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Novel insights of ethylene role in strawberry cell wall metabolism

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ABSTRACT

Due to its organoleptic and nutraceutical qualities, strawberry fruit (*Fragaria x ananassa*, Duch) is a worldwide important commodity. The role of ethylene in the regulation of strawberry cell wall metabolism was studied in fruit from Toyonoka cultivar harvested at white stage, when most changes associated with fruit ripening have begun. Fruit were treated with ethephon, an ethylene-releasing reagent, or with 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene action, maintaining a set of non-treated fruit as controls for each condition. Ethephon treated-fruit showed higher contents of hemicelluloses, cellulose and neutral sugars regarding controls, while 1-MCP-treated fruit showed a lower amount of those fractions. On the other hand, ethephon-treated fruit presented a lower quantity of galacturonic acid from ionically and covalently bound pectins regarding controls, while 1-MCP-treated fruit showed higher contents of those components. We also explored the ethylene effect over the mRNA accumulation of genes related to pectins and hemicelluloses metabolism, and a relationship between gene expression patterns and cell wall polysaccharides contents was shown. Moreover, we detected that strawberry necrotrophic pathogens growth more easily on plates containing cell walls from ethephon-treated fruit regarding controls, while a lower growth rate was observed when cell walls from 1-MCP treated fruit were used as the only carbon source, suggesting an effect of ethylene on cell wall structure. Around 60% of strawberry cell wall is made up of pectins, which in turns is 70% made by homogalacturonans. Our findings support the idea of a central role for pectins on strawberry fruit softening and a participation of ethylene in the regulation of this process.

1. Introduction

Primary plant cell wall is a complex and dynamic structure mainly formed by a cellulose-hemicellulose network embedded into a hydrophilic matrix of pectins, with a minor proportion comprised by structural and polysaccharide-modifying proteins (PMPs) [1,2]. Pectins are also the major component of middle lamella, the mediator of neighbor cells connection [3]. Furthermore, maintenance of cell adhesion is important to protect plant tissues integrity, but some developmental processes as dehiscence, organ abscission, pollen release and fruit ripening require cell separation, which in turn needs modifications of cell wall and middle lamella components. In that sense, it has been proved that the cooperative action of many PMPs is required for cell wall and middle lamella disassembly, being that process crucial to lead fleshy fruits softening [4,5]. Although changes in flesh fruit texture is required for both seeds spread and consumer's acceptance, an extensive postharvest softening drives to a higher susceptibility to pathogens attack, difficulties on shipping and a shortening of fruit shelf life. In this context, the effects of plant growth regulators on fleshy fruits ripening and cell wall disassembly have become a focus of considerable scientific attention [6–8].

Fruits are classified accordingly to the presence (climacteric) or absence (non-climacteric) of an increment on respiratory activity and a peak of ethylene biosynthesis during ripening [9]. The relationship between ethylene production and perception, and gene expression and accumulation of PMPs, has been studied both in

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climacteric and non-climacteric fruits by many research groups [8,10].

In strawberry, it is well known that auxins produced by achenes are essential for receptacle growth and expansion, but also these hormones constitute the key point to delay strawberry fruit ripening [11]. The expression of many genes encoding proteins involved in strawberry cell wall disassembly, as polygalacturonases (PGs) and β-xylosidases (β-Xyls), is down-regulated by treatments with exogenous auxins [12,13]. Nevertheless, the expression of *FaPME1*, a relevant gene in strawberry cell wall metabolism that encodes a pectin methylesterase (PME), is up-regulated by auxins [14]. On the other hand, it has been shown that abscisic acid (ABA) is a positive regulator of strawberry fruit ripening [12,15,16].

Regarding ethylene, a growing number of evidences support its participation in strawberry fruit ripening. Three strawberry cDNAs encoding different ethylene receptors were isolated, and authors reported an increased expression of *FaEtr1*, *FaErs1* and *FaEtr2* during fruit ripening [17]. Furthermore, *FaEtr1* and *FaEtr2* genes were more responsive to exogenous ethylene application in white fruit than in the red ones, in agreement with previous reports [18,19]. In that sense, it has been detected an increase in ethylene production both in ripe strawberries and when expanded fruit progress from green to white color, which suggests that ethylene might act as a signal for the progression of the ripening process [20]. Likewise, several studies have shown that gene expression or enzyme activities of strawberry fruit PMPs respond to ethylene, although the effect of this hormone on the metabolism of cell wall polymers is way far from being clear [12–14,21,22].

Therefore, the present study is aimed to contribute to the knowledge of ethylene's role in the cell wall and middle lamella metabolism of strawberry, an important agronomical crop and a model of non-climacteric fruit within Rosaceae family.

2. Materials and methods

2.1. Fruit material

Strawberry (*Fragaria* × *ananassa* Duch., cv Toyonoka) fruit were obtained from local producers (La Plata, Buenos Aires Province, Argentina). Fruit were harvested at white stage (W) with a mild pink tonality. The peduncle of each fruit was cut at 30 mm from the receptacle base, and fruit were washed, drained, classified according to shape and size, and used for plant growth regulators assays.

2.2. 2-chloroethylphosphonic acid and 1-methylcyclopropene treatments

Whole white strawberry fruit were submerged during 5 min in a solution of 2 mM 2-chloroethylphosphonic acid (ethephon; an ethylene-releasing agent) in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol (both used as surfactants), prepared immediately before use. Control fruit were submerged for the same time in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol. Immersion treatments were applied at room temperature, and then fruit were vertically placed on microcentrifuge tubes with their peduncles in contact with distilled water to avoid dehydration.

In the case of 1-methylcyclopropene (1-MCP; an inhibitor of ethylene perception) treatment, fruit with peduncles in contact with distilled water were treated with this inhibitor (1 μl L^{-1}) in a hermetic container for 10 h at 22 °C, while the control were kept under the same conditions without 1-MCP.

Immediately after treatments, fruit from both assays were placed in a growth chamber at 22 °C during 48 h. After storage, the calyx and peduncle were removed and fruit were cut apart, frozen in liquid nitrogen and stored at −80 °C until use. Thirty fruit were used for each condition and the entire experiment was repeated three times.

