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Ethylene biosynthesis and signaling elements involved in chilling injury and other postharvest quality traits in the non-climacteric fruit of zucchini (*Cucurbita pepo*)

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ABSTRACT

The immature non-climacteric fruit of zucchini is very sensitive to postharvest chilling injury (PCI). Although the fruit produces very low ethylene at harvest, cold storage induces an increase in ethylene production in the refrigerated fruit, and upon rewarming up to room temperature. The production of ethylene after rewarming was proportional to cold damage and found to be higher in the fruit of the cultivars most susceptible to chilling. The effects of the ethylene inhibitor 1-methycyclopropene (1-MCP) has been analyzed using the postharvest quality parameters of nine zucchini cultivars stored at 20 °C and 4 °C for 14 days. We have also determined the evolution of ethylene production in the fruit of these cultivars, and assessed the expression profiles of twelve ethylene biosynthesis and signaling genes in both control fruit and in 1-MCP treated fruit, of two cultivars that responded to 1-MCP. The 1-MCP treatment reduced the rate and delayed the onset of PCI symptoms and reduced fruit weight loss, although only in the fruit of the most chilling-susceptible cultivars, and concomitantly we found a reduction in the respiration rate and in the level of cold-induced ethylene. This data suggests that ethylene is not only a response of the fruit to cold damage, as occurs upon rewarming, but could also play a regulation role in the onset of PCI in this non-climacteric fruit. The genes *CpACO1* and *CpACS1* were found to be involved in the biosynthesis of the cold-induced ethylene, but the role played by *CpACO1* was found to be the key function, since it was induced higher than *CpACS1* in response to cold storage. More over *CpACO1* was the only ethylene biosynthesis gene that was downregulated by the 1-MCP treatment. Some of the ethylene perception and signaling genes, including *CpETR1*, *CpCTR1* and *CpEIN3.1* were also induced in response to cold storage, and were downregulated in response to 1-MCP, indicating that the positive effect of 1-MCP in the fruit of certain zucchini cultivars is also associated with a downregulation of genes involved in ethylene perception and signal transduction pathways.

1. Introduction

Storage at chilling temperatures constitutes one of the most important postharvest technologies to preserve the quality and extend shelf life of fruits and vegetables during postharvest. Tropical and subtropical fruits and vegetables, however, are very sensitive to cold storage, and when stored at low temperatures rapidly develop postharvest chilling injury (PCI). Zucchini is an immature fruit vegetable that is harvested when the fruit has an average length of about 20 cm (Cantwell and Kasmire, 2011), and which is very susceptible to chilling storage, showing irreversible PCI symptoms after just 2–3 days of storage at 5 °C (Martínez-Téllez et al., 2002; Megías et al., 2014). During chilling storage, zucchini fruit suffers pitting, loss of water, softening and increased sensitivity to pathogens, which quickly reduce its market value (Wang, 1994; Serrano et al., 1998). Given that most of the greenhouse-produced zucchini in Spain is exported to other

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European countries, the development of new varieties and technologies that preserve fruit quality during postharvest transportation and storage are priorities for the commercialization of this fruit in Europe and worldwide.

Immature fruit vegetables are considered non-climacteric since they produce little ethylene at harvest and during postharvest storage. However, as also occurs in other non-climacteric fruits (Cooper et al., 1969; Zacarías et al., 2003), the storage of immature fruit of cucumber and zucchini at chilling temperatures induces the production of ethylene (Wang and Adams, 1980, 1982). This cold-induced ethylene is actually induced when refrigerated fruit of zucchini is transferred to room temperature (Mencarelli et al., 1983; Megías et al., 2014), and this rewarming is also required for the induction of ethylene production in cucumber (Wang and Adams, 1982), citrus (McCollum and Mcdonald, 1991; Lafuente et al., 2001) and kiwi (Hyodo and Fukasawa, 1985). We have recently reported that this cold induced ethylene increases gradually during the cold storage of zucchini at 4 °C, and peaks at 7 days of cold storage. Afterwards, ethylene production in the fruit falls gradually until reaching the basal production observed at harvest (Megías et al., 2014). This decline in ethylene production after 7 days of cold storage at 4 °C may be due to a loss of membrane integrity affecting the conversion of ACC to ethylene by the ACO enzyme (Concellón et al., 2005; Zheng et al., 2008).

Ethylene regulates a wide range biochemical, physiological and developmental processes that devalue climacteric and non-climacteric fruits during postharvest storage (Abeles et al., 1992; Barry and Giovannoni, 2007; Li et al., 2010). Ethylene is also responsible for the response of plants to biotic and abiotic stresses, including the postharvest storage of fruit at chilling temperatures (Wang and Ji, 1989; Bleecker and Kende, 2000). Though the role of stress induced ethylene under low temperature in PCI and fruit deterioration has been studied previously, the results obtained are not conclusive. In tomato, overexpression of ethylene response factors (ERFs) improves chilling tolerance (Zhang and Huang, 2010), and the blocking of ethylene biosynthesis or signaling by 1-methylcyclopropene (1-MCP) decreases cold tolerance (Zhao et al., 2009), suggesting that ethylene is an inducer of cold tolerance. More recently, an RNA-Seq analysis in tomato fruit has indicated that induced tolerance to CI in tomato fruit seems to be related to the restoration of ethylene biosynthesis and signaling (Cruz-Mendívil et al., 2015). Reports on banana (Jiang et al., 2004) and nectarine fruit (Dong et al., 2001) also indicate that 1-MCP reduces cold tolerance. Nevertheless in many fruits 1-MCP successfully prevents deterioration of fruit quality by delaying ripening and senescence, but also by inducing cold tolerance, as has been reported for persimmon, loquat and, plum among others (Salvador et al., 2004; Cai et al., 2006; Wang et al., 2010; Singh and Singh, 2012).

The implication of ethylene biosynthesis and signaling in PCI and postharvest deterioration of immature non-climacteric fruits of zucchini or cucumber during their storage at chilling temperatures is unknown. In this paper we have determined the effect of 1-MCP on the evolution of different fruit quality parameters during postharvest storage of the fruit of nine cultivars of zucchini at 20 °C and 4 °C. The production of ethylene and CO_2, and the expression of twelve genes involved in the biosynthesis, perception and signaling of ethylene was also monitored during the postharvest storage of fruits. Results indicate that 1-MCP is able to reduce certain fruit deterioration processes in only some of the cultivars analyzed, and that the improvement in fruit quality due to 1-MCP is associated with a reduction of the fruit respiration rate, a reduction of ethylene biosynthesis mediated by a downregulation of *CpACS1* and *CpACO1*, and a reduction in the expression of ethylene perception and signaling genes. Ethylene is therefore a negative regulator of cold tolerance in zucchini squash.

