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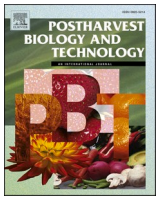

Exogenous abscisic acid mitigates chilling injury in zucchini during cold storage by eliciting a time-dependent shaping of specialized metabolites

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ABSTRACT

Chilling injury is a physiological disorder that appears when zucchini fruit is stored at low temperatures, causing a severe diminution of the quality and nutraceutical value. Abscisic acid (ABA) has been proven to be a key natural agent preventing low-temperature damage. This work aimed to elucidate the changes in exocarp metabolites of zucchini fruit during cold storage and the mechanisms underlying the protective effects of ABA through an untargeted metabolomics approach. A time-dependent metabolic modulation could be observed in response to cold storage, where exogenously applied ABA elicited distinct metabolomic signatures. Supervised statistics were then used to identify methyl jasmonate, heliespirone C, and (indol-3-yl)acetyl-L-phenylalanine as the key compounds in the fruit exocarp having the highest discriminant ability. Noteworthy, the untargeted phenolic profile of zucchini exocarps was also distinctively modulated amid the different treatments. Overall, the implication of ABA in accumulating specialized metabolites, having a dual role in chilling injury mitigation during cold stress and increasing the nutraceutical properties of zucchini fruits, was observed.

1. Introduction

Nowadays, fruit quality maintenance throughout the transport and commercialization period is a crucial concern at an industrial level, being low-temperature storage the most widely used technique to avoid the rapid deterioration of these food products. However, subtropical horticultural crops that are harvested at an immature stage, such as zucchini fruit, suffer a physiological disorder named chilling injury (CI) when are exposed to low temperatures (< 10 ºC) (Carvajal et al., 2011; Valenzuela et al., 2017), affecting to the cell wall and epicuticular wax composition (Carvajal et al., 2015a, 2021), which leads to weight loss (WL), softening and the development of pitting in the fruit surface as its main symptoms. These injuries harm fruit quality and negatively impact the commercial outcome of zucchini, leading to significant economic losses (Ali et al., 2023; García et al., 2020).

Several approaches have been investigated to cope with CI in zucchini fruit, mainly represented by the application of physical treatments, such as temperature preconditioning or the design of edible coatings (Carvajal et al., 2015b; Castro-Cegrí et al., 2023b), as well as the exogenous application of several chemical treatments, like putrescine or γ-aminobutyric acid that induce the GABA shunt pathway, producing ATP and NADH (Palma et al., 2015, 2019), nitric oxide involved in the antioxidant defence system (Jiménez-Muñoz et al., 2021) or abscisic acid, a key molecule in cold tolerance acquisition in several varieties of zucchini fruit. (Carvajal et al., 2017).

Among these, abscisic acid (ABA) has emerged as a promising candidate, as it plays an important role in the tolerance induction against abiotic stress in plants (Guajardo et al., 2016; Rizvi et al., 2022), and it was found effective in the response against chilling injury in fruits (Tang et al., 2022; Zhao et al., 2022). ABA is recognized as a key molecule improving the postharvest cold tolerance of zucchini fruit by regulating the transcriptomic and antioxidant system (Benítez et al., 2022; Castro-Cegrí et al., 2023c), as well as inducing several changes in primary metabolism during cold storage (Castro-Cegrí et al., 2023a). Consequently, depicting the mechanism of action of ABA at a metabolic level would confer robust support to better understand its effectiveness in the maintenance of zucchini fruit quality under postharvest cold storage conditions.

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This work investigates the exogenous application of ABA on zucchini fruit under cold storage from a metabolomic perspective. Adopting a hypothesis-free untargeted profiling strategy will provide a comprehensive overview of the phytochemical reprogramming elicited by ABA application under cold storage. In fact, a dual metabolomic approach will be applied, namely a metabolomics-wide profiling to get insight into the effect of ABA on the biochemical processes triggered in zucchini exocarp, together with a phenolic profiling to shed light on the shift of polyphenol subclasses modulation in response to ABA. Accordingly, determining the untargeted metabolomic profile will shed light on the biochemical mode of action of ABA during cold storage and provide insight into the eventual prospects regarding the nutraceutical properties of zucchini fruits.