2.3. Isolation of cell wall polysaccharides

Cell wall polysaccharides were obtained as Alcohol Insoluble Residues (AIRs) according to díAmour et al. [23] with slight modifications. Five grams of frozen fruit were homogenized with 20 ml of boiling absolute ethanol and refluxed for 30 min. The homogenate was vacuum filtered and the residue was washed three times with 15 ml of absolute ethanol. After this, the residue was dried overnight at 37 °C and weighed. Three independent AIRs extracts were obtained for each treatment and control fruit.

2.4. Extraction and quantification of pectins

Polyuronides were isolated according to díAmour et al. and Nara et al. [23,24] with modifications. A 100 mg aliquot of AIRs was homogenized in 100 ml of distilled water and shaken overnight at 20 °C. The homogenate was vacuum filtered and the solid washed three times with 10 ml of distilled water. The filtrates were pooled and labeled as water-soluble pectins (WSP). The residue was then suspended in 50 ml of 0.05 M sodium acetate containing 0.04 M EDTA, pH = 4.5 and shaken during 4 h at 20 °C. The homogenate was vacuum filtered and the solid washed three times with 10 ml of the same buffer. The filtrates were pooled and labeled as EDTA-soluble pectins (ESP). Finally, the last residue was suspended in 50 ml of 0.05 M HCl and heated with agitation at 100 °C for 1 h. After cooling, the homogenate was vacuum filtered and the residue washed three times with 10 ml of 0.05 M HCl. The filtrates were pooled and labeled HCl-soluble pectins (HSP). Uronic acid concentrations of all fractions were estimated by the m-hydroxydiphenyl method using galacturonic acid (GalA) as standard [25]. Neutral sugars (NS) concentration was estimated by the anthrone method using glucose as standard [23].

2.5. Extraction and quantification of hemicelluloses and cellulose

The washed residue from pectin extraction was shaken for 8 h with 50 ml of 4 M NaOH at 20 °C. The homogenate was vacuum filtered and washed three times with 5 ml of 4 M NaOH. The filtrates were pooled and labeled as hemicellulose fraction. Finally, the remained solid was considered as cellulose. Quantification of hemicelluloses and cellulose was done after complete hydrolysis with 66% v/v H_2SO_4 at 37 °C during 1 h and estimated as glucose by using the anthrone method [23].

2.6. In vitro cell wall swelling

Five milligrams of AIRs were suspended in 4 ml of sterile distilled water and shaken horizontally for 24 h at 25 °C. Tubes were then placed vertically, and cell wall swelling was assessed based on the height of the sedimented AIRs layer [4]. Three independent AIR extracts were used for each condition analyzed.

2.7. RNA isolation and reverse transcription

Total RNA was isolated from 5 g of frozen fruit using the 2-butoxyethanol method [26], treated with DNAase I (Promega) and then purified with chloroform:octanol (24:1). First strand of cDNA was obtained by using the following mixture: 1 μg of total RNA, 0.03 mM dNTPs, 1 μl of Moloney murine leukemia virus RT (200 U μl^{-1}; Promega), 5 μl of 5× reaction buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT, pH = 8.3), 330 pmoles of random primers (Biodynamics S.R.L., Buenos Aires, Argentina) and distilled water up to a total volume of 25 μl. The reaction mixture

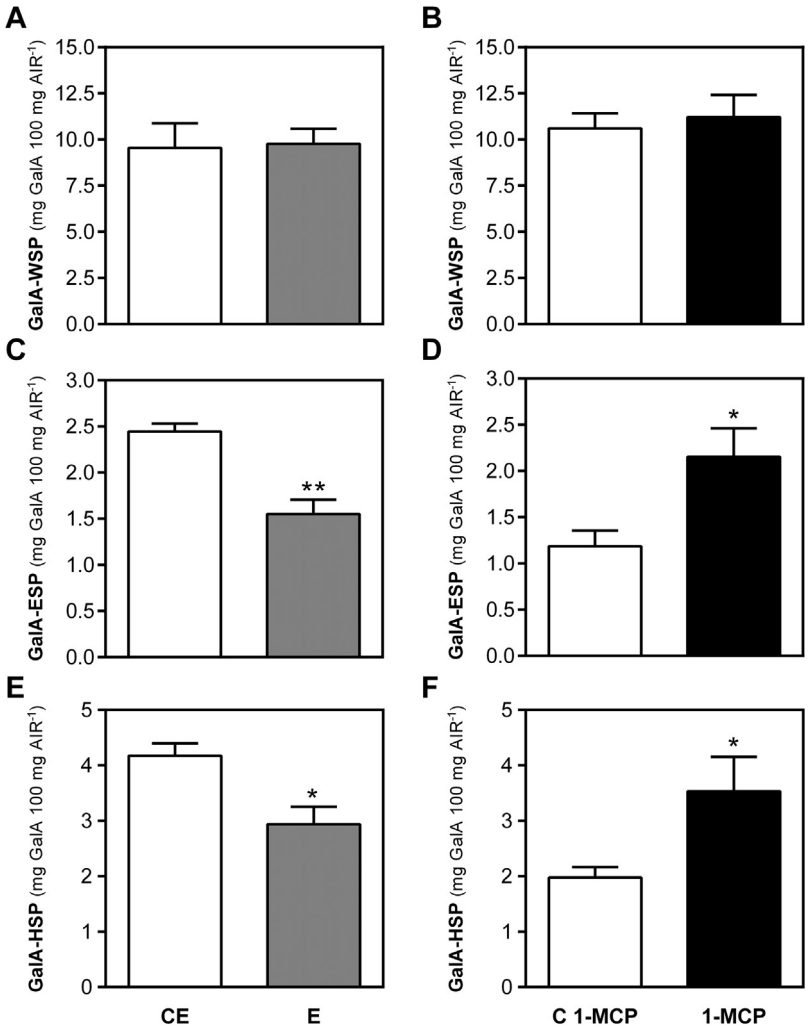

Fig. 1. Effect of ethephon (E) and 1-methylcyclopropene (1-MCP) treatments on content of GalA-WSP per 100 mg of AIR (A,B), GalA-ESP per 100 mg of AIR (C,D) and GalA-HSP per 100 mg of AIR (E,F) compared with controls (CE or C 1-MCP). Bars represent the mean ± SEM of three replicates. Asterisks indicate significant differences between treatments and controls (*: $P < 0.05$; **: $P < 0.01$).

was incubated during 1 h at 38 °C and 5 min at 95 °C. Five independent RNA extractions were performed for each condition evaluated.

