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Specific response of apple skin and pulp tissues to cold stress and 1-MCP treatment

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Abstract

It is now widely accepted that 1-MCP reduces ethylene production and prevents scald disorder in apple skin tissue. However, despite this beneficial effect, very little is known about the effects of 1-MCP on this tissue. This study aimed to determine how this treatment affects ACC metabolism in both skin and pulp tissues in relation to cold storage. Changes in ACC metabolism were monitored in control and 1-MCP treated fruit stored in air and removed after 0, 15, 30, 90 and 150 days of storage. 1-MCP treatment caused an inhibition of ethylene production but also of ACC synthase (ACS) activity and ACC levels both in pulp and skin. Compared to the control, 1-MCP treatment also induced a significant reduction in ACC oxidase (ACO) activity, but the inhibition remained incomplete in both tissues. High levels of MACC were found in 1-MCP treated fruit, showing the presence of a malonyl transferase insensitive to 1-MCP treatment. Collectively, these results showed that apple skin and pulp exhibited similar climacteric behaviour. The results also showed that the different parameters involved in ACC metabolism were differentially inhibited by the 1-MCP treatment during cold storage. ACS was completely inhibited in both tissues, ACO only partially and the treatment was ineffective to prevent MACC accumulation.

Keywords: 1-Methylcyclopronene; Ethylene; ACC metabolism; Cold stress; Apple skin tissue; Apple pulp tissue

1. Introduction

Apple (*Malus* × *domestica* Borkh.) is a climacteric fruit that exhibits a rise in ethylene production and respiration rates during ripening. It has been clearly established that ethylene biosynthesis originates from *S*-adenosyl methionine (SAM) via synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC). ACC synthesis proceeds through the action of ACC synthase (ACS) and the auto stimulation of ethylene synthesis in climacteric fruit. The last step of ethylene biosynthesis, the conversion of ACC to ethylene, is the result of the action of ACC oxidase (ACO). The ACC level can be finally regulated by the rate of its conversion to malonyl-ACC (MACC) through the action of malonyl transferase enzyme (Yang and Hoffman, 1984).

Exposure of pears to low temperature promoted ethylene synthesis in a number of pears cultivars (Knee et al., 1983; Blankenship and Richardson, 1985). However, although ACC levels increase during cold storage, the activity of ACS remains low (Knee, 1987). When the pears are removed and placed at 20 °C, the rate of ethylene and carbon dioxide production sharply increases within 12 h. The activity of ACS and ACC levels also increase rapidly (Knee, 1987). Apples exhibit a somewhat similar response to cold stress. In this fruit, cold stress may also stimulate ACC accumulation, ACS and ACO activity (Larrigaudière and Vendrell, 1993; Lelièvre et al., 1995; Larrigaudière et al., 1997). Activation of ethylene synthesis by cold stress was also observed by Knee et al. (1983) in 'Golden Delicious' apples.

The effect that 1-methylcyclopronene (1-MCP) has on ethylene production in climacteric fruit has already been extensively described (Blankenship and Dole, 2003). This compound is able to influence fruit ripening and improves post storage quality in climacteric fruit (Blankenship and

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Dole, 2003). Although many works describe the beneficial effects of 1-MCP treatment on quality parameters, very little is currently known about the specific effects that this treatment may have on ACC metabolism and especially upon different tissue types such as skin and pulp. It would seem important to gain a better understanding of these specific effects. It is generally accepted that 1-MCP treatment inhibits fruit ripening. As a consequence, apple pulp remains firmer and maintains its acidity during the storage period. 1-MCP treatment also makes it possible to maintain a greener skin colour due to inhibition of ethylene production and reduced chlorophyllase activity (Hershkovitz et al., 2005). 1-MCP also inhibits α-farnasene production and as a result prevents the accumulation of conjugated trienols (Fan et al., 1999a; Watkins et al., 2000; Shaham et al., 2003; Pechous et al., 2005). In less typical cases, 1-MCP treatment may also cause a disorder characterized by diffuse skin browning (DSB), especially in Golden cultivars. The reason for the appearance of this disorder remains unknown but it is possibly associated with ethylene metabolism and senescence-related processes.