2. Material and methods

2.1. Experimental design and fruit material

Healthy and uniform zucchini fruits (*Cucurbita pepo* L. morphotype Zucchini) of the cultivar 'Sinatra' (Clause-Tezier) were provided by the company Fruits & Vegetables La ÑECA S.A.T. The experimental groups included three replicates per treatment and cold storage period, each comprising 6 uniform fruits. Freshly harvested fruits were collected at harvest (T0 time point) and subjected to the treatment, which consisted of dipping the fruit at 20 ºC for 20 min into a 0.5 mM ABA solution or distilled water, for ABA-treatment and control samples, respectively. The concentration of ABA employed was chosen according to previous assays performed in 'Sinatra' zucchini fruits (Carvajal et al., 2017). All fruits were then dried for 2 h after treatment, and further stored in permanent darkness in a temperature-controlled chamber at 4 ºC and 85 – 90% relative humidity (RH) for 1, 5 and 14 days, thus making the T1, T5, and T14 time points, respectively. After sampling, fruit was subjected to quality evaluation (see Section 2.2.) and, finally, the exocarp was completely removed, merged for each experimental group, frozen and powdered in liquid nitrogen, lyophilized, and stored at room temperature until the performance of subsequent analyses.

2.2. Weight loss, chilling injury index and electrolyte leakage determination

Each fruit's weight loss percentage was calculated using the following formula: % weight loss = [(Wi − Wf)/Wi] × 100, being Wi the initial fruit weight and Wf the final fruit weight.

The chilling injury index (CI) was evaluated using a subjective scale of visual symptoms, as previously described (Carvajal et al., 2011). Each fruit was rated according to the following scale: 0, no pitting; 1, slight (≤ 10% of pitting in fruit surface); 2, medium (10 – 20% of pitting in fruit surface); and 3, severe pitting (> 20% of pitting in fruit surface).

Electrolyte leakage was determined in the fruit exocarps. For this purpose, 10 discs were removed from each replicate with an 11-mm diameter stainless-steel cork borer. Five replicates from each experimental group were selected. Each replicate was rinsed with 50 mL of deionized water thrice for 3 min. Then, discs were incubated in 50 mL of deionized water for 30 min and shaken at 100 rpm, and the resulting solution was used to analyse the conductivity at room temperature using a conductometer. Total conductivity was determined after boiling the flasks for 10 min and cooling at room temperature. The electrolyte leakage was expressed as a percentage of total conductivity.

2.3. Untargeted metabolomic profiling of zucchini exocarp via UHPLC/QTOF-HRMS

Lyophilised samples were subjected to solvent extraction before their analytical profiling. Briefly, 50 mg of samples were mixed in 2 mL of methanol/H_2O/formic acid solution (80.0/19.9/0.1; *v/v/v*) and subjected to ultrasound-assisted extraction using a sonication bath for 30 min at room temperature. Samples were centrifuged at 8000 × *g* for 15 min at 4 ºC, and supernatants were syringe-filtered (cellulose membrane, 0.22 μm pore size) into analytical vials. The extraction was carried out in triplicate.

The metabolomic analysis of exocarps was carried out through ultra-high-performance liquid chromatography coupled to quadrupole-time-of-flight high-resolution mass spectrometry (UHPLC/QTOF-HRMS) using a 1290 UHPLC system and an electrospray ionization source (ESI)-equipped QTOF G6550 iFunnel mass spectrometer (Agilent Technologies®, Santa Clara, CA, USA). The detailed protocol for metabolomics data acquisition and compound annotation was previously optimized (Rouphael et al., 2020) and applied as indicated elsewhere, including the citations in the Supplementary File S1.

Acquired data were processed by the software MassHunter Profinder v.10.0 (Agilent®), and compounds annotation was achieved by monoisotopic accurate mass and isotopic pattern (accurate spacing and isotopes ratio), in compliance with the Level 2 of COSMOS Metabolomics Standard Initiative (putatively annotated compounds (Salek et al., 2013)), using the "Find-by-formula" algorithm against the database imported from PlantCyc v.15.5 (Plant Metabolomic Network, PMN, available at http://www.plantcyc.org). To determine the phenolic profile, the database Phenol-Explorer v.3.6 was employed in the same conditions (available at http://phenol-explorer.eu). Metabolomics data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gkad1045, PMID:37971328) with the identifier MTBLS9290.

