Calcium chloride treatment modifies cell wall metabolism and activates defense responses in strawberry fruit (*Fragaria x ananassa*, Duch)

Running title: Molecular determinants on the preservation of strawberry fruit quality by calcium

Silvia E. Langer[a], María Marina[a], José L. Burgos[a], Gustavo A. Martínez[b,c], Pedro M. Civello[b,c], Natalia M. Villarreal[a,*]

[a] *INTECH (CONICET-UNSAM), Instituto Tecnológico de Chascomús, Avenida Intendente Marino km 8,2, B7130IWA, Chascomús, Pcia. Buenos Aires, Argentina*

[b] *INFIVE (CONICET-UNLP), Instituto de Fisiología Vegetal, Diag. 113 N° 495 - C.c 327, 1900 La Plata, Argentina*

[c] *Facultad de Ciencias Exactas, (UNLP), 47 y 115, 1900 La Plata, Argentina*

** Corresponding author: Natalia M. Villarreal nvillarreal@intech.gov.ar Tel +54 2241 424049 Fax +54 2241 424048*

Abstract

BACKGROUND: Fruit dips in calcium ions solutions have been shown as an effective treatment to extend strawberries (*Fragaria x ananassa*, Duch) quality during storage. In the present work, strawberry fruit were treated with 10 g L^{-1} calcium chloride solution and

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treatment effects on cell wall enzymes activities and the expression of encoding genes, as well as enzymes involved in fruit defense responses were investigated.

RESULTS: Calcium treatment enhanced pectin methylesterase activity while inhibited those corresponding to pectin hydrolases as polygalacturonase and ²-galactosidase. The expression of key genes for strawberry pectin metabolism was up- (for *FaPME1*) and down- (for *FaPG1*, *FaPLB*, *FaPLC*, *Fa²Gal1* and *FaAra1*) regulated by calcium dips. In agreement, a higher firmness level and ionically-bound pectins (IBP) amount were detected in calcium-treated fruit compared with controls. The *in vitro* and *in vivo* growth rate of fungal pathogen *Botrytis cinerea* was limited by calcium treatment. Moreover, the activities of polyphenol oxidases, chitinases, peroxidases and ²-1,3-glucanases were enhanced by calcium ions dips.

CONCLUSION: News insights concerning the biochemical and molecular basis of cell wall preservation and resistance to fungal pathogens on calcium-treated strawberries are provided.

Keywords: calcium chloride, strawberry, cell wall metabolism, pectins, fruit defense

INTRODUCTION

Strawberry is a soft fruit which undergoes a rapid loss of firmness during storage leading to mechanical damage as well as increasing fungal infections.[1] In strawberry, fruit softening is mainly caused by cell wall disassembly and middle lamella dissolution, being pectins metabolism a key control point for this process.[2,3] In this sense, the use of more

environment-friendly approaches as heat- and UV-C light treatments to delay softening and to control diseases during postharvest have been assayed in strawberry fruit.[4-6] Moreover, several research groups have reported the beneficial effect of treatments with calcium solutions to maintain strawberry quality. Lara *et al*.[7] informed that 1% w/v calcium chloride dips improved resistance to the necrotrophic pathogen *Botrytis cinerea*, helped treated fruit to retain higher amounts of ionically-bound pectins (IBP) and contributed to preserving structural integrity of cell wall and middle lamella. Hernández-Muñoz *et al*.[8] showed that 1% w/v calcium gluconate dips were effective to decrease surface damage caused by *B. cinerea* and to delay both fungal decay and loss of firmness on treated fruit when compared with controls. In a very interesting work, Zhang *et al*.[9] demonstrated that fruit immersion in 2% w/v calcium chloride solution, effectively inhibited the degradation of IBP as an evident cross-linking between calcium and pectin was observed in treated fruit. Through Atomic Force Microscopy (AFM) assays, authors showed that after thirteen and seventeen days of cold storage, calcium-treated fruit were firmer and showed a larger percentage of wider and longer IBP molecules than controls, respectively.

Despite these valuable contributions, the biochemical and molecular basis underlying the effects of calcium on cell wall preservation and resistance to fungal infection in strawberry fruit has not been explored. Accordingly, the main goal of the present study was to determine the influence of calcium chloride treatment on the expression pattern and activities of genes and enzymes closely related to strawberry cell

wall metabolism and fruit firmness. The effect of calcium dips on defense-related responses in strawberry fruit was also explored.