To determine which specific changes occur in skin and pulp tissue during cold acclimation and how 1-MCP acts in this context, we carried out a series of experiments concerning skin and pulp tissues. Emphasis was placed on ACC metabolism and on the specific behaviour of skin and pulp tissues in relation to 1-MCP treatment. Our objective was to discover (i) the specific climacteric behaviour of skin and pulp tissues, (ii) their specific responses to 1-MCP treatment, and (iii) the effects that cold stress may have on ACC metabolism in both tissues.

2. Material and methods

2.1. Plant material

'Golden Smoothee' (*Malus* × *domestica* Borkh.) apples were obtained from Lleida (Spain) during summer 2003. Tests were conducted on a block of trees planted in 1975. Orchard characteristics were as follows: conventional fertilization system, Franco class soil texture (according to USDA classification) and Pajam-2 rootstock. Fruit was harvested at the optimal commercial date and following local recommendations established by packinghouses (firmness, soluble solid concentration and acidity values). Fruit was selected on the basis of size and the absence of defects, and stored in experimental chambers (22 m^3) for 5 months in air at 1 °C and 90% RH.

2.2. 1-MCP treatment

Smartfresh™ (Agrofresh Inc., Rohm and Haas, Spring House, PA, USA) was used to release 1-MCP on the day of harvest according to the manufacturer's recommendations. Portions of 1-MCP were weighed in a flask in powder form (for 625 nl l^{-1}, 1 g m^{-3} chamber). Water was added to release 1-MCP (water temperature 35 °C; Smartfresh™: water ratio 1:5) and the flask was immediately sealed and the contents stirred for 10 min to homogenize the mixture. The flask was then taken to the storage chamber, which had a previously set temperature of 0.5 °C. The flask cover was then rapidly removed to start the treatment. 1-MCP treatment was carried out overnight (18 h) in the same chamber that was used for storage. Prior to establishing the storage conditions the chamber was thoroughly aerated.

2.3. Determination of ethylene production

To determine ethylene production, fruit was kept in a room at 20 °C. The rate of ethylene production rate was determined on three replicates of two apples each. Fruit was placed in 1500 ml flasks and continuously ventilated with humidified air at a flow rate of approximately 1.5 l h^{-1}. Ethylene production was measured by taking gas samples of effluent air from respiration jars, using a 1 ml syringe. Gas samples were injected into a gas chromatograph (Hewlett-Packard 5890 Series II, Barcelona, Spain) equipped with a FID detector and an alumina column (1.5 m × 3 mm). Gas analyses were conducted isothermally at 100 °C. N_2 carrier, air and H_2 flows were 45, 400 and 45 ml min^{-1}, respectively. The injector and detector were kept at 120 and 180 °C, respectively.

2.4. Determination of ACC and MACC levels

For the determination of ACC, a 3 g pulp longitudinal slice or skin samples were extracted with 80% ethanol and the ACC content assayed according to the method of Lizada and Yang (1979). ACC levels were expressed as nmol ACC g^{-1} fresh weight. MACC was measured by analyzing the ACC content of an extract hydrolyzed as described by Hoffman et al. (1982). Data were expressed as nmol MACC g^{-1} fresh weight.

2.5. Determination of ACC synthase and ACC oxidase activities

To determine ACS activity, 10 g of pulp (3 g of skin) were frozen with liquid nitrogen, triturated and homogenized in 10 ml 200 mM Tricine buffer (pH 8.5), 10 mM DTT, 20 μM pyridoxal 5-phosphate and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenate was filtered through two layers of miracloth and centrifuged at 18,000 rpm for 20 min at 4 °C, and a 2.5 ml aliquot was loaded into a Sephadex G-25 column (PD 10, Pharmacia, Madrid, Spain) equilibrated with 10 ml 5 mM Tricine buffer (pH 8), 1 mM DTT, 2 μM pyridoxal 5-phosphate. The enzyme was eluted with 3.5 ml of the same buffer. 1.5 ml of enzyme extract was incubated for 2 h at 25 °C with 200 mM Tricine buffer (pH 8) and 100 μM of *S*-adenosyl-L-methionine (SAM). The reaction was then stopped with 100 mM $HgCl_2$. One millilitres of this reaction was then mixed and stirred with 100 μl of NaOCl and NaOH satured (2:1, v/v). After 2 min, a 1 ml gas sample

was taken with a syringe. Gas samples were injected into a gas chromatograph and results were expressed as nmol ACC h^{-1} mg $protein^{-1}$.