2. Material and methods

2.1. Plant material and experimental design

Eight commercial hybrids (Sinatra, Janto, Natura, Victoria, Cronos, Celeste, Blas and Alexander) and one traditional Spanish cultivar (Muc-16) were used to evaluate the effects of 1-MCP on zucchini postharvest storage. Plants from the different cultivars were grown in the same field trial under standard greenhouse conditions in Almería, Spain. Fruits of uniform length (18–20 cm) were harvested in the middle of the productive period in order to minimizing the effects of environmental conditions.

The fruit of each cultivar was randomly divided in 4 batchs. Two of the batchs were treated with 2.4 ml L^{-1} of 1-MCP at 12 °C for 48 h, and the other two treated with air under the same conditions and used as control. This concentration was selected from a previous assay in which the cultivar Sinatra was treated with 0.6, 1.2 and 2.4 mL L^{-1} of 1-MCP (data not shown). After the treatment, one treated and one untreated batch from each cultivar were stored for a total of 14 days in controlled chambers at either 4 °C and 75–80% relative humidity (RH), or 20 °C and 75–80% RH. At 7 and 14 storage days, fruit was transferred to room temperature (RT) and all parameters were evaluated and samples processed after 6 h of acclimation at 20 °C. A total of 12 fruits were analyzed per treatment, storage temperature and cultivar. For ethylene and CO_2 production measurements and for gene expression analyses the 12 fruits were combined in 4 replications of 3 fruit each. However, PCI, weight loss, firmness and colour were determined in all 12 fruits. For gene expression analysis, 3 replicate samples were taken from the exocarp of 3–4 fruits, frozen in liquid nitrogen and stored at −80 °C until use.

We had already observed that ethylene is induced in the refrigerated fruit of zucchini upon transferring the fruit to RT. To ascertain whether ethylene is also produced in the cold chamber before rewarming, we performed an experiment with the fruit of four cultivars. Fruit from each cultivar was stored for 7 and 14 days at 4 °C. After the storage period, fruit was stored in hermetic containers for 6 h for the accumulation of ethylene, but half of the fruit was kept inthecoldchamberat 4 °C andhalfwasmovedtoanotherchamberat 20 °C. Three replications of 4 fruits each were used for each ethylene accumulation method, storage temperature and cultivar.

2.2. Ethylene and CO_2 production

Ethylene and CO_2 production was determined at 0, 7 and 14 days of storage. Twelve fruits were analyzed for each time, temperature and treatment, i.e. 4 replicates of 3 fruits each. Once removed from storage chambers, fruit was enclosed in sealed 10-L containers for 6 h at 4 °C or 20 °C, depending on the experiment. After this incubation period, gas samples were taken and ethylene content was determined three times by gas chromatography in a Varian 3900 GC fitted with a flame ionization detector (FID). In the same way, CO_2 was measured three times with headspace analyzers Check mate II (Dansensor). Ethylene production and respiration rate were expressed as nL g^{-1} 6 h^{-1} and mg kg^{-1} 6 h^{-1}, respectively.

2.3. Evaluation of weight loss, firmness, chilling injury and color

The percentage of weight loss during storage was assessed by weighing 12 individual fruits at 0, 7 and 14 days after harvesting for each treatment and cultivar. The percentage of weight loss of each fruit was calculated according to the following equation:

$$\%\text{Weight loss} = \frac{W_i - W_f}{W_i} \times 100$$

where W_i and W_f are the initial and final fruit weights, respectively.

Fruit firmness was determined in 12 fruits for each treatment, conservation time and cultivar, by using a Stable Micro Systems Texture Analyzer TA.XT-Plus. A 4 mm-diameter probe was used and penetration was conducted at a speed of 1 mm/s to a depth of 10 mm. Firmness was measured three times in a transversal section in the distal region of the fruit, as this part softens faster during postharvest storage.

To assess PCI we have evaluated the surface of the fruit affected by pitting and the severity of pitting symptoms. The fruit surface affected by pitting was used to classify each fruit according to the follow scale: 0 = no damage, 1 ≥ 5% damage, 2 = 6–15% damage, 3 = 16–25% damage, 4 = 26–50% damage, and 5 ≥ 50% damage (Megías et al., 2014). To assess the severity of pitting symptoms severity, the scale was 0 = no damage, 1 = very superficial damage, 2 = superficial damage, 3 = moderate damage, 4 = severe damage, 5 = very severe damage. These two parameters were evaluated in 12 fruits per treatment, storage time and cultivar at 0, 7 and 14 days of storage. The final PCI index was the average of both parameters.

Fruit color was assessed by the parameters L^*, a^* and b^*, determined individually for each fruit using the CIE Lab System of a "Konica-Minolta CR-410" colorimeter. Three determinations were performed on opposite sides of each fruit. The Hue angle index (arctan = (b^*/a^*) and Chroma $(a^{*2} + b^{*2})^{1/2}$ were also calculated. Results are the mean SE of 12 fruits per treatment, storage time and cultivar.

2.4. Gene expression analysis by quantitative RT-PCR

Gene expression analysis was performed in three replicated samples per treatment, storage time and cultivar. Each replication was the result of an independent extraction of total RNA from 3 different fruits. Samples were obtained from fruits stored at different storage temperatures and then rewarmed for 6 h at RT (20 °C). For each sample, portions of exocarp of 3–4 fruits were homogenized together, frozen in liquid nitrogen, and stored at —80 °C. Total RNA was extracted from each sample according to Total RNA Mini kit (Bio-Rad). The remains of DNA in RNA samples were eliminated by digestion with RQ1 RNAse free DNAse (Promega). Before cDNA synthesis, we verified the absence of DNA in RNA and cDNA samples by PCR amplification with primers for *CpACS4*. cDNA was synthesized from 1 mg of total RNA using iScript reverse Transcription Supermix for RT-qPCR (Bio-Rad). The expression of genes was evaluated through quantitative RT-PCR by using the Rotorgene thermocycler (Qiagen) and iTaq Universal SyBR Green Supermix (Bio-Rad). Table S1 shows the different corresponding primers used for q-PCR. The q-PCR primers were designed from the 3' non-coding regions of each gene by using the *Primer Express v 2.0*(Applied Biosystem) software. To avoid possible cross-amplification, and before any q-PCR experiment, the size of the PCR products for each pair of primers was tested in agarose gels. Relative expression of each gene was determined by the comparative Ct (*Cycle Threshold*) method using *C. pepo Elongation Factor 1*-a (EF-1A) and *ACTIN*(*ACT*) genes as internal standards. To use this method, we first demonstrated that the efficiency of amplification for each amplicon was roughly equivalent, regardless of the amount of template cDNA. The absolute value of the slope of DCt (Ct of the target gene — Ct of the reference gene) versus serial dilutions of cDNA for a given sample must be less than 0.1. The relative expression of each gene to a calibrator sample was calculated using the formula 2^{-DDCt}, where DDCt is the difference between the DCt of each sample and the DCt of the calibrator sample.