2.4. Data processing and statistical analysis

Once annotation was completed, acquired raw data were processed by the software Mass Profiler Professional v. 15.1 (Agilent®) before their multivariate statistical analysis. For this purpose, the abundance of identified features was transformed at log2 values and normalized at the 75th percentile (Salehi et al., 2018). Then, compound abundance was baselined with respect to the median abundance values of all samples to perform an unsupervised hierarchical cluster analysis (HCA), obtaining a fold change (FC)-based heatmap (Euclidean distance, Ward's linkage rule). Later, to determine the differentially modulated metabolites, a fold change analysis was performed (FC cut-off = 2) in combination with one-way analysis of variance (ANOVA), setting a significance value of $\alpha = 0.05$, followed by the *post hoc* Tukey's honest significant difference test, applying the Bonferroni correction.

After that, a supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed using the SIMCA v.16.0 software (Umetrics®, Malmo, Sweden). The quality of the obtained models was evaluated according to their goodness-of-fit (R^2) and goodness-of-prediction (Q^2) parameters, assuming a predictability threshold of $Q^2 > 0.5$. Furthermore, models were statistically validated by cross-validation analysis of variance (CV-ANOVA, significance level, $\alpha = 0.05$), and overfitting was excluded by the performance of permutation test (n = 200). The OPLS analysis was combined with variable importance in projection (VIP) analysis to identify the metabolites with the highest influence on the discrimination within the models, the so-called VIP markers, setting a VIP score threshold of 1.0.

The interpretation of the results for the full metabolic profile obtained through PlantCyc annotation was performed on differential metabolites with respect to the initial conditions (T0) (FC > 2, one-way ANOVA $p < 0.05$) using the Omics Dashboard utility from PMN Pathway Tools (Stanford, CA, USA) (Karp et al., 2010). In the case of the phenolic profile, a semi-quantification approach was performed by grouping the annotated compounds from the Phenol-Explorer database into their corresponding subclasses and conducting the quantification of each subclass according to a representative analytical standard (Rocchetti et al., 2017). All standards were employed to construct the calibration curves under the same analytical conditions described earlier. The standards employed for semi-quantification were: cyanidin

(anthocyanins), (+)-catechin (flavanols), luteolin (flavones and other flavonoids), quercetin (flavonols), (+)-sesamin (lignans), ferulic acid (phenolic acids), tyrosol (tyrosols and other low-molecular-weight compounds) and resveratrol (stilbenes). All standards were HPLC-grade and purchased from Extrasynthase® (cyanidin, (+)-catechin, luteolin, (+)-sesamin, tyrosol, and *trans*-resveratrol) and Sigma-Aldrich® (quercetin and ferulic acid). Results were expressed as mg of standard equivalents per gram of dry weight (DW), i.e.: cyanidin equivalents (CyE) for anthocyanins, catechin equivalents (CaE) for flavanols, luteolin equivalents (LE) for flavonols, sesamin equivalents (SE) for lignans, tyrosol equivalents (TE) for low-molecular-weight (LMW) and other polyphenols, ferulic acid equivalents (FE) for phenolic acids, and resveratrol equivalents (RE) for stilbenes.

The results from fruit quality parameters (n = 3) and the semi-quantification of phenolic compounds (n = 6) were expressed as the mean ± standard deviation. In both cases, a one-way ANOVA was performed ($\alpha = 0.05$), followed by Duncan's *post hoc* test by the software SPSS v. 25.0 (IBM®, New York, US).

3. Results

3.1. The effect of cold storage on the quality parameters of zucchini fruit and the influence of ABA treatment

The endogenous content of ABA was increased after treatment in exocarp, observing the largest differences at 1 d of cold storage, as shown by Castro-Cegrí et al. (2023a). The results for zucchini fruit quality parameters, including weight loss (WL), chilling injury index (CI), and electrolyte leakage (EL) are shown in Fig. 1. According to the results, both storage time and ABA treatment significantly affected all quality parameters. Over time, an increasing trend in WL and EL was observed from the beginning, whereas the increase in CI was significantly observed from 5 days of cold storage (Fig. 1B). Concerning treatments, control samples exhibited significantly higher rates of EL from the initial time with respect to ABA. In contrast, statistically significant increases of all parameters were reported in the control over time (Fig. 1). Indeed, the highest damages were noted at 14 days with 12% WL, 2.2% CI, and 14.7% EL for control samples (Fig. 1). In all cases, at 14 days, ABA effectively maintained the postharvest fruit quality by promoting a significant decrease reduction of these parameters of 31.2% for WL, 66.4% for CI, and 23.4% for EL with respect to control fruit. In general, the preservative effect of ABA on zucchini fruit was significantly reported from the beginning of the cold storage period and was increased over time, revealing a sustained action on postharvest fruit quality.