MATERIALS AND METHODS

Fruit material and calcium chloride treatment

Three hundred strawberry fruit (*Fragaria* × *ananassa*, Duch, cv Aroma) were harvested at commercial ripening stage (80-90% red) from local producers (La Plata, Buenos Aires Province, Argentina) and transported to the laboratory. The peduncle of each fruit was cut at 30 mm from the receptacle base, and fruit with no visible damage were classified according to size and shape. Two hundred and thirty fruit were put in fourteen plastic trays. The following treatments were applied on a set of almost 115 fruit (seven trays) for each condition: 0.5 h of incubation in distilled water at room temperature (25 ± 1 °C) (Control or C) and, 0.5 h of incubation in 10 g L^{-1} $CaCl_2$ at room temperature (Calcium chloride-treated fruit or Ca). Fruit were dried in air at room temperature for 2 h. Fruit from three trays per each condition (C and Ca) were taken after each treatment (0 days or 0 d) and used to measure firmness. Then, samples were cut into eights, frozen in liquid nitrogen and stored at -80 °C until use. Fruit from four trays per each condition (C and Ca) were stored for 8 days at 4 °C and then incubated during 2 days at 20 °C (shelf condition) (8 + 2 d). Subsequently, firmness was measured and fruit were cut into eights, frozen in liquid nitrogen and stored at -80 °C until use.

Firmness

Fruit firmness was measured with a texture analyzer (TA.XTPlus, Stable Micro Systems Texture Technologies), using a 25 mm diameter cylinder Perspex. Fruit were compressed 0.8 mm and the maximum force (in Newton, N) developed during the test was recorded. Two measures were made for each fruit, with a rotation of 180° angle between measures.

Cell wall polysaccharides

Measurements were performed according to Villarreal *et al.*[10] with slight modifications. Five grams of frozen fruit were homogenized with 20 mL of absolute ethanol and boiled with reflux during 30 min. The homogenate was vacuum filtered and the residue was washed three times with 15 mL of absolute ethanol. The residue was dried for 12 h at 37 °C and weighed. Three independent strawberry cell wall extracts (expressed as Alcohol Insoluble Residues or AIRs) were obtained for control and calcium chloride-treated fruit at the final time of assay (8 + 2 d). Afterward, 50 mg of AIRs were homogenized in 50 mL of distilled water and shaken overnight at 20 °C. The homogenate was vacuum filtered and the solid was washed three times with 5 mL of distilled water. Filtrates obtained were pooled and named as water-soluble pectins (WSP). The residue was subsequently suspended in 25 mL of 0.05 M sodium acetate which contained 0.04 M EDTA, pH= 4.5 and shaken at 20 °C for 4 h. The vacuum filtered homogenate was saved and the solid was washed three times with 2.5 mL of the same buffer. Filtrates were pooled and named as EDTA-soluble pectins (ESP). Then, the residue was suspended in 25 mL of 0.05 M HCl and heated at 100 °C with agitation for 1 h. The homogenate was cooled and

vacuum filtered, and the residue was washed three times with 2.5 mL of 0.05 M HCl. Filtrates were pooled and named as HCl-soluble pectins (HSP). Uronic acid concentration of each fraction was evaluated by the m-hydroxydiphenyl method using galacturonic acid (GalA) as standard.

Finally, the washed residue from pectin extraction was mixed with 50 mL of 4 M NaOH and shaken during 8 h at 20 °C. The vacuum filtered homogenate was washed three times with 5 mL of 4 M NaOH and filtrates were joined and named as hemicellulose fraction. Samples were hydrolyzed with 660 mL L^{-1} H_2SO_4 at 37 °C for 1 h and hemicelluloses content was estimated as glucose with the anthrone method.[11]

Neutral sugars (NS) concentration was measured over total pectins (extracted from 100 mg of AIR in a similar way of HSP extraction) by the anthrone method.

Cell wall enzymes activity

Three independent extracts using 5 g of frozen strawberries for calcium-treated and controls fruit were prepared at each time of assay, and activity of each extract was measured twice. Measurements of cell wall enzymatic activities were performed as previously fully described.[12]

Data for total pectin methylesterase (PME; EC: 3.1.1.11) activity was expressed as µmol of demethylated galacturonic acid (GalA) generated per second and per kilogram of fruit. Results for polygalacturonase (PG; EC: 3.2.1.15) activity were expressed as nmol of galacturonic acid released per second per kilogram of fruit. ² -galactosidase (² -Gal; EC: 3.2.1.23) activity results were shown as nmol of p-nitrophenol released per second per

kilogram of fruit. Finally, ±-arabinofuranosidase (±-Ara; EC: 3.2.1.55) activity was indicated as nmol of 4-nitrophenol released per second per kilogram of fruit.