2.5. Statistical analysis

Data were treated for multiple comparisons by analysis of variance (ANOVA), followed by least significant difference test (LSD) with significance level $p \leq 0.05$. Sources of variation were storage time and treatment for each cultivar. ANOVA was

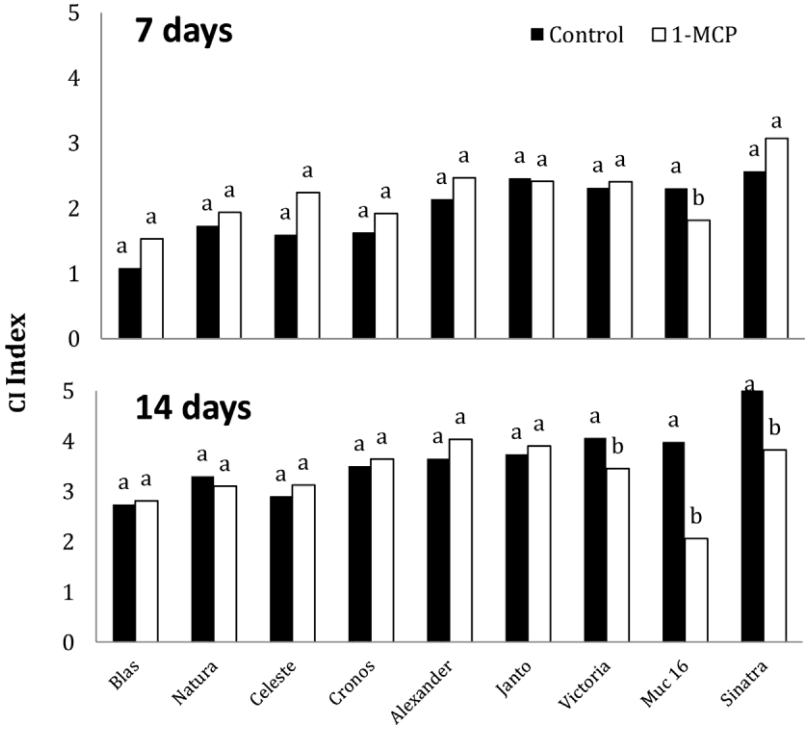

Fig. 1. Comparison of postharvest chilling injury (PCI) in Control fruit with that of 1-MCP treated fruit of nine cultivars of zucchini after 7 and 14 days of storage at 4 °C. The results represent the mean of three independent replicates for each sample. The different lower-case letters indicate significant differences according to the treatments for each storage time and each cultivar (p-value < 0.05).

performed using the statistical software Statgraphic Centurion XVI (STATGRAPHICS. Statpoint Technologies, Inc., Warrenton, VA). Normality of distribution was verified using Kolmogorov–Smirnov test, when the assumption of normality failed, the variables were transformed. Weather transformation was not possible non-parametric Kruskal–Wallis test was used to compare differences between groups, with significance level at <0.05.

3. Results

3.1. Effect of 1-MCP on the postharvest quality of zucchini fruit stored at two temperatures

Figs.1 and 2 show the effects of storage temperatures (20 °C and 4 °C) and 1-MCP treatment on PCI and weight loss of zucchini fruit from nine cultivars after 7 and 14 days of storage. Cold storage induced the onset of PCI in the fruit surface in the form of pitting and skin dimpling, with the fruit of some cultivars (Victoria, Sinatra and Muc-16) more severely affected by PCI than the others (Fig. 1). The effect of 1-MCP on PCI depended on the particular cultivar. The treatment was effective in reducing PCI in the most severely affected fruits: Victoria, Muc-16 and Sinatra, but had no effect on the fruit of the other cultivars (Fig. 1).

The percentage of fruit weight loss was correlated with PCI, suggesting that this postharvest parameter is a good indicator of chilling damage in zucchini. The fruit of Victoria, Muc-16 and Sinatra, which were the fruits most severely affected by PCI, lost the most weight during postharvest (Fig. 2). After 7 and 14 days of storage, the refrigerated fruit lost significantly more weight than the fruit stored at 20 °C, consistent with the fruit of all nine cultivars (Fig. 2). The percentage of fruit weight loss was not altered by 1-MCP in Blas, Natura Cronos and Alexander, but it was reduced significantly in the fruit of Victoria, Muc-16 and Sinatra (Fig. 2). which were the most severely affected by cold damage.

The loss of fruit firmness also varied between cultivars but was not dependent on storage temperature, neither was it correlated with PCI and weight loss (Table S2). In some cultivars, we have even found a slight increase in fruit firmness during storage at both 20 °C and 4 °C. The effect of 1-MCP on fruit firmness was negligible in the fruit of most of the cultivars after 14 days of storage (Table S2). At 4 °C the treatment had no effect on fruit firmness, and at 20 °C 1-MCP only reduced firmness loss in the fruit of Blas, Sinatra and Victoria (Table S2).

Regarding color parameters, no differences were detected in the zucchini fruit of the different cultivars in response to cold storage or 1-MCP treatment (Table S3).

3.2. Effect of 1-MCP on ethylene production in zucchini fruit stored at two temperatures

Zucchini is an immature fruit that produces little ethylene at harvest and during its postharvest storage at non-chilling temperatures (Fig. 3). However, storing the fruit at chilling temperatures (4 °C) induces a burst of ethylene which peaks at 7 days of cold storage, but which falls to its original low level after 14 days of cold storage (Fig. 3). We have previously demonstrated that this cold-induced ethylene is mainly produced once the fruit is removed from the refrigerated chambers and is rewarmed at RT for a few hours (Megías et al., 2014). The data in Fig. 3 are from fruit that were stored for 7 and 14 days in controlled chambers at 4 °C and 20 °C, and then maintained at 20 °C for 6 h to accumulate ethylene. As indicated by Megías et al. (2014), we have observed a high inter-cultivar variability for this cold-induced ethylene production. In the refrigerated fruit of those cultivars that were severely affected by cold damage (Victoria, Muc-16 and Sinatra), ethylene production was more highly induced at 7 days of storage (Fig. 3). Therefore, ethylene production in fruit stored at 4 °C seems to be proportional to PCI and weight loss (Fig. 3).