For ACO activity, 10 g of pulp (3 g of skin) was frozen with liquid nitrogen, triturated and homogenized in 20 ml (10 ml in skin) 0.1 M Tris–HCl buffer (pH 7.4), 10% glycerol, 30 mM Na-ascorbate, 5 mM DTT and 1% (w/v) PVP. The homogenate was filtered through two layers of miracloth and centrifuged at 15,900 rpm for 20 min at 4 °C, and a 2.5 ml aliquot was loaded into a Sephadex G-25 column (see above) equilibrated with 20 mM Tris–HCl buffer (pH 7.4), 10% glycerol, 3 mM Na-ascorbate and 1 mM DTT. An enzyme extract (500 μl) was then mixed with 10 μM $FeSO_4$, 3 mM sodium bicarbonate and 50 μM ACC. The mixture was then aired and incubated for 20 min at 26 °C. After this time, a 1 ml gas sample was taken using a syringe. This was then injected into a gas chromatograph and the results were expressed as nmol ethylene h^{-1} mg $protein^{-1}$.

Protein measurements were performed according to Bradford (1976). Data on enzyme activity represent the mean of four individual fruits.

2.6. Data processing and statistical analysis

For the figures, data were analyzed for significant differences by applying variance analysis (ANOVA) using the SAS (Statistical Analysis System) statistical package and were subjected to mean separation by the LSD (least significant difference) test ($P < 0.05$).

3. Results

3.1. Effects of 1-MCP on ethylene production

At harvest, fruit produced very low levels of ethylene and only a slight increase was observed after 11 days at 20 °C (Fig. 1A). Storage of fruit at low temperature (1 °C) increased the peak of ethylene production and reduced the time required to reach the onset of the autocatalytic ethylene rise (Fig. 1B). As generally observed, the 1-MCP treatment clearly inhibited ethylene production during the shelf life period, both after 90 and 150 days of storage (Fig. 1B and C).

3.2. Effects of 1-MCP on ACS activity in skin and pulp during storage

Changes in ACS activity were monitored during cold storage, both in skin and pulp tissue in relation to 1-MCP treatment (Fig. 2). Very low levels of ACS activity were found at the beginning of storage (time 0 days). In control fruits the activity then rapidly increased throughout storage, similarly in skin and pulp up to 90 days. Later, ACS activity increased in pulp but remained stable in skin tissue. As expected, 1-MCP clearly inhibited ACS activity in both skin and pulp tissue.