RNA extraction, cDNA synthesis and Real-time PCR assays

Total RNA was isolated from 5 g of frozen strawberries using the 2-butoxyethanol method,[13] treated with DNAase I (Promega) and purified with chloroform:octanol (24:1). Five independent RNA extractions were performed for control and calcium chloride-treated fruit. The first strand of cDNA for each sample was obtained as described in Langer *et al*.[12] Primers sequences, sizes of amplified fragments and accession numbers are listed as supporting information (Table S1). The amplification reactions for Real Time PCR assay were performed using Fast Start Universal SYBR Green Master Rox 2X (Roche) according to the instructions of the manufacturer, in a Step One Plus Real-Time PCR System (Applied Biosystems), using the same conditions described in Langer *et al*.[12] The relative expression level corresponding to mean of five biological replicates was normalized against the expression level of *FaGAPDH1* gene (showing constant expression level throughout all conditions analyzed). Relative expression levels were calculated conforming to the method described by Pfaffl[14] and expressed in arbitrary units ± SEM

***Botrytis cinerea* growth rate assay and fruit infection**

B. cinerea strain B05.10 from the IIB-INTECH Fungal Culture Collection IFCC 458/02, was used. Prior to inoculation, mycelium was grown at 24 °C in solid Czapek-Dox medium (50 g glucose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.05 g $FeSO_4.7H_2O$, 20 g agar, pH= 5.5-6.0).

In vitro assay: A growth curve was performed in a previous work.[10] As a result, assays were performed using 0.2 mg mL^{-1} of AIRs for each condition studied and using a 4 mm^2 agar plug which was transferred from the edge of a 5-day-old actively growing culture of *B. cinerea* to the solidified plates with 8 g L^{-1} agar. *Botrytis* growth areas were measured after 24, 72 and 120 h. Three independent AIRs extractions from control and calcium-treated fruit were used, and three technical replicates of each of them were made. Sizes of the fungus growth areas were measured using the Image-Pro®Plus software (Media Cybernetics Inc., San Diego, CA).

In vivo assays: For sporulation, plates were kept from 7 to 10 d in darkness at room temperature. Then, conidia were harvested with sterile water containing 0.02 mL L^{-1} Tween-20, filtered and counted with a hemocytometer. The inoculum concentration was tuned to 2 x 10^4 conidia per mL with PDB medium, complemented with 10 mM KH_2PO_4 and 10 mM sucrose. Prior to inoculations, conidia were incubated for 2-3 h at room temperature without shaking. Strawberries at ripe stage were disinfected with 2 mL L^{-1} of a commercial NaClO solution (55 g of active Cl_2 L^{-1}) and twenty-four fruit for control and calcium chloride treatment were used. For inoculation, two 10 µL droplets were placed on the surface of each fruit and incubated at 20 °C in darkness during 6 days. Fruit were observed daily, photographed with a digital camera and lesions were evaluated as the percentage of mycelia on the inoculation zone; incipient lesion beyond the inoculation zone + mycelia; moderate lesion beyond the inoculation zone + mycelia and putrefaction symptoms.

Defense enzyme activity

All activity measures were performed according to Pombo *et al.*[6] with some modifications, using 5 g of frozen strawberries for each assay.

For chitinase (Chi; EC: 3.2.1.14) and ² -1,3-glucanase (² -1,3-gluc; EC: 3.2.1.6) activities 5 g of strawberries were homogenized in an Omnimixer with 30 mL of 10 mM sodium acetate buffer, 20 g L^{-1} PVP, pH= 5.0. The mixture was stirred at 4 °C for 3 h and then centrifuged for 30 min at 12,000 xg.

For chitinase, 2,210 µL of supernatant were mixed with 739 µL of 2 g L^{-1} chitin azure (Sigma). The reaction mixture was incubated at 37 °C with stirring, 710 µL aliquots were taken at 0, 2, 4 and 6 h (stopping the reaction with the addition of 178 µL of 2 N HCl and frozen with $N_{2\ (l)}$) and absorbance was measured at 575 nm.

For ² -1,3-glucanase activity, 1,050 µL of extract were mixed with 350 µL 10 g L^{-1} laminarin azure (Sigma). The reaction mixture was incubated at 37 °C with stirring and aliquots of 300 µL were taken at 0, 7, 15 and 22 h. The reaction was immediately stopped by adding 750 µL of ethanol, frozen with $N_{2(l)}$ and absorbance was measured at 575 nm.