2.8. Real-time PCR assays

First strand cDNA from each condition as described above was used for Real-time PCR assays. Sequences of primers used, sizes of amplified fragments, accession numbers, and EC numbers are listed in Table STI. The amplification reactions were performed using FastStart Universal SYBR Green Master Rox 2X (Roche) according to the manufacturer's instructions, in a Step One Plus Real-Time PCR System (Applied Biosystems). PCR conditions were: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s; and a melting curve from 60 °C to 95 °C at 0.3 °C increments. A dilution series was built to estimate the amplification efficiency of each primer pair, using a cDNA from strawberry fruit samples as template. Each reaction was performed in triplicate, and a negative water control was included in each run. Fluorescence was measured at the end of each extension step. In each experiment, the relative expression level corresponds to mean of five biological replicates, normalized against the expression level of *FaGAPDH1* gene (with constant expression level throughout all condition analyzed). Expression levels were calculated according to the method described by Pfaffl [27] and expressed in arbitrary units ± SEM.

2.9. Pathogens strains, growth conditions and growth rate analysis

Botrytis cinerea strain B05.10 and *Rhizopus stolonifer* of the IIB-INTECH Fungal Culture Collection IFCC 458/02 and IFCC 365/00, respectively, were used in all the experiments. Both fungi were routinely maintained in potato-dextrose agar slants at 4 °C. Prior to inoculation, mycelium of both fungi were grown in solid Czapek-Dox medium (50 g glucose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.05 g $FeSO_4 \cdot 7H_2O$, 20 g agar, pH = 5.5–6.0) at 24 °C. Preliminary assays were done to optimize the amount of AIRs to be added for each pathogen (growth curve, Fig. S1). For this, a 4 mm^2 agar plug was transferred from the edge of a 5-day-old and 2-day-old actively growing culture of *B. cinerea* and *R. stolonifer*, respectively, to the solidified plates with agar 0.8% (w/v) and 0.02, 0.1, 0.2 or 2 mg ml^{-1} of AIRs from control fruit. Plates were then cultivated at 24 °C, and the fungus growth area was measured after 24, 48 and 72 h for *B. cinerea* (Fig. S1A) or 24 and 48 h for *R. stolonifer* (Fig. S1B). Further experiments were performed using 0.2 mg ml^{-1} of AIRs of each condition analyzed, and the fungus growth areas were measured after the same times for each pathogen. Three independent AIRs extractions were performed for each treatment and control fruit, and five technical replicates of each of them were made. The size of the *B. cinerea* and

R. stolonifer growth areas were measured and determined using the Image-Pro®Plus software (Media Cybernetics Inc., San Diego, CA).

2.10. Statistical analysis

Data for AIRs, pectins, hemicelluloses and cellulose content, as well as relative expression and fungi growth area were analyzed by Student's *t*-test at a significance level of 0.05.

3. Results

3.1. Ethylene effects on strawberry cell wall composition

To determine the influence of ethylene on cell wall composition, cell walls (as Alcohol Insoluble Residues, AIRs) were prepared from ethephon- and 1-MCP-treated fruit as well as controls, and sequentially fractionated to yield water soluble pectins (WSP), ionically (ESP) and covalently (HSP) bound pectins, base-soluble hemicelluloses and an insoluble cellulose residue.

The analyses of AIR content revealed no statistical differences among ethephon- and 1-MCP-treated fruit regarding each control (Fig. S2A and B).

As regards pectins, no differences in the content of galacturonic acid (GalA) from WSP were found neither in ethephon-treated fruit regarding control, nor 1-MCP-treated fruit in comparison with control (Fig. 1A and B, respectively). However, ethephon treatment caused a significant reduction both in GalA content from ionically (37%) and covalently (30%) bound pectin regarding not treated fruit (Fig. 1C and E, respectively). On the other hand, fruit which underwent 1-MCP treatment showed a significant increment in GalA (almost 45%) in both pectins fractions (ESP and HSP) in comparison with controls (Fig. 1D and F, respectively).

When neutral sugar content was studied, we detected that the level of NS in WSP was significantly higher in fruit treated with ethephon regarding no-treated fruit (Fig. 2A), and the opposite was detected for 1-MCP-treated fruit when compared with control (Fig. 2B). Moreover, NS in HSP was statistically lower in 1-MCP-treated fruit regarding control (Fig. 2D). Accordingly, we detected an NS/GalA ratio <0.8 in each condition evaluated (Fig. S3A and B). Moreover, ethephon-treated fruit showed a higher NS/GalA ratio regarding controls, and the opposite situation was observed in 1-MCP-treated fruit (Fig. S3A and B).

Regarding hemicelluloses, we detected a significantly higher content of this cell wall fraction on ethephon-treated fruit, while 1-MCP-treated fruit had a lower amount of hemicelluloses when compared with controls (Fig. 3A and B, respectively). The cellulose content was higher in ethephon-treated fruit, while a significant reduction of this cell wall fraction was found in 1-MCP-treated fruit as regards controls (Fig. 3C and D, respectively).

Additionally, the percentage of pectins, hemicellulose and cellulose from AIRs of strawberry treated-fruit (ethephon and 1-MPC) and controls was calculated. We detected an average of 60% of pectins, 10% of hemicellulose and 30% of cellulose (Fig. S3C). Strawberries treated with ethephon showed a lower percentage of pectins and a higher percentage of hemicelluloses and cellulose in relation to controls, while the opposite was found in 1-MCP-treated fruit.

We also analyzed the ethylene effect on strawberry cell wall swelling by an *in vitro* assay, and found a lower swelling of AIR from ethephon-treated fruit regarding controls, while an opposite effect was observed in samples from 1-MCP-treated fruit (Fig. 4A and B respectively).

3.2. Relative expression of strawberry cell wall genes

The effects of ethephon and 1-MCP treatments on cell wall composition above described, suggested an influence of ethylene on the cell wall metabolism. Therefore, we decided to analyze the effect of this hormone on the expression of a set of genes involved in cell wall metabolism of strawberry fruit.