The treatment with 1-MCP promoted a reduction of ethylene production in all the cultivars (Fig. 3). In the fruit stored at 20 °C this reduction was almost inappreciable, but in the fruit stored at 4 °C there was a significant reduction in ethylene production at both 7 and 14 days of storage (Fig. 3). In the 1-MCP treated fruit of certain cultivars, including Natura, Janto, Cronos, Muc-16 and

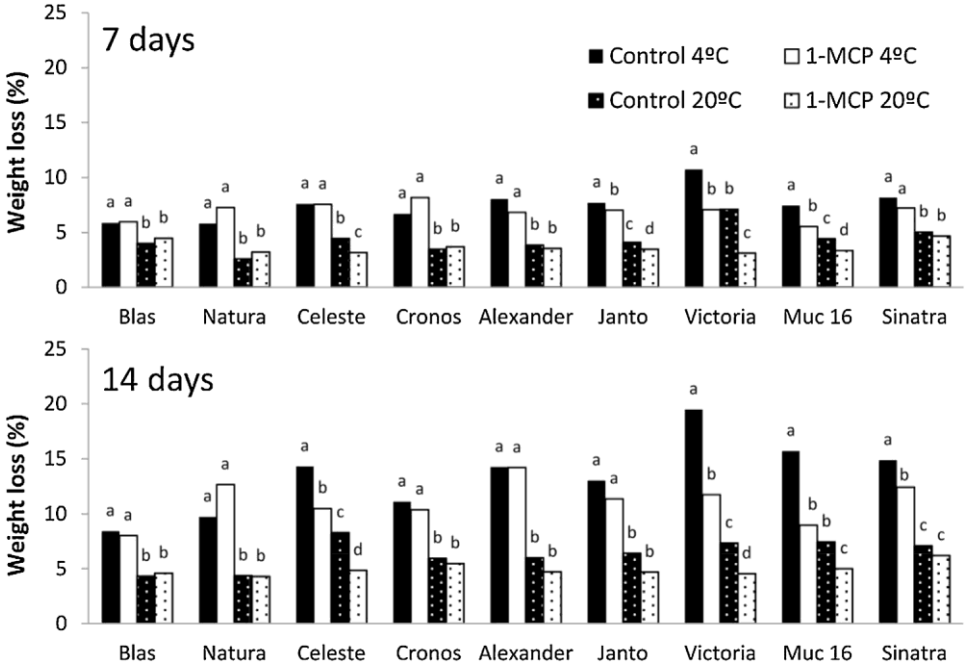

Fig. 2. Comparison of weight loss in control and 1-MCP treated fruit of nine cultivars of zucchini after 7 and 14 days of storage at 20 °C and 4 °C. The results represent the mean of three independent replicates for each sample. The different lower-case letters indicate significant differences between treatments for each storage time and cultivar (p-value < 0.05).

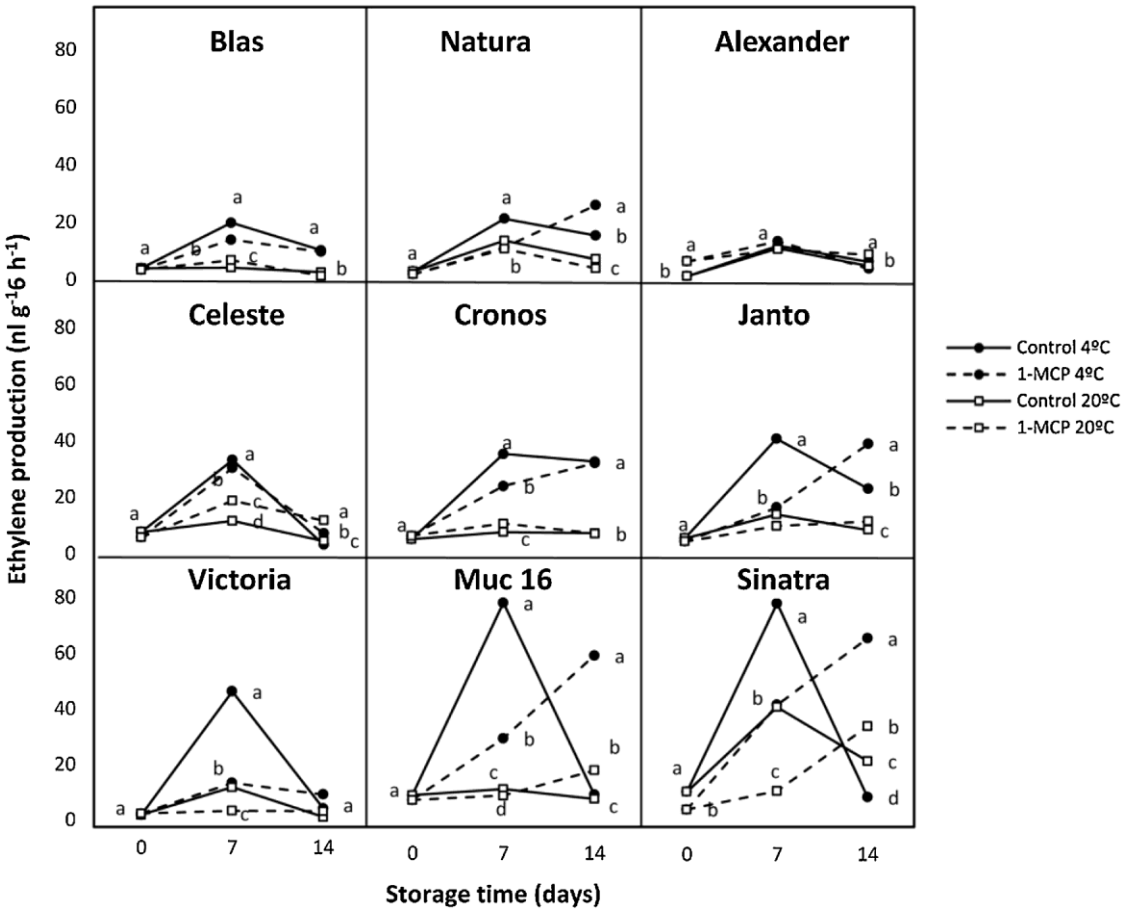

Fig. 3. Comparison of ethylene production in control and 1-MCP treated fruit of nine cultivars of zucchini stored at 20 °C and 4 °C for 0, 7 and 14 days. For ethylene accumulation, after storage at each temperature, the fruit was incubated for 6 h in hermetic containers at room temperature (20 °C). The results represent the mean of three independent replicates for each sample. The different lower-case letters indicate differences between treatments at each storage time for each cultivar (p-value < 0.05).