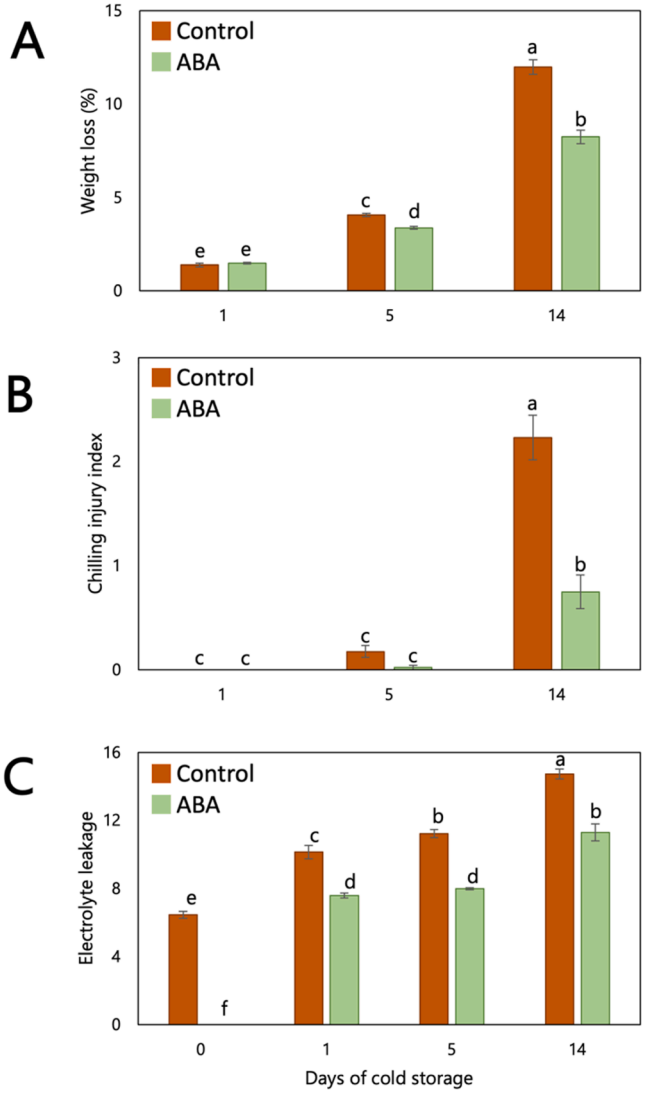

Fig. 1. Changes in percentage of weight loss (WL), chilling-injury index (CI) and electrolyte leakage (EL) of zucchini control and ABA-treated fruits at 0, 1, 5 and 14 days of cold storage. Data are presented as the mean, and vertical bars correspond to standard deviation. Different letters indicate significant statistical differences according to Duncan's test ($p < 0.05$).

3.2. Application of multivariate statistics to decipher the hierarchical effects of cold storage and ABA on the metabolic profile of zucchini exocarp

An untargeted metabolomic analysis by UHPLC/QTOF-HRMS approach was carried out to elucidate the behavior of fruit exocarps of zucchini during the cold storage period at a metabolome-wide level, and the mechanisms underlying the protective effects attributed to ABA. The application of this high throughput approach enabled the determination of 1747 putatively annotated chemical entities in the exocarps of zucchini fruit, and the full list of annotated compounds is provided in Table S1, including their abundance, retention time (min), mass (u), and the molecular formula. Overall, these data were subjected to further multivariate statistical analysis to interpret the effect of cold storage and ABA treatment on the metabolome of zucchini fruit.

Firstly, an unsupervised hierarchical cluster analysis (HCA) was performed to naively elucidate the influence of both factors (cold storage time and ABA treatment) on the metabolic profile of zucchini fruit (Fig. 2). As observed, the cold storage period was revealed to play the highest contribution to the modulation of the metabolic profile of exocarps, whereas the effect associated with ABA was found to be a secondary factor, as it did not reflect a defined profile. The fold change-based heatmap indicates that different sets of compounds were found increased after 14 days of cold storage with respect to those found increased at either 0, 1, or 5 days of storage, thus ruling the establishment of two subclusters that grouped the 14-day time point apart from those at 0, 1, and 5 days (Fig. 2).