For polyphenol oxidase (PPO; EC: 1.10.3.1) and peroxidase (POD; EC: 1.11.1.7) activities, 5 g of strawberries were homogenized in an Omnimixer with 0.02 M Na_2HPO_4, 0.08 M NaH_2PO_4, 1 mL L^{-1} Triton X-100, 1 M NaCl, 30 g L^{-1} PVPP, pH= 6.0. The mixture was stirred for 1 h and then centrifuged at 12,000 xg for 20 min.

For PPO, 400 µL of the supernatant were mixed with 950 µL of 0.02 M Na_2HPO_4, 0.08 M NaH_2PO_4 pH= 6.0 and 150 µL of 20 mM pyrocatechol. The mixture was incubated at 30 °C and the enzymatic activity was evaluated by measuring the OD increase at 410 nm.

For POD, 100 µL of extract were mixed with 0.02 M Na_2HPO_4, 0.08 M NaH_2PO_4 pH= 6.0, 2 mM pyrogallol and 4 mM H_2O_2. The mixture was incubated at 30 °C and the enzymatic activity studied by measuring the increase of OD at 470 nm.

Statistical analysis

Results for RIAs and polymers content were analyzed by Student's t-test. Data corresponding to the rest of experiments was analyzed by One-way ANOVA and means compared with Tukey as post-test (except for *in vitro B. cinerea* growth where Dunnett was used as post-test) at $p < 0.05$.

RESULTS

Fruit firmness, cell wall amount and composition

In contrast with control fruit, which flesh firmness decreased after storage, calcium chloride-treated fruit showed an arrestment on postharvest softening (Fig. 1A). Moreover, calcium-treated fruit were around 15% firmer than controls after 8 days at 4 °C + 2 days at 20 °C (8 + 2 d) (Fig. 1A).

As differences in firmness levels were detected after cold + shelf storage, for calcium chloride-treated and control fruit, these samples were used to study the effect of treatment on cell wall (as Alcohol Insoluble Residues, AIRs) content and composition. As a result, no differences in AIRs amount between control and treated fruit were observed (Fig.

1B), although differences were detected when AIRs from both groups of fruit were sequentially fractionated.

Considering pectins, the most remarkable result was observed in ionically bounded pectins (ESP) as its amount of GalA was 25% higher in treated fruit than in controls (Fig. 1D). A slightly higher (10%) content of galacturonic acid (GalA) from WSP was shown in calcium chloride-treated fruit compared to controls (Fig. 1C) and no differences in covalently bounded pectins (HSP), as well as hemicelluloses content, were evident (Fig. 1E and Supp. Fig. 1A, respectively). Additionally, a significantly greater amount of neutral sugars was detected in total pectins extracted from AIRs of calcium-treated fruit in relation to controls (Fig. 1F).

Activity of cell wall enzymes and the expression of encoding genes

The responses to calcium chloride treatment of pectin methylesterase and polygalacturonase activities as well as encoding genes were evaluated. Total PME activity increased after eight days at 4 °C + two days at 20 °C both for calcium chloride-treated and control fruit (Fig. 2A). Nevertheless, treated fruit showed a higher PME activity in relation to controls both at 0 days (0 d) and after cold + shelf storage (8 + 2 d) (Fig. 2A). Concomitantly, a higher accumulation of *FaPME1* transcript was observed in calcium-treated fruit at 0 days compared with controls (Fig. 2B). It is worth to mention, that the effect of calcium treatment on the degree of esterification (DE) of pectins was also analyzed and, accordingly with PME activity results, it was detected a lower DE on treated fruit than in controls both at 0 d and at 8 + 2 d (Fig. 2C).

Calcium chloride treatment significantly decreased total PG activity both previous (0 d) and after cold + shelf storage (8 + 2 d) when compared with not treated fruit (Fig. 2D). A down-regulation on *FaPG1* gene expression was observed in calcium-treated fruit compared to controls after treatment (0 d, Fig. 2E).

The effect of calcium chloride dips on the expression pattern of a family of pectate lyase (PL, EC: 4.2.2.2) genes (*FaPLA*, *FaPLB* and *FaPLC*) was also studied. A decrease on *FaPLA* expression was detected after storage (8 + 2 d) both for control and treated fruit but no differences on the expression levels between treated and control fruit were observed (Fig. 2F). Interestingly, calcium chloride-dips provoked a significant down-regulation of *FaPLB* expression both prior and after storage (Fig. 2G) and a lower accumulation of *FaPLC* transcripts than controls immediately after treatment (Fig. 2H).