Sinatra, cold-induced ethylene was not reduced at 14 days of storage, as occurred in the refrigerated control fruit, but continued increasing at 14 days of storage (Fig. 3). These data suggest that 1-MCP also delayed the ethylene production peak in the fruit of certain cultivars until over 14 days of cold storage (Fig. 3).

The reduction and delay in the production of chilling-induced ethylene could explain the beneficial effect of 1-MCP in

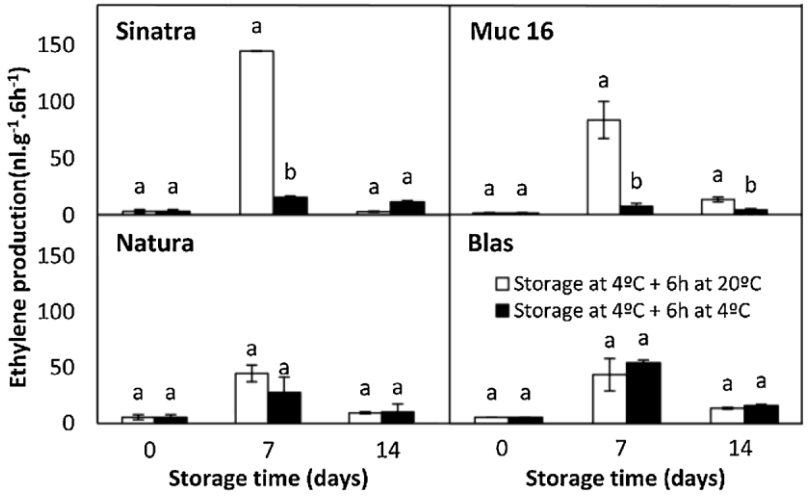

Fig. 4. Ethylene production in the fruit of four cultivars of zucchini. All the fruit was stored for 0, 7 and 14 days at 4 °C, and then stored for 6 additional hours at 4 °C or 20 °C for ethylene accumulation. The results represent the mean of three independent replicates for each sample. The different lower-case letters indicate differences between the two forms of accumulating ethylene (at 4 °C or 20 °C for 6 h) for each storage time and cultivar (p-value < 0.05).

alleviating chilling injury and weight loss of the fruit during postharvest storage at 4 °C. When cold-induced ethylene was measured after rewarming the refrigerated fruit at RT for a few hours, PCI symptoms were already visible in the refrigerated chambers. It is therefore likely that 1-MCP performs its action in the storage chambers, inhibiting the action of the basal ethylene in fruit stored at either 4 °C or 20 °C. To test this hypothesis we have compared the production of ethylene in the fruit of 4 cultivars, two with a low production of cold-induced ethylene and cold damage that did not respond to 1-MCP (Blas and Natura), and two with a high production of cold-induced ethylene and cold damage that responded to 1-MCP treatment (Muc-16 and Sinatra). The fruit was refrigerated for 7 and 14 days at 4 °C. In some fruit ethylene production was measured in the chamber at 4 °C for 6 additional hours, and in others by taking the fruit out of the cold chambers and rewarming it at 20 °C for 6 h before ethylene measurements. Results are shown in Fig. 4. At the storage temperature of 4 °C the fruit of all cultivars has a basal ethylene production that is slightly induced at 7 days of cold storage. In the more chilling sensitive cultivars Sinatra and Muc-16, the rewarming of the fruit at RT (20 °C) triggers ethylene production from 16-20 to more than 100 ng g^{-1} FW 6 h^{-1} (Fig. 4). It seems that cold storage is able to accumulate transcripts or enzymes involved in ethylene biosynthesis which are activated once the fruit is taken out from the cold storage chambers. However, in Natura and Blas, ethylene production remains very low, even after rewarming the fruit (Fig. 4).

3.3. Effect of 1-MCP on respiration rate of zucchini fruit stored at two temperatures

During storage at 20 °C, the respiration rate (RR) of the control fruit of the different cultivars fell progressively from around 60 ml CO_2 g^{-1} 6 h^{-1}at harvest to about 20 ml CO_2 g^{-1} 6 h^{-1} at 14 days of storage (Fig. 5). However, at 4 °C this reduction was not so effective, since the RR of refrigerated fruit was higher than that of fruit stored at 20 °C (Fig. 5).

1-MCP treatment reduced the RR in the fruit of all cultivars when stored at both 4 °C and 20 °C (Fig. 5). This reduction was particularly significant in those zucchini cultivars that were more susceptible to PCI damage, as they produced more CO_2 during cold storage at 4 °C (Fig. 5). Therefore, the reduction in the RR of zucchini fruit has always been associated with a reduction in PCI symptoms and better postharvest performance in response to 1-MCP treatment and in concordance with the utilized cultivar.

3.4. Transcriptional regulation of ethylene biosynthesis and signaling genes in response to cold storage and 1-MCP

To study the molecular mechanisms involved in ethylene biosynthesis and signaling in response to cold and 1-MCP treatments, we have assessed the expression profile of twelve ethylene genes in zucchini fruit, seven involved in biosynthesis, and five in the ethylene signaling pathway. For this purpose the cultivars Muc-16 and Victoria were selected because their fruits