First, we analyzed the ethylene effect on the relative expression of genes that have been shown to be relevant in strawberry pectins metabolism. The expression of *FaPME1* (encoding a pectin methylesterase) was significantly down-regulated by ethephon treatment, while fruit treated with 1-MPC had a 50% increase of *FaPME1* expression regarding controls (Fig. 5A and B, respectively). On the other hand, ethephon up-regulated *FaPG1* (which encodes a polygalacturonase) gene expression as regard control, and the opposite was detected in 1-MCP-treated fruit (Fig. 5C and D, respectively). Regarding the effect of ethylene on *FaPLA* (which encodes a putative pectate lyase), no significant differences were observed neither on ethephon- nor 1-MCP-treated fruit when compared with controls (Fig. 5E and F, respectively).

Second, we analyzed the effect of ethylene on the expression of some relevant genes for strawberry hemicelluloses metabolism. The expression of *FaXyl1* (encoding a β-xylosidase), *FaEGase1* (encoding a putative endoglucanase) and *FaXTH1* (encoding a putative xyloglucan endotransglycosylase/hydrolase) was significantly down-regulated by ethephon treatment when compared with controls (Fig. 6A, C and E), and the opposite effect was observed when fruit were treated with 1-MCP (Fig. 6B, D and F).

Additionally, the ethylene effect on the expression pattern of genes involved in galactans and arabinans metabolism was studied. We detected a down-regulation of *FaAra1* (which encodes a putative arabinofuranosidase) when strawberry fruit were treated with ethephon (Fig. 7A), while a significant up-regulation of that gene was observed in 1-MCP-treated-fruit (Fig. 7B). In the case of *FaGal1* and *FaGal2* (encoding β-galactosidases) ethephon treatment significantly up-regulated the expression of both genes (Fig. 7C and E), and the opposite was observed in 1-MCP treated fruit, in comparison with controls (Fig. 7D and F).

3.3. Strawberry pathogen's growth on isolated cell wall material

Since ethylene modifies strawberry cell wall composition and possibly its structure (as it was shown in Section 3.1), we decided to study the capacity of *B. cinerea* and *R. stolonifer* to grow on agar plates containing cell wall material (AIRs) from fruit treated with ethephon or 1-MCP, and corresponding controls, as the only carbon source (Fig. 8). The radial growth of *B. cinerea* and *R. stolonifer* was significantly higher on plates containing AIRs from fruit treated with ethephon regarding control, almost at every incubation time that was evaluated (Fig. 8A and B). On the other hand, when AIRs from fruit treated with 1-MCP were used as carbon source, the mycelial growth area of *B. cinerea* and *R. stolonifer* was significantly lower than controls (Fig. 8C and D).

4. Discussion

It has been proposed that strawberry fruit softening is closely related to pectins solubilization and depolymerization, rather than to hemicellulose or cellulose catabolism [28,29–31]. In the present work, we study the effect of ethylene on strawberry cell wall metabolism through two approaches: fruit treatment with an ethylene releasing agent (ethephon), and treatment with a competitive inhibitor of ethylene action (1-MCP).

We detected that ethephon treatment significantly reduced the content of galacturonic acid (both in ESP and HSP fractions) from

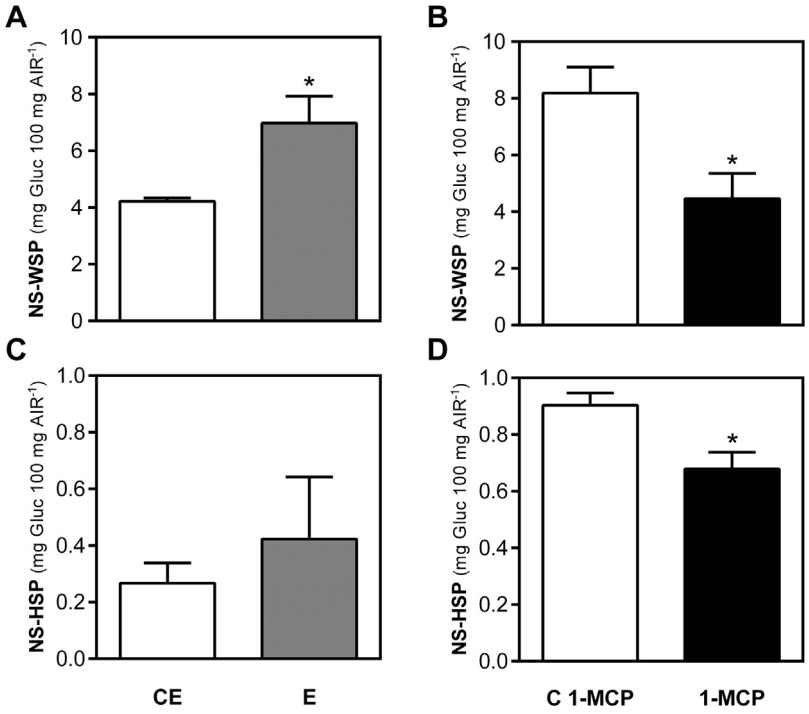

Fig. 2. Effect of ethephon (E) and 1-methylcyclopropene (1-MCP) treatments on content of NS-WSP per 100 mg of AIR (A,B) and NS-HSP per 100 mg of AIR (C,D) compared with controls (CE or C 1-MCP). Bars represent the mean ± SEM of three replicates. Asterisk indicates significant differences between treatments and controls (*: $P < 0.05$).