The action of calcium chloride dips on the metabolism of pectins side chains was also evaluated. An inhibition on ² -galactosidase (EC: 3.2.1.23) activity was observed on calcium chloride-treated fruit compared to controls after assay (0 d), then activity decreased for controls remaining at the same level of treated fruit (Fig. 3A). In agreement, a down-regulation on *Fa² Gal1* expression was detected at 0 days for treated fruit and then, transcript levels decreased and were equal for both groups of fruit (Fig. 3B). No differences on the expression of *Fa² Gal4* were observed between calcium-treated fruit and controls at any time assayed (Fig. 3C).

Considering arabinans metabolism, although *FaAra1* was significantly down-regulated at 0 days in calcium chloride-treated fruit compared to controls (Fig. 3E), no effect on total ±-arabinofuranosidase (±-Ara) activity was observed by treatment (Fig. 3D).

The effect of calcium chloride-dips on the expression of genes encoding a putative ² -xylosidase (*FaXyl1*, EC 3.2.1.37), xyloglucan endo-transglycosylase (*FaXTH1*, EC 2.4.1.207) and an expansin (*FaExp2*) was also studied. It was shown a decrease in the expression levels for the three genes considered after cold + shelf storage for both groups of fruit (Supp. Fig. 1B-D). Nevertheless, no differences in the accumulation of any transcript at any time of assay between calcium-treated and controls fruit were evident (Supp. Fig. 1B-D).

***In vitro* and *in vivo Botrytis cinerea* growth**

Growth of *Botrytis cinerea* mycelia on agar plates containing AIRs from calcium chloride-dipped or control fruit as the only carbon source was evaluated. As a result, the radial growth of *B. cinerea* on plates containing AIRs from treated fruit (Ca^{2+} 8 + 2 d) was significantly lower than non-dipped (C 8 + 2 d) fruit at each time evaluated (24, 72 and 120 h post inoculation, hpi) (Fig. 4). In order to discard an inhibitory effect of calcium chloride *per se*, agar plates containing 10 g L^{-1} $CaCl_2$ + AIR from treated fruit (Ca^{2+} 8 + 2 d) were included. As a result, it was shown a higher rate of growth of *Botrytis* (reaching Petri dish edge at 72 hpi) on these plates when compared to those containing AIR from controls and calcium-treated fruit (Fig. 4).

The effect of calcium chloride treatment on fruit resistance to *B. cinerea* infection was also studied. Fruit from calcium-treated and controls were inoculated with a suspension of fungal conidia and then stored at 20 °C for 6 days. Two days post-inoculation (dpi), 81% of controls and 57% of calcium-treated fruit showed incipient mycelia in the inoculation zone (Fig. 5A). At 4 dpi, 17% of controls fruit showed mycelia in the inoculation zone, 45% incipient lesion beyond the inoculation zone + mycelia, 29% moderate lesion beyond the inoculation zone + mycelia and 5% putrefaction symptoms, while percentages of fruit with the same kind of lesions after calcium chloride treatment were 38%, 50%, 7% and 2%, respectively (Fig. 5B). At 6 dpi calcium chloride-treated fruit continued showing a reduction on the severity of fungal infection (21% mycelia in the inoculation zone, 21% incipient lesion beyond the inoculation zone + mycelia, 52% moderate lesion beyond the inoculation + mycelia and 2% putrefaction symptoms) compared with controls (5%, 14%, 67% and 10% respectively) (Fig. 5C).

Defense responses

An enhancement in polyphenol oxidase activity was detected in calcium chloride-treated fruit in comparison with controls just after treatment (0 days). At 8 days at 4 °C + 2 days at 20 °C (8 + 2 d), values in treated tissues decreased and reached similar levels to those of controls (Fig. 6A).

Total peroxidase activity increased between 0 and 8 days at 4 °C + 2 days at 20 °C both in controls and treated fruit. Nevertheless, calcium-treated fruit showed a significantly higher POD activity than control group both prior and after cold + shelf storage (Fig. 6B).

An increase in chitinase activity was detected in calcium chloride-treated fruit at 0 days. After 8 days at 4 °C + 2 days at 20 °C, levels on enzyme activity remained at similar values in both groups (Fig. 6C).

For ² -1,3-glucanase, the enzymatic activity in calcium chloride-dipped fruit was higher than in the corresponding controls after storage (8 + 2 d, Fig. 6D). No differences in phenylalanine ammonia lyase (PAL) activity were observed between controls and treated fruit at any time analyzed (Supp. Fig. 1E).