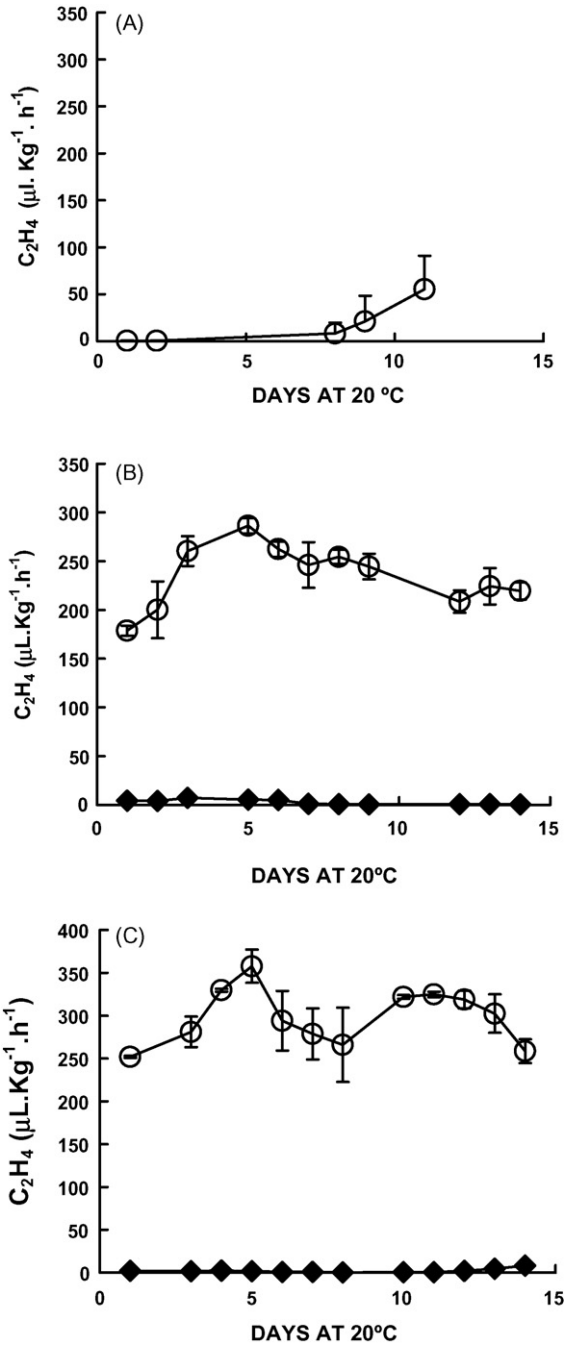

Fig. 1. Ethylene production in 'Golden Smoothee' apples during ripening at 20 °C after harvest (A) and effects of 1-MCP treatment on ethylene production during ripening after 90 days (B) and 150 days (C) of storage at 1 °C. (◯) Control fruit and (♦) fruit treated with 0.625 μl l^{-1} 1-MCP. Values for ethylene production represent means of three replicates of two fruit each ±S.D.

3.3. Effects of 1-MCP on ACO activity in skin and pulp during storage

In control fruits and after a 2-week lag period, a sharp increase in ACO activity was observed in both skin and pulp tissues and up to 90 days (Fig. 3). Later and in contrast to the result observed for ACS, ACO activity remained stable in

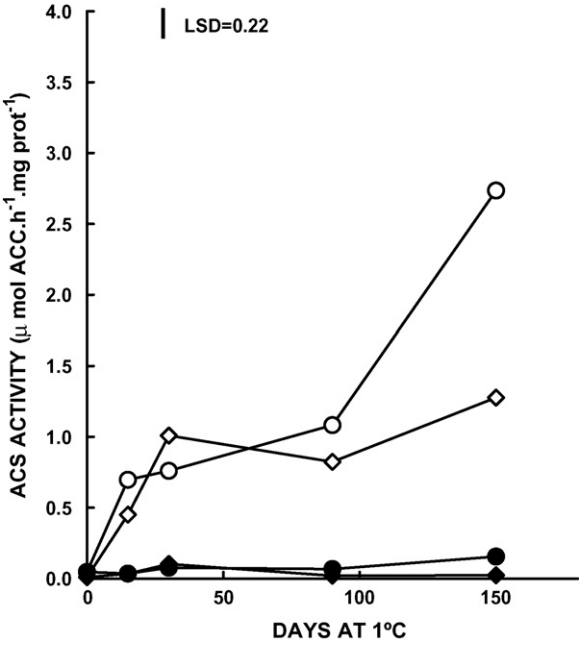

Fig. 2. Effects of 1-MCP treatment on ACC synthase activity in skin and pulp of 'Golden Smoothee' apples at harvest and after 15, 30, 90 and 150 days of storage at 1 °C. (◯) Control fruit (pulp), (●) fruit treated with 0.625 μl l^{-1} 1-MCP (pulp), (◊) control fruit (skin), and (♦) fruit treated with 0.625 μl l^{-1} 1-MCP (skin). Each point represents the mean for four different fruits. $LSD_{(ACS)} = 0.22$ ($P < 0.05$).

