from tissue located at various distances from the seed (or seed cavity in seedless fruit): a, inner pulp close to the seed base; b, mid-section pulp 0.5 cm from seed base; c, mid-section pulp 1.0 cm from seed base; d, outer pulp close to the blossom end. Values have been normalized to section d of seeded fruit, arbitrarily set to 1. Vertical bars represent ±standard deviation of three replicates.

3 weeks at 5 °C following 3 d shelf-life (except the first measurement after 7 d at 5 °C) (Fig. 4A). 1-MCP-treated fruit exhibited the significantly lowest EC levels during cold and shelf-life storage (Fig. 4A). In seedless fruit ethylene treatment increased EC levels during 15 d in cold storage, but later in storage these differences disappeared compared with control fruit (Fig. 4B). 1-MCP-treated seedless fruit exhibited the lowest EC levels as in the seeded fruit (Fig. 4A, B). In avocado fruit, EC has been shown to serve as a good indicator of membrane permeability, and to be highly correlated with ethylene production, softening, and CI symptoms (Montoja *et al.*, 1994; Hershkovitz *et al.*, 2005). Similar effects of 1-MCP on ion-leakage reduction have been found in apple and pear fruits (Larrigaudiere *et al.*, 2003; Pesis *et al.*, 2007).

Expression of PaETR *in avocado*

The transcript level of *PaETR* was examined in both seeded and seedless fruit of ripe control avocado. Samples were taken from tissue located at various distances from the seed or seed cavity (sections a–d, Fig. 5) towards the blossom end. *PaETR* expression in seeded fruit was highest closest to the seed base and gradually decreased towards the blossom end of the fruit. By contrast, the expression of *PaETR* transcript in seedless avocado fruit was similar in tissue sections a–c and dropped to very low levels at the blossom end (Fig. 5).

The *PaETR* gene from avocado is highly homologous to *LeETR4* from tomato fruits. *LeETR4* transcript has been previously shown to be up-regulated in ripening fruit (Tieman *et al.*, 2000) and to have a specialized role in modulating ethylene responses, including fruit maturation (Kevany *et al.*, 2007). In avocado fruit, *PaETR* transcript is up-regulated at the onset of ripening and hyper-regulated by exogenous ethylene (V Hershkovitz *et al.*, unpublished data). The expression of this gene in control seeded mesocarp was highest close to the base of the seed and decreased gradually towards the blossom end (Fig. 5). This pattern did not exist in seedless avocado and was correlated with endogenous levels of ethylene evolution in seeded fruit (Table 3). The highest *PaETR* transcription levels may represent higher ethylene-mediated receptor degradation, as has been found for tomato (Kevany *et al.*, 2007). Therefore, it may suggest that mesocarp close to the seed base in seeded avocado is more responsive to ethylene than the mesocarp in seedless fruit.

Based on the present findings, a model is proposed to describe the interactive effects of germination processes, cold stress, and ethylene on enhancement of mesocarp discoloration (Fig. 6). The model shows that enhancement of mesocarp discoloration in avocado pulp close to the base of the seed most likely results from embryo growth. We propose that ethylene, produced by mature avocado pulp, triggered embryo growth but not its germination, since seed

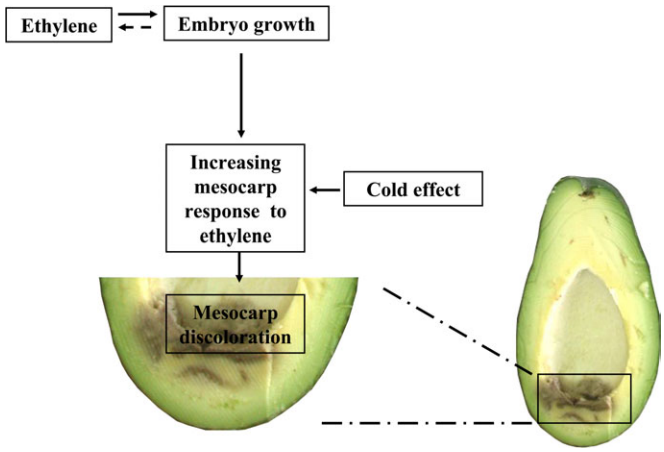

Fig. 6. Working model describing the interactive effects of ethylene, cold stress, and embryo growth on mesocarp discoloration in seeded avocado fruit. Solid arrows correspond to direct interaction and the dotted line indicates indirect evidence.

germination occurred even in the presence of 1-MCP. Cold exposure and embryo growth most likely enhanced discoloration, since discoloration occurred only in the cold, while the embryo developed also at 20 °C. Ethylene by itself did not enhance the chilling injury in seedless avocado, further supporting the assumption that it is embryo growth that acts to enhance the chilling injury. Embryo growth acts via increasing mesocarp response to ethylene which is higher close to the base of the seed. This conclusion is supported by the findings that PPO and *PaETR* expression are higher near the seed base. This gradient did not appear in seedless fruits. Higher levels of receptors transcript are correlated with enhanced ethylene response (Kevany *et al.*, 2007). The fact that *PaETR* is higher near the seed base only following storage when seed started to grow and not immediately after harvest (V Hershkovitz *et al.*, unpublished data), further supports the conclusion that embryo growth is the trigger for enhancing tissue sensitivity and not the germination. Since embryo growth preceded ethylene production it could be further suggested that the initial seed germination might be a signal for ethylene production in avocado fruit which leads to increased growth and enhances mesocarp response to ethylene.

Conclusions

Results of the present study provide evidence for the involvement of avocado seed germination and ethylene in CI. First, ethylene treatment increased CI-induced mesocarp discoloration, which was located close to the base of the seed in seeded fruit, and did not influence CI in seedless fruit (Table 1, Fig. 2). Secondly, it is not the seed itself that accelerates the CI but the germination processes that occur after harvest, which were shown to be augmented by ethylene. Thirdly, ethylene is not necessary for the germination processes but significantly increases the size of the embryo, a phenomenon that persisted even when the seeds were removed from the avocado fruit. Finally, the gradual distribution of PPO activity and *PaETR* expression in seeded avocado indicates that germination processes increase the mesocarp response to ethylene. Together, these findings support the involvement of embryo growth and ethylene in CI-induced mesocarp discoloration.

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