DISCUSSION

Besides turgor pressure, pectins metabolism is central to determine strawberry fruit texture.[3;15;16] Pectin is a structural heteropolysaccharide, consisting of homogalacturonan (HG) and type I and type II rhamnogalacturonan regions (RG-I and RG-II, respectively).[16] PMEs catalyzed the de-esterification of galacturonic acid from HG backbone, allowing calcium bridges formation and also providing the substrate of pectinases such as PGs and PLs.[2,17] Besides HG´s structure preservation, it has been shown that the side-chain conformation of the RG-I are tightly related to fruit firmness, being the study of ² -Gal and ±-Ara activities a matter of interest.[18,19]

The ability of calcium ions treatments to preserve strawberry cell wall structure has been studied by others research groups mainly through physicochemical approaches as cell wall mono- and polysaccharides composition, transmission electron microscopy, nuclear magnetic resonance, high-performance liquid chromatography and atomic force microscopy.[7,9]

In the present work, the biochemical and molecular basis which might explain the efficacy of calcium ions treatments to preserve strawberry cell wall structure were explored.

Similarly to results reported for strawberry fruit dipped in 1% (w/v) calcium gluconate and 2% (w/v) calcium chloride,[8,9] higher firmness levels in calcium chloride-treated fruit were observed after 8 days of cold + 2 days of shelf storage comparing with non-treated fruit (Fig. 1A). When total cell wall content was analyzed, no differences between treated and control fruit were observed (Fig. 1B). Nevertheless, calcium dips affected cell wall composition, especially increasing the amount of ionically-bound pectins (IBP or ESP) as well as neutral sugars content on pectins branch chains when compared with controls (Fig. 1D and F). In that sense, it has been reported that strawberry uptake of exogenous calcium ions increases the amount of chelate-soluble pectins, thus enhancing cell wall stability and preventing the dissolution of the middle lamella.[7;9] Notably, Zhang *et al*.[9] informed that, after cold storage, strawberries from cv Sijichun showed cross-linked structures and lower degradation of linear, branched and polymeric structures than not treated fruit. In addition, larger percentages of longer and wider IBP molecules (length e 800 nm and width e 90 nm) were observed in calcium-treated group as regards controls.

Besides maintaining cell wall structure through Ca^{2+}-dimethyl esterified pectin networks, it has been suggested that calcium ions are able to preserve pectin structure by the inactivation of pectinases.[9,20] To our knowledge, the present work constitutes the first one reporting the effect of calcium on relevant enzyme activities for strawberry pectin

metabolism such as pectin methylesterase,[21] polygalacturonase,[22] ²-galactosidase[23] and ±-arabinofuranosidase.[18] A positive regulation of total PME activity by calcium was detected, being this result confirmed by assaying the degree of pectins esterification (Fig. 2A and C). On the other hand, calcium dips were able to inhibit both total polygalacturonase and ²-galactosidase activities (Fig. 2D and Fig. 3A, respectively).

In strawberry, cell wall enzymes are typically encoded by a gene family.[23-26] Accordingly, we were interested to study the effect of calcium ions on the expression of those genes specifically expressed in fruit and/or those which were functionally characterized trough antisense technologies and proved to be relevant to determine fruit firmness (*FaPME1*[26], *FaPG1*[22;27], *FaPLB, FaPLC*[25,28], *FaGal1*, *FaGal4*[19,23] and *FaAra1*[18]). We detected an up-regulation of *FaPME1* and a significant down-regulation of *FaPG1*, *FaPLB*, *FaPLC*, *FaGal1* and *FaAra1* on calcium-treated fruit regarding controls (Fig. 2E, G, H; Fig. 3B, E). Notably, the most pronounced calcium effects both in enzymatic activities and gene expression were found prior to cold + shelf storage (0 days). Accordingly, we suggest that higher fruit firmness and a more preserved cell wall structure of calcium-treated fruit after storage (8 + 2 d), is closely related to the down-regulation of genes involved in fruit pectin disassembly (*FaPG1*, *FaPLB, FaPLC*, *Fa² Gal1*), as well as an up-regulation of *FaPME1* just after assay. Thus, calcium ions would be maintaining ionically-bound pectins structure and middle lamella through an up-regulation of PME activity and its related gene expression, allowing a higher number of Ca^{2+}-pectin bridges to

be formed and, simultaneously down-regulating the activity and gene expression of well-known pectin degrading enzymes.

When the ability of *Botrytis cinerea* to growth both in a medium containing isolated cell walls (AIRs) as well as on the surface of fresh fruit was evaluated, a reduction on the growing area an also in the percentage and severity of lesions were detected in calcium-treated fruit in comparison with controls (Fig 4 and Fig 5, respectively). Lower *in vitro* rate growth and *in vivo* infection of *Botrytis* might be explained by the effect of calcium on the strengthened of cell wall (which would limit the accessibility of pathogen's hydrolases to its plant substrates), and/or through an activation of fruit defense responses. Indeed, Lara *et al*.[7] suggested that the improved strawberry resistance to fungal attack could arise from increased biosynthesis of protective proteins by calcium treatment.