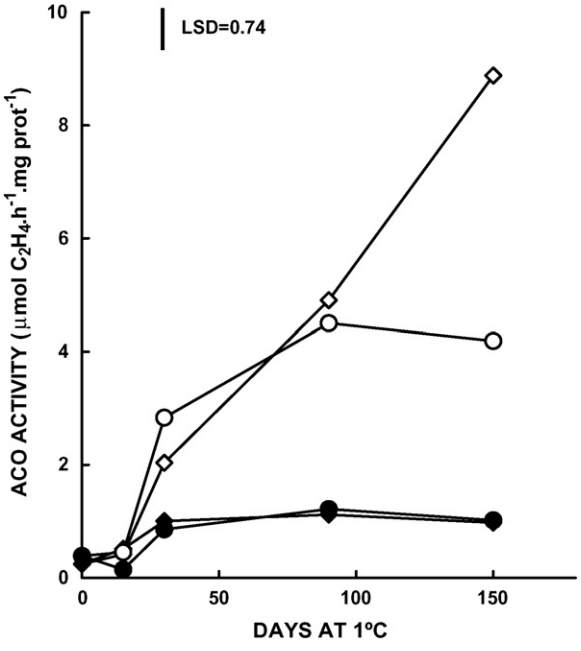

Fig. 3. Effects of 1-MCP treatment on ACC oxidase activity in skin and pulp of 'Golden Smoothee' apples at harvest and after 15, 30, 90 and 150 days of storage at 1 °C. (◯) Control fruit (pulp), (●) fruit treated with 0.625 μl l^{-1} 1-MCP (pulp), (◊) control fruit (skin), and (♦) fruit treated with 0.625 μl l^{-1} 1-MCP (skin). Each point represents the mean for four different fruits, $LSD_{(ACO)} = 0.74$ ($P < 0.05$).

pulp but increased in skin tissue. 1-MCP treated apples exhibited less ACO activity than control fruit, both in skin and pulp. However and in contrast to ACS, a significant residual ACO activity was observed during all the experimental period.

3.4. Effects of 1-MCP on ACC and MACC levels in skin and pulp during storage

In control fruit, ACC content rapidly accumulated during the first day of storage, peaking at 15 days. It then decreased in both skin and pulp (Fig. 4A). In general, lower levels of ACC were found in pulp than in skin. Very low levels of ACC

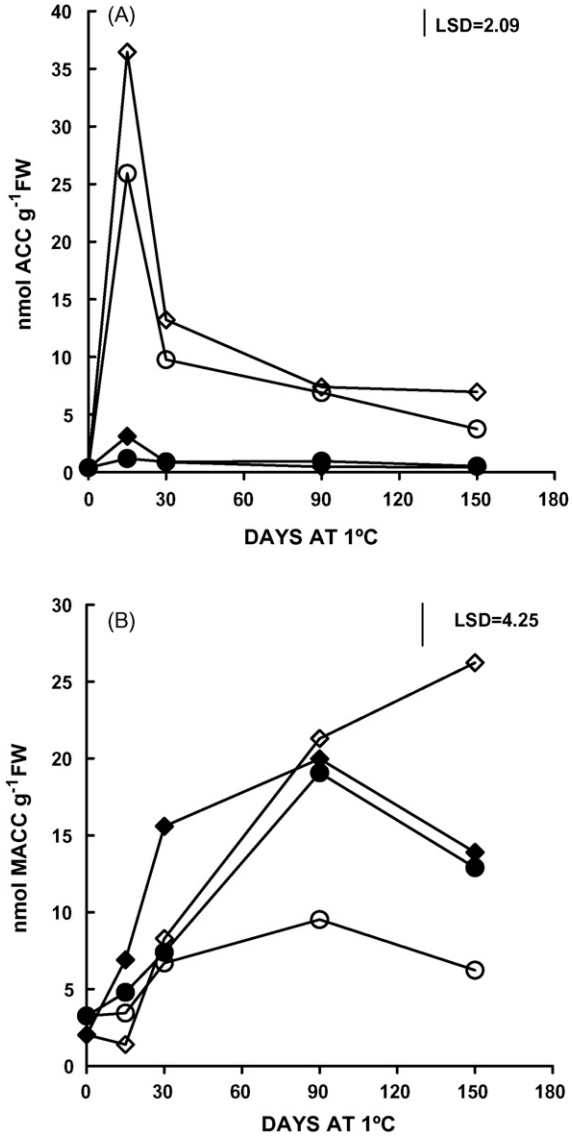

Fig. 4. Effects of 1-MCP treatment on ACC levels (A) and MACC levels (B) in skin and pulp of 'Golden Smoothee' apples at harvest and after 15, 30, 90 and 150 days of storage at 1 °C. (◯) control fruit (pulp), (●) fruit treated with 0.625 μl l^{-1} 1-MCP (pulp), (◊) control fruit (skin), and (♦) fruit treated with 0.625 μl l^{-1} 1-MCP (skin). Each point represents the mean for four different fruits. $LSD_{(ACC)} = 2.09$, $LSD_{(MACC)} = 4.25$ ($P < 0.05$).

were found throughout storage in both skin and pulp when fruit was treated with 1-MCP.

Different behaviour was observed for MACC accumulation (Fig. 4B). In control fruit, MACC levels significantly increased after a 15-day lag period in both pulp and skin until 1 month of storage. Skin and pulp tissues subsequently exhibited different behaviour. While MACC levels remained constant in pulp tissue, a progressive increase was observed for skin tissue. Somewhat surprisingly, the 1-MCP treatment did not inhibit MACC accumulation either in pulp or in skin. In general, higher levels of MACC were found in skin, especially during the first day of storage.