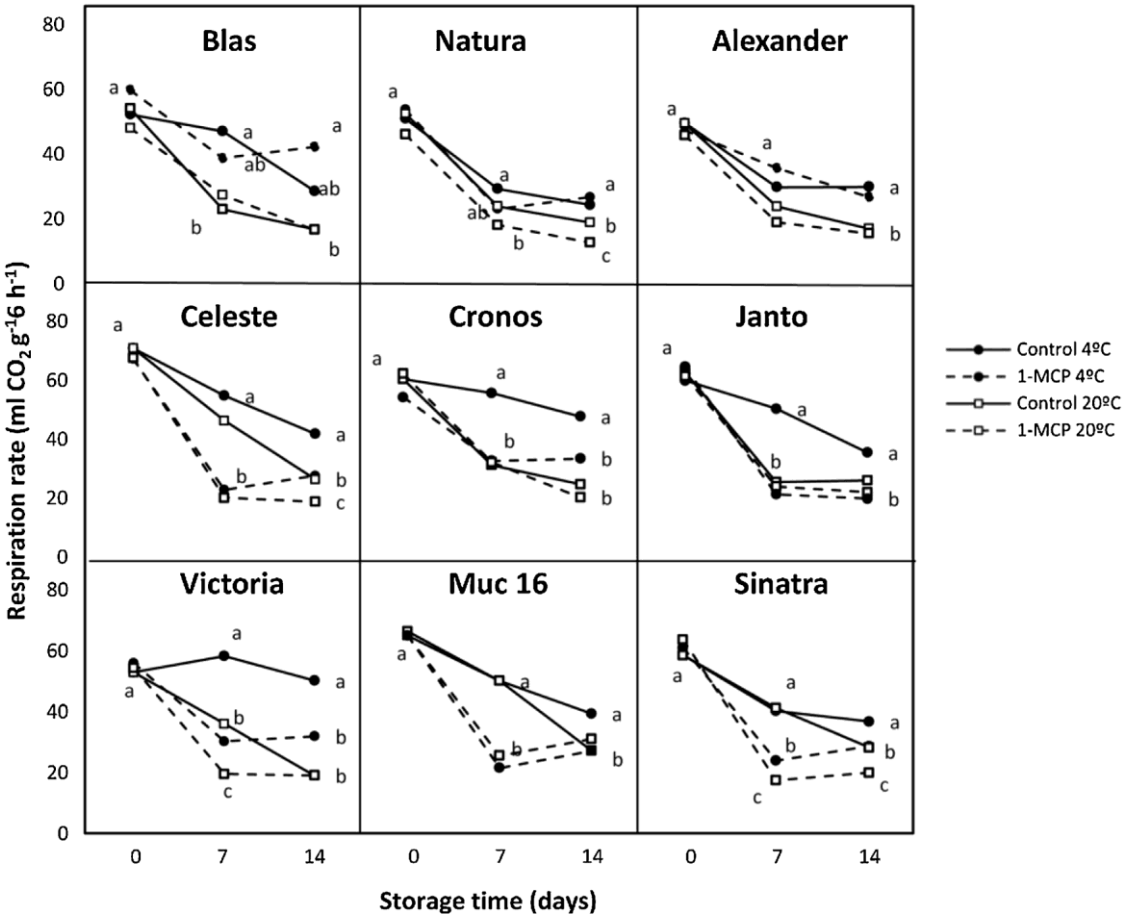

Fig. 5. Evolution of CO_2 production in control and 1MCP-treated fruit of nine cultivars of zucchini stored for 0, 7 and 14 days at 20 °C or 4 °C, and then rewarmed at 20 °C for 6 h before measurements. The results represent the mean of three independent replicates for each sample. The different lower-case letters indicate the differences between treatments for each storage time and cultivar (p-value < 0.05).

were highly susceptible to cold storage and ethylene was highly induced in response to cold storage; both responded to 1-MCP treatment thereby reducing PCI severity.

Fig. 6 shows the expression patterns of ethylene biosynthesis genes in the fruit of Muc-16 and Victoria throughout the storage period of 14 days at both 20 °C and 4 °C. Two of the analyzed genes (*CpACS2* and *CpACS7*) were not detected under the conditions of the study (data not shown). The genes C*pACS4* and *CpACS6* barely showed differences between fruits stored at 20 °C and 4 °C (Fig. 6). However, storage at 4 °C promoted *CpACO1* and *CpACS1* expression in both cultivars, suggesting that they are both involved in the production of cold-induced ethylene in zucchini (Fig. 6). *CpACS5* was the only gene that was clearly induced at 20 °C in the fruit of both cultivars (Fig. 6). Given that this induction of *CpACS5* at 20 °C was not correlated with a real production of ethylene, it is likely that this induction does not occur concomitantly with an upregulation of *ACO* genes in the fruit. In fact the expression of *CpACO1* was very low at 20 °C and was drastically induced by cold storage in both cultivars analyzed (Fig. 6).

1-MCP did not alter the expression of *CpACS1*, *CpACS4*, *CpACS5* and *CpACS6* (Fig. 6), however the expression of *CpACO1* was significantly reduced in the refrigerated fruit of both Muc-16 and Victoria (Fig. 6), indicating that ethylene biosynthesis during the storage of zucchini fruit at chilling temperatures, and accordingly its response to 1-MCP, is probably regulated by *ACO.*

The expression profiles of two ethylene perception (*CpETR1* and *CpERS1*) and three ethylene signaling (*CpCTR1*, *CpEIN3.1* and *CpEIN3.1*) genes during zucchini fruit postharvest is shown in Fig. 7. *CpCTR1* and *CpEIN3.2*, were upregulated by chilling in both cultivars, although in Muc-16 *CpETR1* and *CpEIN3.2* were also slightly induced by chilling, suggesting that cold-induced ethylene production in zucchini is accompanied by an induction of the ethylene perception and signaling machinery.

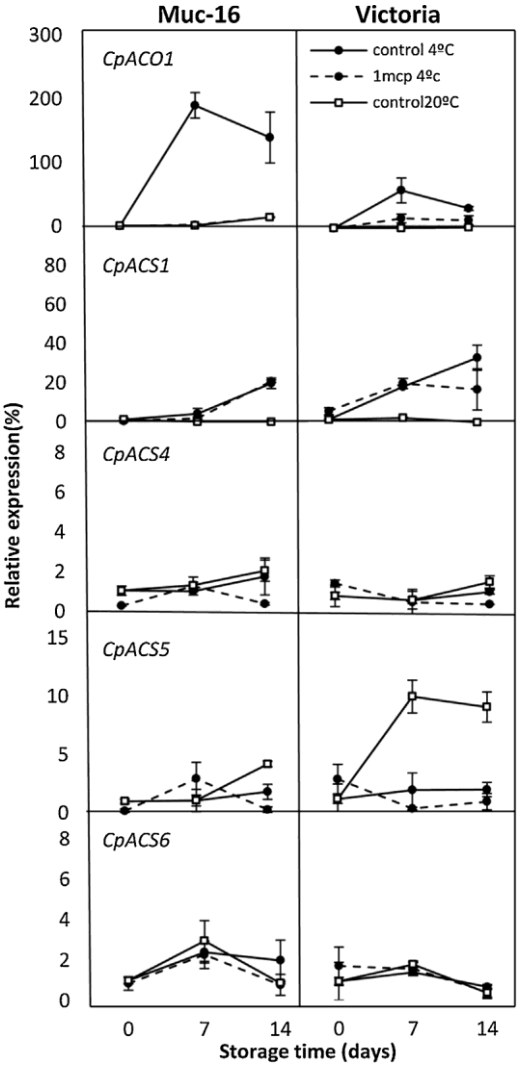

Fig. 6. Expression profiles of ethylene biosynthesis genes in control and 1-MCP treated fruit of Muc-16 and Victoria. Control fruit of the two cultivars was stored for 0, 7 and 14 days at 4 °C and 20 °C, and then rewarmed at 20 °C for 6 h before collecting the exocarp material that was used for gene expression analyses. The 1-MCP treated fruit was only stored at the chilling temperature of 4 °C. Transcript levels for each gene were assessed by quantitative RT-PCR. Data were standardized with the expression at harvest. Results represent the mean and standard deviation of three independent replicate samples for each cultivar, treatment and storage time.