Hence, the effect of calcium ions on a set of strawberry enzymatic activities well documented as related to plant defense against pathogens invasion[1] was studied. The enzyme activity of polyphenol oxidase, peroxidase and chitinase increased over levels found in controls after treatment (0 days); while higher activity of ² -1,3-glucanase was observed in treated fruit compared with control after cold + shelf storage (Fig. 6). Although an enhancement of defense activities in strawberry fruit has been informed for others postharvest treatment such as UV-C irradiation[6] and methyl jasmonate[29], the present work is the first one providing biochemical information about calcium effect on strawberry resistance mechanisms to fungal pathogens.

As a final remark, it is worth to consider that calcium ions might be acting directly on the activation of these defense enzymes, but also treated fruit could be responding to a plant elicitor generated through the increase on PME activity by calcium. In this sense, it has been reported that the overexpression of *FaPME1* gene in woodland *Fragaria vesca* generate partially demethylated oligogalacturonides (OGAs) with eliciting capacity.[30] In addition and, through metabolomics and transcriptomics studies, authors showed that the generation of these partially demethylated OGAs might reinforce the plant defense system and also play an active role in fruit development.[31] In this way, although further studies are needed in order to confirm this hypothesis, it could be possible that the up-regulation of *FaPME1* by calcium dips elicits the defense responses observed in treated fruit, thus contributing to restrict fungal pathogen's invasion and preserving strawberry fruit quality.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Figure legends

Fig. 1 Effect of calcium chloride treatment on strawberry fruit firmness (A), cell wall content per Kg of fruit (B), GalA-WSP (C), GalA-ESP (D), GalA-HSP (E) and neutral sugars (F) content per Kg of AIR. C 0 d, Ca^{2+} 0 d: control and calcium chloride treated fruits prior storage; C 8 + 2 d, Ca^{2+} 8 + 2 d: control and calcium chloride treated fruits after 8 days at 4 °C + 2 days at 20 °C. Different letters indicate statistically significant differences at $p < 0.05$ (One-way ANOVA and Tukey for A and Student's t-test for B-F).

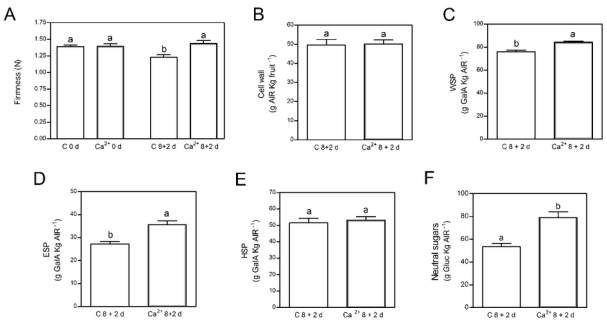
Fig. 2 Calcium chloride effect on PME activity and *FaPME1* gene expression (A, B), degree of pectin esterification (% DE, C), PG activity and *FaPG1* gene expression (D, E), *FaPLA*, *FaPLB* and *FaPLC* gene expression (E, F, G). Different letters indicate significant differences among mean values ($p < 0.05$, ANOVA, Tuckey).

Fig. 3 Effect of calcium chloride dips on ²-Gal activity, *Fa²Gal1* and *Fa²Gal4* gene expression (A, B, C), ±-Ara activity and *FaAra1* gene expression (D, E). Different letters indicate significant differences among mean values ($P < 0.05$, ANOVA, Tuckey).

Fig. 4 *In vitro Botrytis cinerea* growth on agar plates containing AIRs from control (C), $CaCl_2$ treated fruit (Ca^{2+}) and $CaCl_2$ + AIR from treated fruit (10 g L^{-1} $CaCl_2$ + Ca^{2+}) after cold + shelf storage (8 + 2 d). Asterisks indicate significant differences between treatments and controls (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, One-way ANOVA, Dunnett).

Fig. 5 Effect of calcium chloride dips on strawberry' susceptibility to *Botrytis cinerea* infection after 2, 4 and 6 days post inoculation (dpi, A, B and C respectively). Different grade of damage: mycelia on the inoculation zone (white bar); incipient lesion beyond the inoculation zone + mycelia (light gray bar); moderate lesion beyond the inoculation zone + mycelia (dark gray bar) and putrefaction symptoms (black bar).