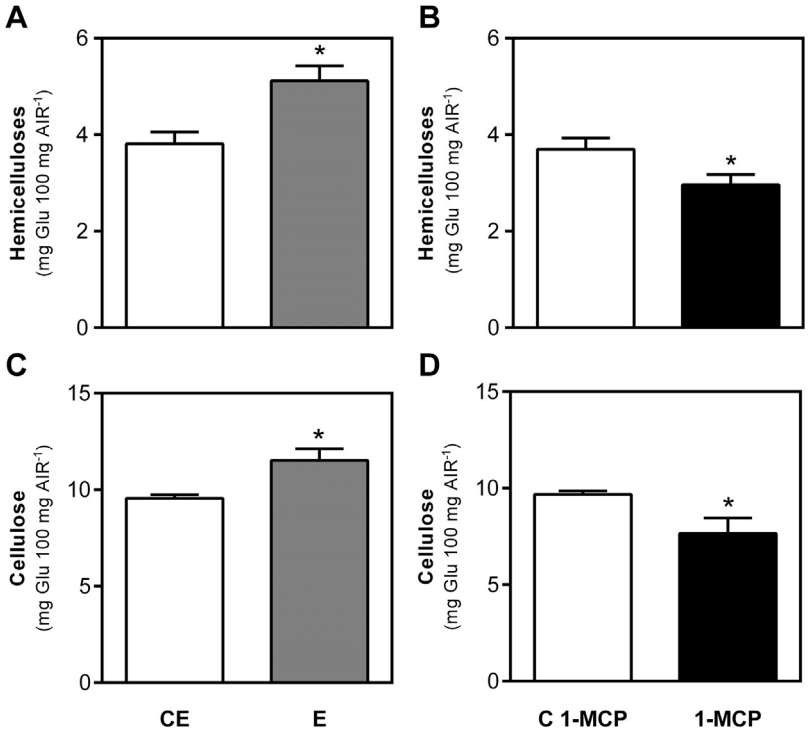

Fig. 3. Effect of ethephon (E) and 1-methylcyclopropene (1-MCP) treatments on content of hemicellulose (as mg Glu per 100 mg of AIR) (A,B) and cellulose (as mg Glu per 100 mg of AIR) (C,D) compared to controls (CE or C 1-MCP). Bars represent the mean ± SEM of three replicates. Asterisk indicates significant differences between treatments and controls (*: $P < 0.05$).

strawberry pectins, while a contrast effect was detected with 1-MCP treatment (Fig. 1C–F). On the other hand, ethephon treatment raised both hemicelluloses and cellulose amounts regarding non-treated fruit, and the opposite was found with 1-MCP treatment (Fig. 3A–D).

In addition, the higher NS/GalA ratio values detected on ethephon-treated fruit in comparison with controls suggest that the ethylene would be involved in the modulation of strawberry pectins metabolism, possibly through a higher hydrolysis of homogalacturonans and a lower hydrolysis of side chains, although an

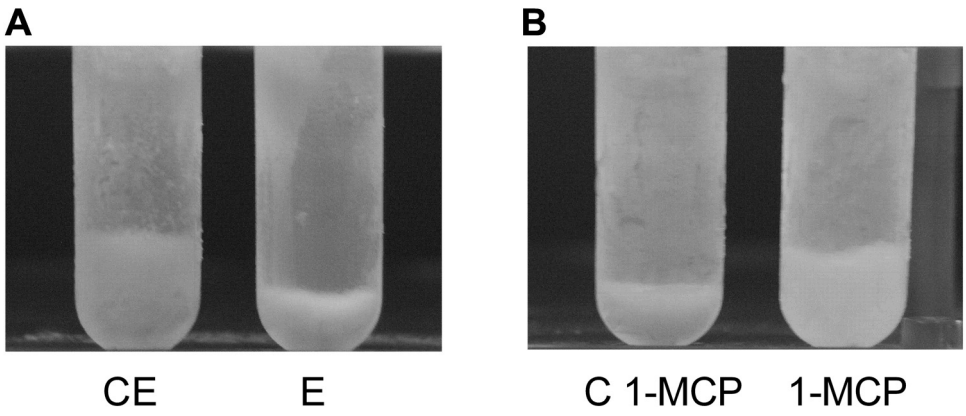

Fig. 4. Effect of ethephon (E) and 1-methylcyclopropene (1-MCP) treatments on AIR swelling compared wiht controls (CE or 1-MCP) (A,B). Image is representative of three replicates.

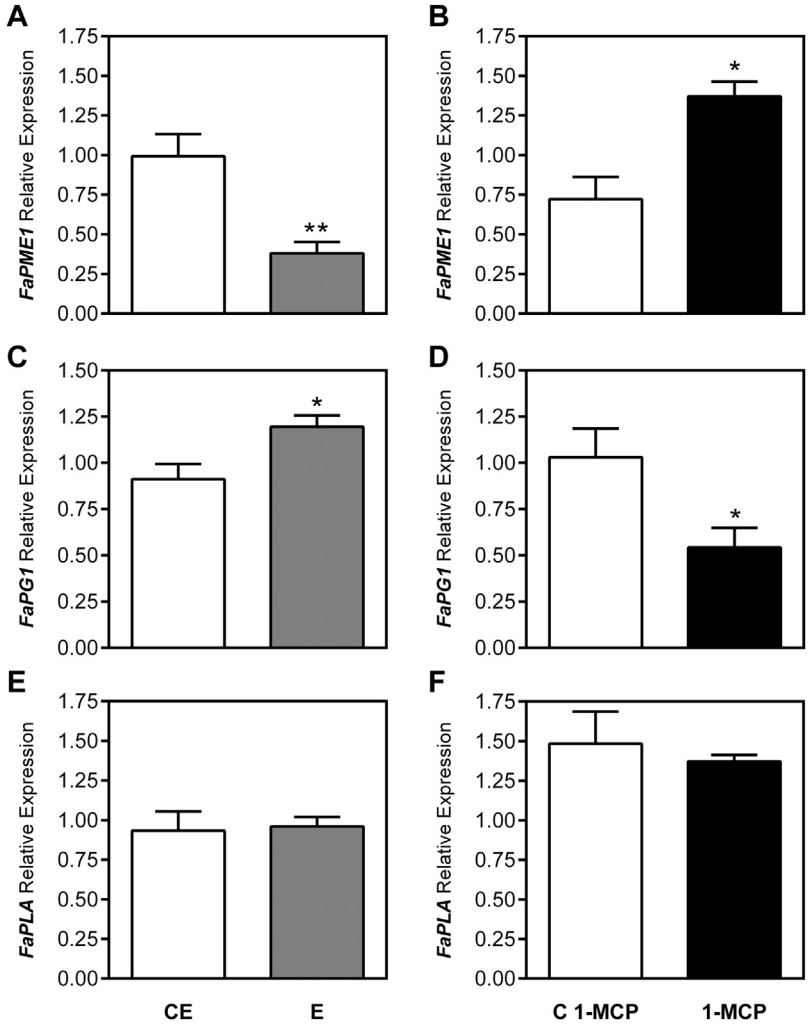

Fig. 5. Effect of ethephon (E) and 1-methylcyclopropene (1-MCP) treatments on *FaPME1* (A,B), *FaPG1* (C,D) and *FaPLA* (E,F) expression. Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls (CE or C 1-MCP). Bars represent the mean ± SEM of five replicates. Asterisks indicate significant differences between treatments and controls (*: $P < 0.05$; **: $P < 0.01$).

effect on pectins biosynthesis should not be discarded (Figs. 1 and 2 and S3). In that sense, the only studies about fruit cell wall biosynthesis have been performed in tomato, and reported that this process keeps going throughout the fruit ripening process [32,33].