Once the effect of cold storage period was featured, a supervised Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) was performed for each time point to better address the influence of the postharvest treatment with ABA on the metabolome of zucchini fruit (Fig. 3). The obtained models demonstrated that fruits treated with ABA exhibit a different metabolic profile compared to control fruits throughout the whole cold storage period. Noteworthy, all models from days 1, 5, and 14 present high-quality parameters in terms of goodness-of-fit (R^2 = 0.998, 0.998, and 1.000, respectively) and goodness-of-prediction (Q^2 = 0.831, 0.590, and 0.698, respectively) (Fig. 3A, B, and C, respectively). To identify the metabolites that contributed the most to the discrimination between treatments (control vs. ABA), a VIP analysis was performed for each OPLS-DA model, and the most

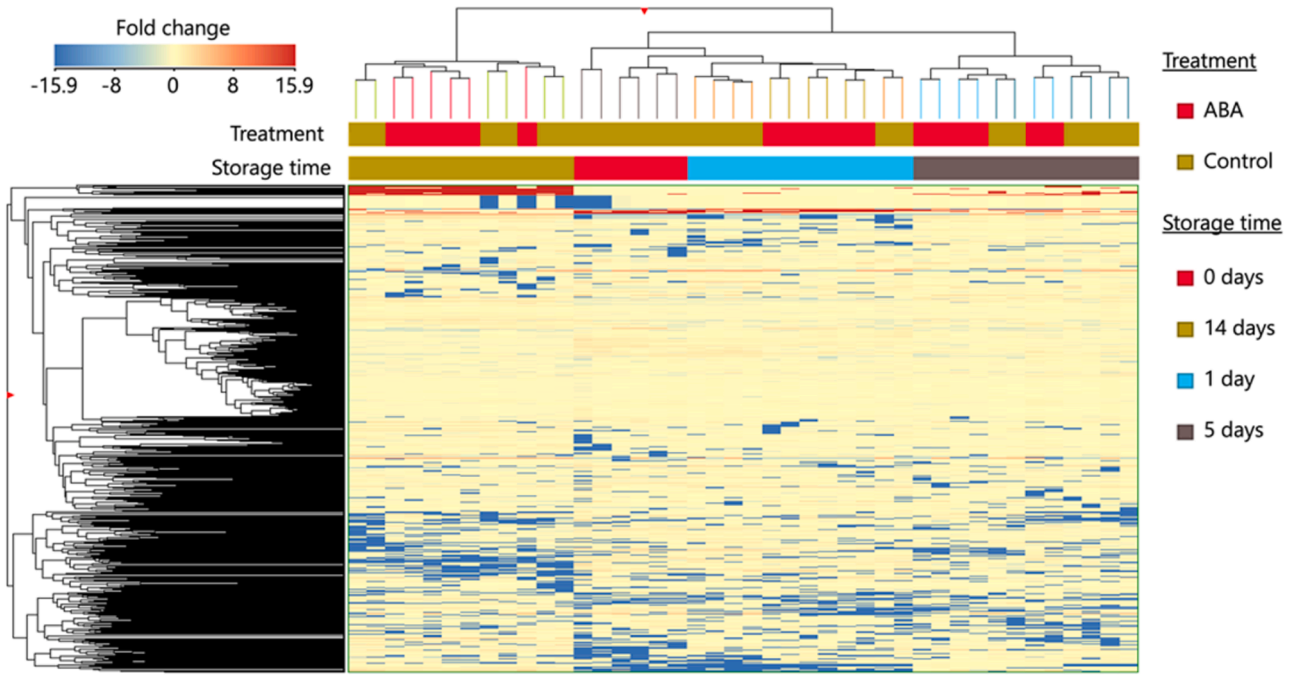

Fig. 2. Hierarchical cluster analysis of the untargeted metabolic profile of exocarps from zucchini fruits under cold storage (at 0, 1, 5, and 14 days) untreated (control) or treated with ABA (ABA). Clustering was performed according to the fold change-based heatmap, baselined to the median values of all samples (Euclidean distance, Ward's algorithm).

significant VIP markers (VIP scores > 1.9) were incorporated into a Venn diagram to detect the signature metabolites found at each time point (Fig. 3D). The full list of VIP markers (VIP score > 1.0) is provided in Table S2, together with their logFC values.