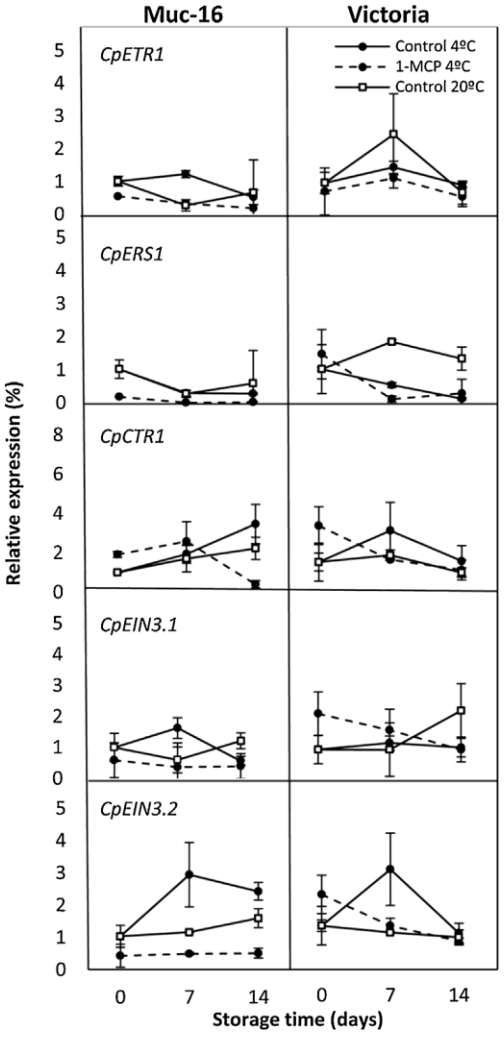

Fig. 7. Expression profiles of ethylene perception and signaling genes in control and 1-MCP treated fruit of Muc-16 and Victoria. Control fruit of the two cultivars was stored for 0, 7 and 14 days at 4 °C and 20 °C and then rewarmed at 20 °C for 6 h before collecting the fruit exocarp for gene expression analyses. The 1-MCP treated fruit was only stored at 4 °C. Transcript levels for each gene were assessed by quantitative RT-PCR. Data were standardized with the expression at harvest. Results represent the mean and standard deviation of three independent replicate samples for each cultivar, treatment and storage time.

The 1-MCP treatment did not considerably alter the expression of ethylene perception and signaling genes, although most of them were downregulated in response to the treatment (*CpETR1, CpCTR1, CpEIN3.1* and *CpEIN3.2*), especially in the cultivar Muc-16 (Fig. 7). Therefore, the positive effect of 1-MCP on zucchini quality during chilling storage was not only associated with a downregulation of ethylene biosynthesis, but also with an inhibition of genes involved in ethylene perception and signal transduction pathway.

4. Discussion

The postharvest storage of zucchini fruit from nine cultivars at the chilling temperature of 4 °C produced a very characteristic symptomatology known as postharvest chilling injury (PCI), including the occurrence of pitting in fruit skin and increased weight loss in comparison with fruit stored at 20 °C. The involvement of ethylene in the development of PCI was analyzed in this paper by comparing ethylene production and the expression of ethylene biosynthesis and signaling genes during postharvest fruit storage at 4 °C and 20 °C, and by determining the effects of 1-MCP on fruit quality, ethylene production, and gene expression profiles.

4.1. The production of ethylene and the expression of ethylene genes in response to cold storage and 1-MCP treatment

We have previously demonstrated that storage of zucchini fruit at chilling temperatures induces a burst of ethylene in the fruit once it is rewarmed for a few hours at RT (Megías et al., 2014). The induction of this ethylene depends on chilling storage time, peaking at 7 days of cold storage. After the seventh day the ethylene biosynthesis machinery must be damaged since the production of ethylene is progressively reduced to its initial low value at 14 days of cold storage (Megías et al., 2014). The level of chilling-induced ethylene at 7 days is proportional to the level of cold damage (PCI and weight loss), with a higher production of cold-induced ethylene in the fruit of those cultivars that were the most severely affected by cold damage (Fig. 3). We have previously argued that this chilling-induced ethylene is not responsible for the occurrence of PCI since PCI symptoms are visible in the cold chamber before fruit rewarming and ethylene burst induction (Megías et al., 2014). Differences in ethylene production between cultivars upon rewarming may be the consequence of PCI, resulting in increased production of ethylene in the fruit of those cultivars that were the most severely affected by PCI (Victoria, Sinatra and Muc-16).

The induction of ethylene production during chilling storage has been demonstrated in different climacteric and non-climacteric fruit, including cucumber, kiwi, citrus and pear, among others (Wang and Adams, 1982; Hyodo and Fukasawa, 1985; McCollum and Mcdonald, 1991; Lafuente et al., 2001). The induction of ethylene production is always associated with an accumulation of transcripts for the ethylene biosynthesis enzymes ACS or ACO (Lelievre et al., 1997; Zacarías et al., 2003; Maul et al., 2011; Lado et al., 2015). In tomato, *ACS* genes are upregulated in response to cold storage, but two *ACOs* are downregulated in a chilling-susceptible genotype and two *ACOs* are upregulated in a chilling-tolerant genotype, indicating that *ACO* is the key step controlling ethylene biosynthesis in tomato fruit during cold storage (Cruz-Mendívil et al., 2015). In zucchini, the only ethylene biosynthesis genes that were found to be induced by cold storage were *CpACS1* and *CpACO1*, but the *CpACO1* regulatory function appears to play a key role in chilling induced ethylene production, since *CpACO1* was induced by cold in the two analyzed cultivars Muc-16 and Victoria (Fig. 6). Thus, the accumulation of ACO protein appears to be responsible for the production of cold-induced ethylene after rewarming. The downregulation of *CpACO1* and ethylene in response to 1-MCP treatment also supports this conclusion. *CpACS1*, and three additional *ACS* genes analyzed in this paper, did not change their expression profile in response to 1-MCP, which indicates that 1-MCP controls ethylene biosynthesis by modulating *ACO* rather than by modulating the expression of *ACS* in zucchini squash. As also proposed by Lado et al., (2015) in grapefruit, the correlation between PCI symptoms and *CpACO1* expression suggests that this gene expression can be used as a PCI marker, and that ACO induction during postharvest storage of fruits is related to PCI rather than to cold storage.