In the present work, we detected an average of 60% of pectins, 10% of hemicellulose and 30% of cellulose on strawberry cell wall (Fig. S3C). We decided to study the effect of ethylene on cell wall swelling, as this process has been related to several chemical characteristics and particular properties of middle lamella and cell wall components. A lower swelling was observed in AIRs from ethephon-treated fruit, while the opposite effect was detected in AIRs from 1-MCP treated fruit regarding controls (Fig. 4). These

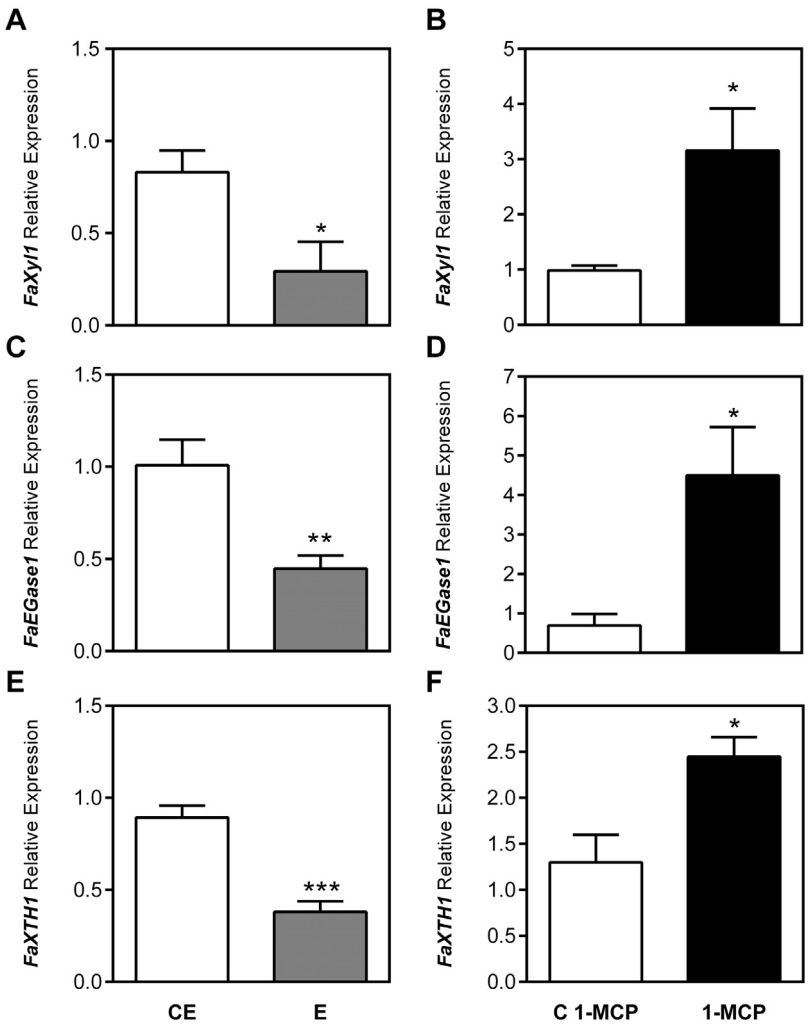

Fig. 6. Effect of ethephon (E) and 1-methylcicloropene (1-MCP) treatments on *FaXyl1* (A,B), *FaEGase1* (C,D) and *FaXTH1* (E,F) expression. Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared wiht controls (CE or C 1-MCP). Bars represent the mean ± SEM of five replicates. Asterisks indicate significant differences between treatments and controls (*: $P < 0.05$; **: $P < 0.01$, *** $P < 0.001$).

findings could be explained considering that cell wall swelling has been associated not only with higher pectin solubility, but also with a higher length of pectin side branches [34]. The treatment of kiwi cell walls with Na_2CO_3 caused hydrolysis of pectins side chains and induced a greater swelling [34]. Furthermore, Brummell [35] suggests that greater cell wall swelling may also be associated to a loosening of the cellulose-xyloglucan network as we detected on 1-MCP treated fruit (Fig. 3B and D).

Considering pectins metabolism in strawberry, it has been reported HSP depolymerization during ripening of fruit from Toyonoka cultivar, possibly due to an activation of PMPs as polygalacturonases [28]. Moreover, two independent studies performed in anti-sense transgenic strawberry plants with a down-regulated expression of a pectate lyase [29] and polygacturonase [31], rendered firmer fruit when compared with wild type.

Pectin methylesterases catalyze homogalacturonans de-esterification within primary cell wall, middle lamella and the corners of cell junctions [3]. On one hand, PMEs facilitate PGs action (cell wall disassembly and cell to cell adhesion disruption), but on the other hand, PMEs promote Ca^{2+}-bridges between galacturonic acid residues from HGs (cell wall and neighbor cells adhesion stabilization) [3,36,37]. In this context, PME activity constitutes a key control point for both the assembly and disassembly of pectin networks. *FaPME1* is specifically expressed in strawberry fruit, showing two expression peaks when the receptacle is white with green achenes and at turning stage, being the gene expression low when receptacle is white with some rosy spots or completely red [14]. Regarding ethylene regulation, Northern-blot assays showed that exogenous ethylene treatment decreased *FaPME1* mRNA accumulation in ripe fruit, and the opposite was observed when fruit were treated with 1-MCP [14]. Our results in white-pale pink Toyonoka strawberry fruit are in agreement with those observed by Castillejo et al. [14] in ripe fruit, and fully support the fact of a negative regulation of *FaPME1* expression by ethylene (Fig. 5A and B).

Polygalacturonases catalyze the hydrolysis of the α-1,4 glycosidic bonds between galacturonic acid residues within HGs [38]. In strawberry fruit, Northern-blot assays performed in Camarosa cultivar showed that *FaPG1* gene expression is down-regulated by ethylene [12], which was confirmed in the present work by Real Time PCR assays in Toyonoka cultivar (Fig. 5C, D). Ours results about ethylene effect on *FaPME1* and *FaPG1* expression could explain at least partly, the lower amount of GalA-ESP and GalA-HSP detected in strawberry fruit treated with ethephon, in comparison with controls, and the higher amount of those fractions on 1-MCP-treated fruit regarding controls.