4. Discussion

The effect of 1-MCP on ethylene production depends on the species and varieties of fruit (Blankenship and Dole, 2003). Generally, 1-MCP treatment clearly inhibits ethylene production in apples e.g. 'Fuji', 'Red Delicious' and 'Granny Smith' (Fan et al., 1999b). The present work confirmed this inhibition in 'Golden Smoothee' apples. Although this result is not new, this work offers the advantage of the precision with which skin and pulp tissues specifically participated in this kind of inhibition. Furthermore, our results allowed us to define how these tissues particularly responded to cold stress.

In a previous work (Larrigaudière and Vendrell, 1993), we showed that cold temperature may promote ethylene biosynthesis and induce climacteric behaviour in 'Granny Smith' apples. Significant differences in the regulation of ethylene biosynthesis between skin and pulp tissue were also found by Lara and Vendrell (2003) in this cultivar. In other cultivars, such as 'Royal Gala' and 'Starking Delicious', different types of behaviour were observed, but in all the cases ethylene production clearly increased following cold stress (Larrigaudière et al., 1997). Our results showed that 'Golden Smoothee' apples also followed this general behaviour and that the key enzyme appeared to be ACS. This cold-induced activation of ACS was previously described in 'Passe Crassane' pears (Lelièvre et al., 1997) and apples (Tian et al., 2002). Cold storage promotes the accumulation of ACS and ACO mRNA, which triggers the ethylene burst observed after removal from storage.

As previously observed in 'Gala' apples (Lu and Toivonen, 2003), our results showed that 1-MCP treatment clearly inhibited ACS activity in 'Golden Smoothee' apples. This inhibition was significant in both pulp and skin tissues and was probably the result of the inhibition of the basic ethylene levels and of the signalling pathway that occurs even in cold conditions. This result also showed that 1-MCP acted both in skin and pulp tissues, a result that – for the first time to our knowledge – establishes the presence of ethylene receptors in both tissues. The question therefore centres on the physiological reason for the presence of ethylene receptors in skin. In pulp, ethylene is directly linked to ripening and permits the fruit to acquire its organoleptical quality. In skin, ethylene is likely needed for colour changes and for the accumulation of volatiles in order to achieve ripening.

Very high levels of ACO activity were observed in control fruits during cold storage. The increase in ACO was noted only after a 2-week lag period that corresponds to the time necessary to induce the climacteric process in accordance to the McMurchie model (McMurchie et al., 1972). Both skin and pulp exhibited a typical climacteric behaviour. However, after 90 days ACO activity only increased in skin tissue. This result is likely the reflection of different regulatory processes between tissues during cold storage.

As for ACS, the increase in ACO activity observed under cold conditions was clearly inhibited by the 1-MCP treatment. However, significant residual activity was observed in both tissues. This result reflected the specific behaviour of ACO enzyme with reference to 1-MCP treatment. Residual ACO activity may be due to higher levels or higher turnover of ethylene receptors. This result also highlighted the difference existing between ACS and ACO in reference to 1-MCP treatment. Inhibition of ethylene production is mainly the consequence of the inhibition of ACS activity. Because of the maintenance of ACO activity, all the stress factors that may activate ACS activity such as wounding or senescence will restore the ethylene production in 1-MCP treated fruits.

Surprisingly, when we consider the inhibitory effects that the 1-MCP treatment had on ethylene production, ACC levels, ACS and ACO activities; significant and even higher MACC levels were found in 1-MCP treated fruits. This result showed that ACS was only partially inhibited by the 1-MCP treatment, especially in skin. It also showed that the enzyme malonyl transferase was, at least in part, ethylene-independent and not linked to the ethylene signalling pathway regulated by 1-MCP.

Collectively, the results presented in this work showed that apple skin and pulp exhibited similar climacteric behaviour. They also showed that the different parameters involved in ACC metabolism were differentially inhibited by the 1-MCP treatment during cold storage. The inhibition of ethylene production in 1-MCP treated fruits is mainly due to the inhibition of ACS activity but also to the maintenance of a 1-MCP-insensitive malonyl-transferase activity. Further work is needed to better understand this last process.

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