Fig. 6 Calcium chloride effects on polyphenol oxidase (PPO), peroxidase (POD), chitinase (Chi) and ² -1,3-glucanase (² -1,3-Gluc) activities. Different letters indicate statistically significant differences at $p < 0.05$ (One-way ANOVA and Tukey).



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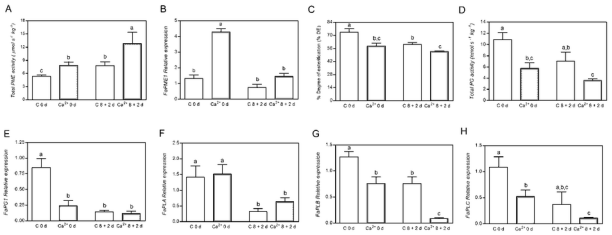

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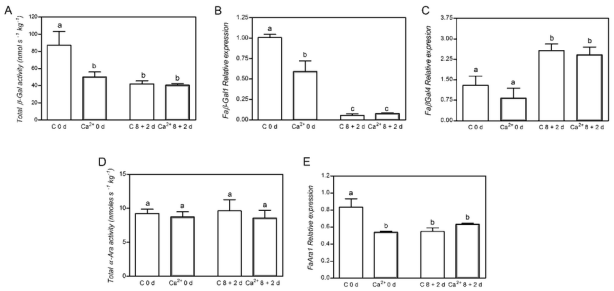

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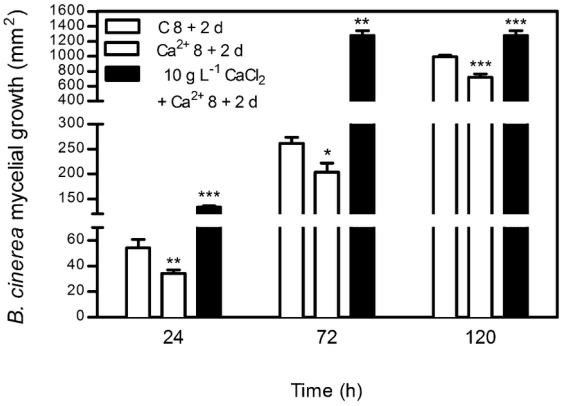

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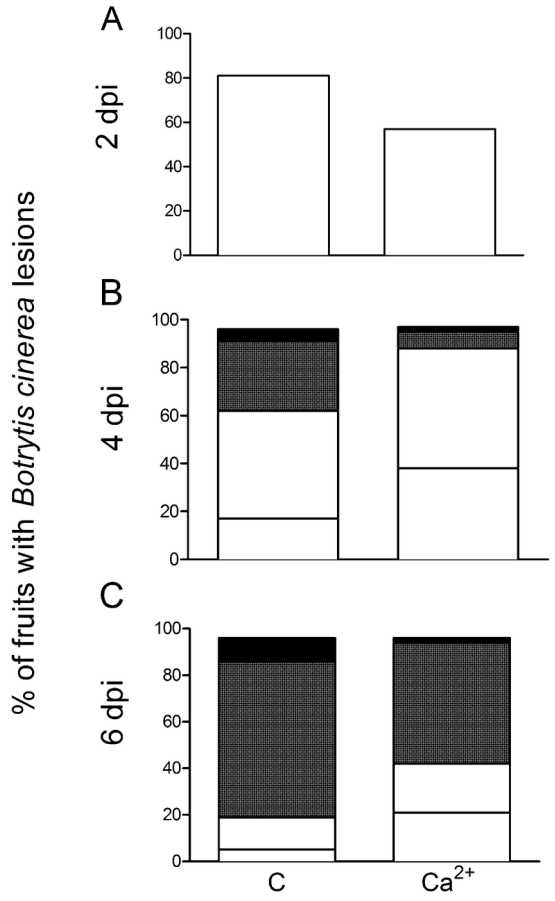

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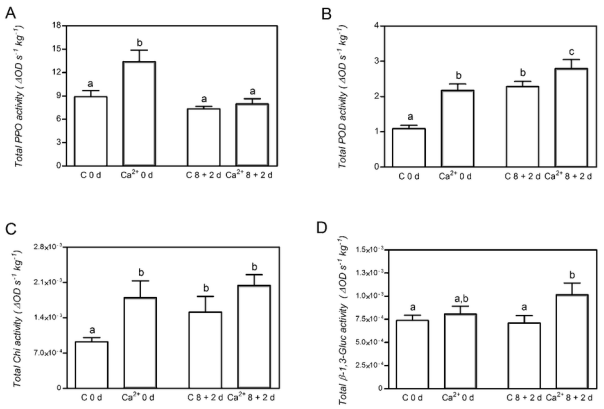

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