The metabolism of hemicelluloses involves, among others, β-xylosidases (β-Xyl), endo-glucanases (EGases), and Xyloglu-

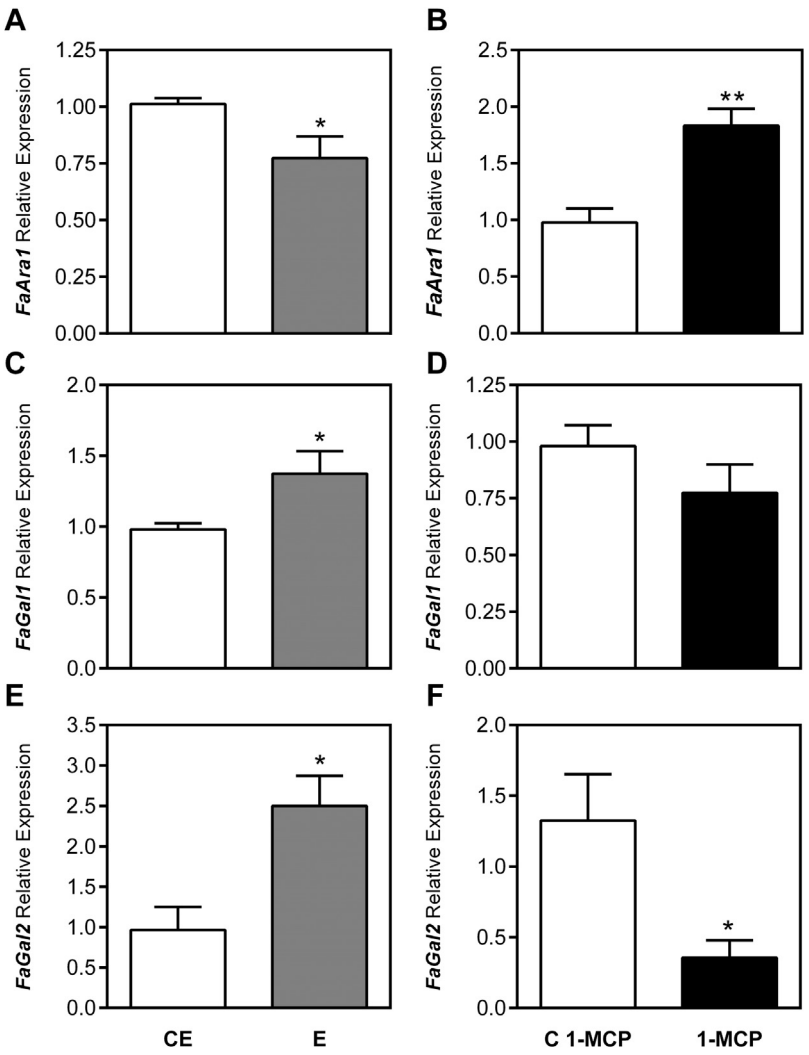

Fig. 7. Effect of ethephon (E) and 1-methylcyclopropene (1-MCP) treatments on *FaAra1* (A,B), *FaGal1* (C,D) and *FaGal2* (E,F) expression. Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls (CE or C 1-MCP). Bars represent the mean ± SEM of five replicates. Asterisk indicate significant differences between treatments and controls (*: $P < 0.05$; **: $P < 0.01$).

can endotransglycosylase/hydrolases (XTH) enzymes. β-Xyl are enzymes that catalyze the hydrolytic cleavage of β-1,4-glycosidic bonds from xylo-oligosaccharides to release xylose [39]. It has been reported that total β-xylosidase activity as well as the expression of *FaXyl1* and the accumulation of its encoded protein, are associated to ripening and softening processes in strawberry fruit [40]. EGases has been proposed to have β-1,4-glucan bonds of xyloglucans as the most common substrates, although non-crystalline cellulose regions should be also considered [41]. In strawberry, *FaEGase1* mRNA accumulation has been strongly associated with fruit ripening [42]. XTHs are enzymes involved in the remodeling of plant cell wall hemicelluloses, and they could exhibit both xyloglucan endotransglycosylase (XET) and/or xyloglucan endo-hydrolase (XEH) activities [43]. In a previous work, we had cloned *FaXTH1* cDNA and bioinformatics and phylogenetic analysis suggested that the putative enzyme FaXTH1 would have XET as exclusive activity, suggesting that this protein might be involved in strawberry hemicellulose reinforcement instead of disassembly [44]. That idea was in line with the fact that *FaXTH1* mRNA accumulation was significantly lower during fruit ripening of strawberry cultivars with a high softening rate like Toyonoka, when compared with fruit from firmer cultivars like Camarosa and Selva [44].

Previously, we detected that treatment with ethephon caused an inhibition both on total β-xylosidase and endoglucanase activities of Toyonoka cultivar, while the opposite was observed with 1-MCP treatment [21]. The results found in the present work regarding *FaEGase1* gene expression constitute to our knowledge, the first report of ethylene effect on endoglucanase mRNA accumulation in strawberry fruit. The higher hemicellulose content found in fruit treated with ethephon, and the lower amount of these components detected in fruit treated with 1-MCP, are related with the down- and up-regulation of *FaXyl1* and *FaEGase1* exerted by ethephon and 1-MCP, respectively (Fig. 6A–D). On the other hand, regarding ethylene effect on *FaXTH1* expression, the down-regulation of this gene expression by ethephon found in the present work, might contribute to decrease the integrity of strawberry hemicellulose network (Fig. 6E and F).