The induction of ethylene biosynthesis after rewarming was also accompanied by the upregulation of genes involved in the ethylene perception and signaling pathway, including *CpETR1*, *CpCTR1* and *CpEIN3.2*. An increase in expression patterns of ethylene signal transduction elements in response to low temperature has been reported in pear (El-Sharkawy et al., 2003), peach (Begheldo et al., 2008), kiwifruit (Yin et al., 2009), and loquat (Wang et al., 2010), among others. In pear and peach, the induction of these ethylene signaling genes is associated with fruit ripening. This induction of ethylene receptors and signaling genes was concomitant with PCI in more cold susceptible cultivars of loquat (Wang et al., 2010). Ethylene signal transduction elements are known to be upregulated by ethylene in zucchini (Manzano et al., 2013; Martínez et al., 2013) and other species (Costa et al., 2010), suggesting that the upregulation of ethylene perception and signaling genes could be the consequence of the burst of ethylene production induced upon rewarming in the Muc-16 fruit. The downregulation of the ethylene receptor and signal transduction genes *CpETR1*, *CpCTR1*, *CpEIN3*.1 and *CpEIN3.2* in 1-MCP treated fruit also indicates that these genes are regulated by ethylene. Ethylene receptor and signaling genes are known to be induced upon ripening and are regulated by ethylene treatment, being repressed by 1-MCP treatment in other fruits, although some of them were found to be constant (Leclercq et al., 2002; El-Sharkawy et al., 2003; Adams-Phillips et al., 2004; Dal Cin et al., 2006; Kevany et al., 2007; Wiersma et al., 2007; Tatsuki et al., 2009; Yang et al., 2013). Our results regarding the expression of ethylene receptors and signaling genes are in accordance with ethylene production, which is both reduced and delayed in response to 1-MCP in many of the cultivars analyzed (Fig. 3). This delay in ethylene production may be caused by the lack of response to ethylene, due to 1-MCP binding on the receptors. Since ethylene receptors and CTRs are considered negative regulators of ethylene (Tieman et al., 2000), the transcriptional repression in 1-MCP treated fruit would be expected to confer high ethylene sensitivity. The levels of ethylene receptors and CTR-like proteins, however, are not necessarily correlated with gene expression. Yang et al. (2013) has also proposed that the binding of receptors with 1-MCP may decrease receptor protein degradation, which is normally triggered by ethylene binding, and therefore decrease the sensitivity of fruit to ethylene signaling.

4.2. 1-MCP enhances the postharvest fruit quality of certain zucchini cultivars under cold storage, whilst preventing ethylene biosynthesis and respiration

1-MCP is widely used as a postharvest treatment to enhance the quality of refrigerated fruit in a number of species. It has been reported that the inhibition of ethylene signaling by 1-MCP reduces PCI severity in avocado (Pesis et al., 2002), persimmon (Salvador et al., 2004), pineapple (Selvarajah et al., 2001), loquat (Cai et al., 2006), melon (Ben-Amor et al., 1999) and plum (Candan et al., 2008), suggesting that ethylene is a negative regulator of cold tolerance. In other species, however, including apricot (Dong et al., 2002), banana (Jian et al., 2004), nectarine (Dong et al., 2001), and

tomato (Zhang and Huang, 2010; Biswas et al., 2014), ethylene seems to be an inductor of chilling tolerance since 1-MCP reduces the quality of refrigerated fruit. In zucchini, the effects of 1-MCP were highly dependent on the cultivar considered, but had no negative effect on fruit quality. In the most chilling-susceptible cultivars Muc-16, Sinatra and Victoria, 1-MCP reduced the visible symptoms of PCI on the fruit surface and diminished fruit weight loss, although these quality parameters were not altered by the treatment in the remaining cultivars (Figs. 1 and 2).

The higher quality maintenance of 1-MCP treated zucchini refrigerated fruit was found to be associated with a decline in fruit respiration rate in all the cultivars analyzed, a physiological parameter that was reported to be correlated with PCI in zucchini (Balandran-Quintana et al., 2003; Zheng et al., 2008) as well as in other fruits such as plum (Zapata et al., 2014; Khan et al., 2011), kiwifruit (Antunes and Sfakiotakis, 2002) and cucumber (Hakim et al., 1999). This was particularly true in the fruit of the most chilling susceptible zucchini cultivars (Fig. 5), which were those whose fruit quality responded better to 1-MCP treatment. The combination of 1-MCP and cold storage was also able to decrease the respiration rate of the mature fruit of *Cucurbita maxima*, concomitantly with a prevention of fruit quality devaluation (Massolo et al., 2013), indicating that a reduction in the respiration rate is related to a delay in the onset of fruit senescence in mature and immature fruit of *Cucurbita*.

Since 1-MCP was able to reduce PCI symptoms and fruit weight loss in the most chilling-susceptible zucchini cultivars, concomitantly with a reduction in ethylene production and ethylene biosynthesis and signaling genes, it is likely that ethylene is not only the consequence of PCI in the fruit but could also play a role in the onset of PCI in this non-climacteric fruit. Ethylene would therefore be a negative regulator of cold tolerance in zucchini, which has also recently been demonstrated regarding cold and freezing tolerance in *Arabidopsis* (Shi et al., 2012) and other species. In accordance with this conclusion we have observed that ethylene production is not only strongly induced after fruit rewarming in the most chilling sensitive cultivars Muc-16 and Sinatra, but is already induced in the refrigerated chamber (Fig. 4), when PCI symptoms occur. This ethylene production under cold stress (before rewarming) could be responsible for the onset of PCI symptoms in zucchini. However, since this ethylene production was lower in the more chilling-susceptible cultivars (Sinatra and Muc-16), which were the only ones that responded to 1-MCP, it is likely that the cultivar-dependent response to 1-MCP is more probably to be due to differences in ethylene sensitivity in each of the cultivars. Differences in ethylene sensitivity have been detected among zucchini cultivars (Manzano et al., 2013); but whether those differences are maintained after development of the fruit, would require further analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2015.11.001.

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