Additionally, the effect of ethylene on galactans and arabinans metabolism was evaluated. Both types of polysaccharides are found in side chains of pectins and hemicelluloses [45]. It has been reported that both total α-L-arabinofuranosidase (α-Ara) activity as well as gene expression of three α-Ara genes are associated to texture differences between strawberry cultivars [46]. Regarding galactans metabolism, it has been reported the expression of two β-galactosidases genes both in strawberry fruit and vegetative tissues

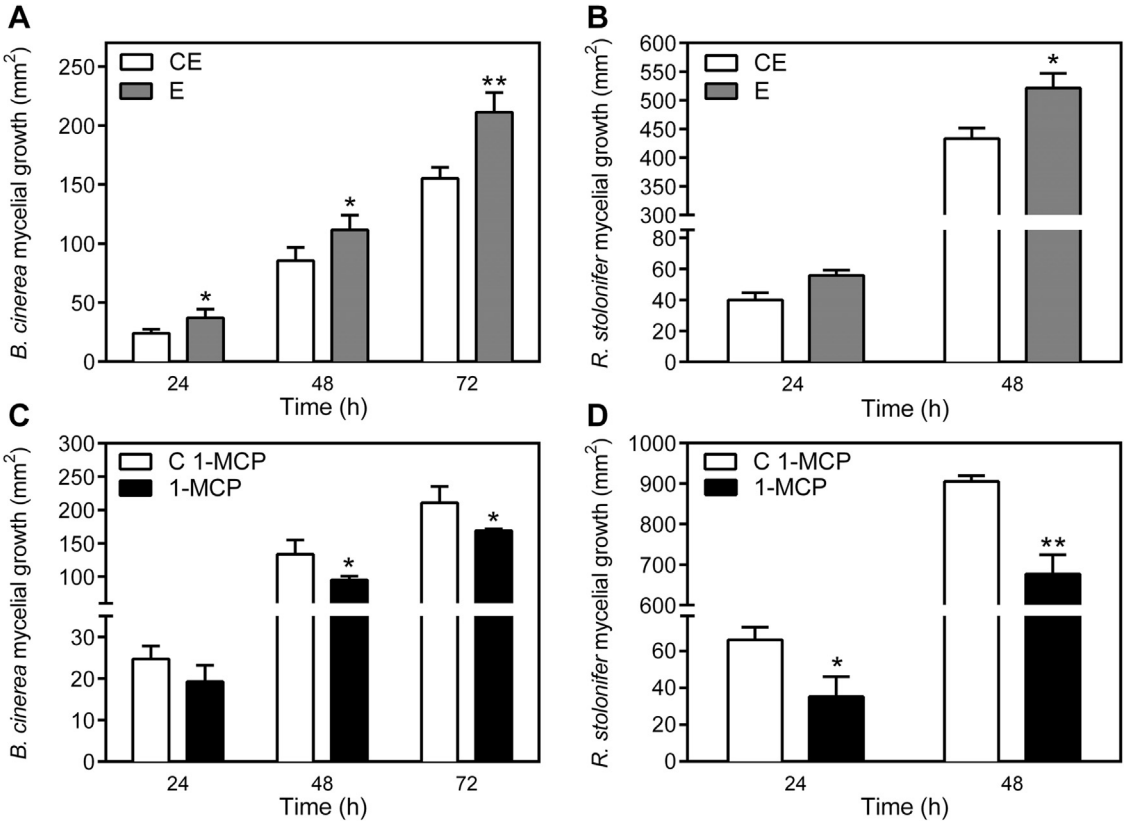

Fig. 8. Growth of *B. cinerea* and *R. stolonifer* on plates containing 0.8% (w/v) agar and 0.2 mg ml^{-1} of AIRs from fruit treated with ethephon (E) and controls (CE) (A,B), as well as fruit treated with 1-methylcicloprope (1-MCP) and controls (C 1-MCP) (C,D) as the only carbon source. Bars represent the mean ± SEM of three replicates. Asterisks indicate significant differences between treatments and controls (*: $P < 0.05$; **: $P < 0.01$).

[47]. Residues of arabinose and galactose are mainly located in side branches of type I rhamnogalacturonans, components of covalently bound pectins [48]. In strawberries, higher amounts of arabinose in relation to galactose were detected and it has been reported that loss of arabinose residues occurs at higher rates than those of galactose during strawberry fruit ripening [49,50]. In this sense, the removal of arabinose could be probably more relevant than the removal of galactose in the loss of neutral sugars. Accordingly, the reduced expression of *FaAra1* in fruit treated with ethephon might have a higher influence on the side chains metabolism than the increased expression of *FaGal1* and *FaGal2*, and thereby would correlate with a higher amount of NS detected in fruit treated with ethephon (Fig. 7).

Plant cell wall constitutes the first physical barrier to phytopathogenic organisms [51]. Indeed, pathogens release cell wall-degrading enzymes to break down this barrier and to use its components as nutrient source, before accessing to cytoplasm [4,52,53]. Postharvest life and commercialization of strawberry fruit is limited by gray mold and leak diseases caused by *Botrytis cinerea* and *Rhizopus stolonifer* [54,55]. It has been reported that transgenic tomato fruit with suppressed polygalacturonase (*LePG*) and expansin (*LeExp1*) expression differs in cell wall composition and integrity from wild type fruit [4]. Through fungal biomass measure assay (using a monoclonal antibody that recognizes a mycelium-localized epitope), authors showed that pathogen growth is reduced in liquid cultures containing cell wall from transgenic fruit (-PG -Exp) compared with cultures supplied with wild type cell walls [4]. In that sense, the reduction of pectins amounts caused by ethephon treatment, would promote the accessibility of the fruit cell wall substrates to *B. cinereaís* and *R. stolonifer's* polysaccharides-degrading enzymes, which is reflected in a higher rate of growth of these pathogens. These observations are confirmed through the opposite results observed for 1-MCP treatment (Fig. 8).

5. Conclusions

To our knowledge, the present work constitutes the first study of the ethylene's effect on the amounts of the main cell wall components and the expression of several genes involved in cell wall modification in strawberry fruit. We detected that white fruit treated with ethephon has a higher content of hemicelluloses and cellulose and a lower amount of galacturonic acid both in ESP and HSP fractions. Considering that around 60% of strawberry AIRs is formed by pectins, which in turns include 70% HGs, our results suggest that at early stages of strawberry fruit ripening, ethylene might act promoting HGs hydrolysis on one hand and inhibiting the catabolism of hemicelluloses and cellulose on the other, possibly through an up-regulation of polygalacturonase gene expression and a down regulation of hemicellulose-degrading genes. Moreover, even when ethephon-treated fruit had higher NS contents regarding controls, we detected that ethylene up-regulates the expression of two genes coding β-galactosidases, which might contribute to reduce galactans content on lateral chains and thus to increase the porosity of strawberry cell wall and middle lamella, facilitating polygalacturonase access to its substrates. The last hypothesis is supported by the higher necrotrophic pathogens' growth on plates containing AIRs from ethephon-treated fruit regarding controls.

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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.plantsci.2016.06.018.

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