Interestingly, 3 metabolites were found as discriminant at all time points, i.e.: methyl jasmonate, heliespirone C, and (indol-3-yl)acetyl-L-phenylalanine. Indeed, all three metabolites were found to increase in ABA-treated samples with respect to control: logFC = 1.57 – 2.00 for methyl jasmonate, logFC = 1.36 – 2.42 for heliespirone C, and logFC = 1.95 – 7.09 for (indol-3-yl)acetyl-L-phenylalanine (Table S2). These results enabled pointing out the metabolites as signature markers of the exocarp of zucchini fruit treated with ABA under cold storage. The VIP markers reported for individual time points reveal a great metabolic variety of exocarp under ABA treatment (Fig. 3D). The increasing number of exclusive discriminant compounds (15 at day 1, 24 at day 5, and 32 at day) suggest an ABA- and time-dependent coordinate metabolic modulation of zucchini fruit.

Considering the short-term discriminant metabolites (exclusively observed at day 1) glucosinolates and derivatives were widely identified, as shown for 2-[(7′-methylsulfanyl)heptyl]malate, (*E*)-5-(methylsulfanyl)pentanal oxime, and 3-[(4′-methylsulfanyl)butyl]malate, which were slightly accumulated in ABA-treated exocarps (logFC = 0.22 – 0.25; Table S2). Besides these stress-related specialized metabolites, the short-term application of ABA promoted the up-accumulation of salicylate 2-*O*-β-*D*-glucoside (logFC = 0.21) and the amino acids hypoglycine A (logFC = 0.28), and N^5-methyl-L-glutamine (logFC = 0.54), as well as the amine acetylcholine (logFC = 1.62).

Regarding the mid-term VIP markers (those exclusively found at day 5), a wide variety of phenolic compounds were identified, showing a general down-regulation due to ABA treatment (Fig. 3D), as observed for the lignan (-)-bursehernin (logFC = −0.28), the anthocyanin cyanidin 5-*O*-β-D-glucoside 3-*O*-β-D-sambubioside (logFC = −0.65), and the flavonoids isorhamnetin 3-*O*-(6"-*O*-feruloyl)-glucoside and 3,7-dimethylquercetin (logFC = −0.65 and −0.34, respectively; Table S2). Moreover, two phytosterols exhibited increased levels in ABA-treated exocarps, namely campest-5-en-3-one and stigmasterol (logFC = 1.29 and 0.10, respectively), whereas two lipids were found down-accumulated:

1–18:3–2–18:3-digalactosyldiacylglycerol and 1-linoleoyl-2-oleoyl-phosphatidylcholine (logFC = −0.19 and −1.13, respectively; Table S2).

Finally, concerning long-term VIP markers (those exclusively found on day 14), a high heterogeneity of compounds was recorded (Fig. 3D). Essentially, an overall down-accumulation of polyphenols and glucosinolates in response to ABA treatment was observed, as it was shown for the lignan (+)-sesaminol 2-*O*-beta-D-glucoside (logFC = −4.34), and the glucosinolates 4-hydroxybutylglucosinolate, desulfoglucobrassicin, and glucobrassicin (logFC = −0.83 – −0.46). In the same way, phytohormones and analogues were found down-accumulated by ABA, as found for *trans*-tuberonic acid, gibberellin A_5, kinetin-9-*N*-glucoside, and the strigolactone (+)-5-deoxystrigol (logFC = −1.60 – −0.71; Table S2). In contrast, the ABA derivative β-D-glucopyranosyl abscisate was found to accumulate (logFC = 1.68). On the contrary, lipid metabolites showed a slight accumulation, especially fatty acids and derivatives, such as tetradecan-1-ol, palmitaldehyde, arachidonate, linoleate, and di-homo-γ-linoleate (logFC = 0.23 – 0.47; Table S2).

3.3. The modulation of biosynthetic metabolism of zucchini exocarp under cold storage in response to ABA treatment

To get insight into the modulation of the biosynthetic metabolism of zucchini fruit under cold storage treated with ABA over time, a selection of significantly differential compounds was performed, according to the statistical significance (one-way ANOVA, $p < 0.05$) and fold change values (FC > 2; FC < −2) with respect to the initial conditions (day 0). A total of 316 compounds satisfied both criteria and were subjected to pathway analysis. The full list of significant compounds is provided in Table S3, together with their logFC values and the chemical ontology provided by the PlantCyc database. The results for the modulation of metabolic biosynthesis allowed us to decipher the metabolic fingerprint triggered by cold storage and ABA treatment with respect to the initial conditions, featuring the clear effect of the storage period and, secondarily, that attributed to ABA (Fig. 4).

Regarding general biosynthesis, secondary metabolism showed the highest positive modulation, followed by fatty acid, lipid metabolism,

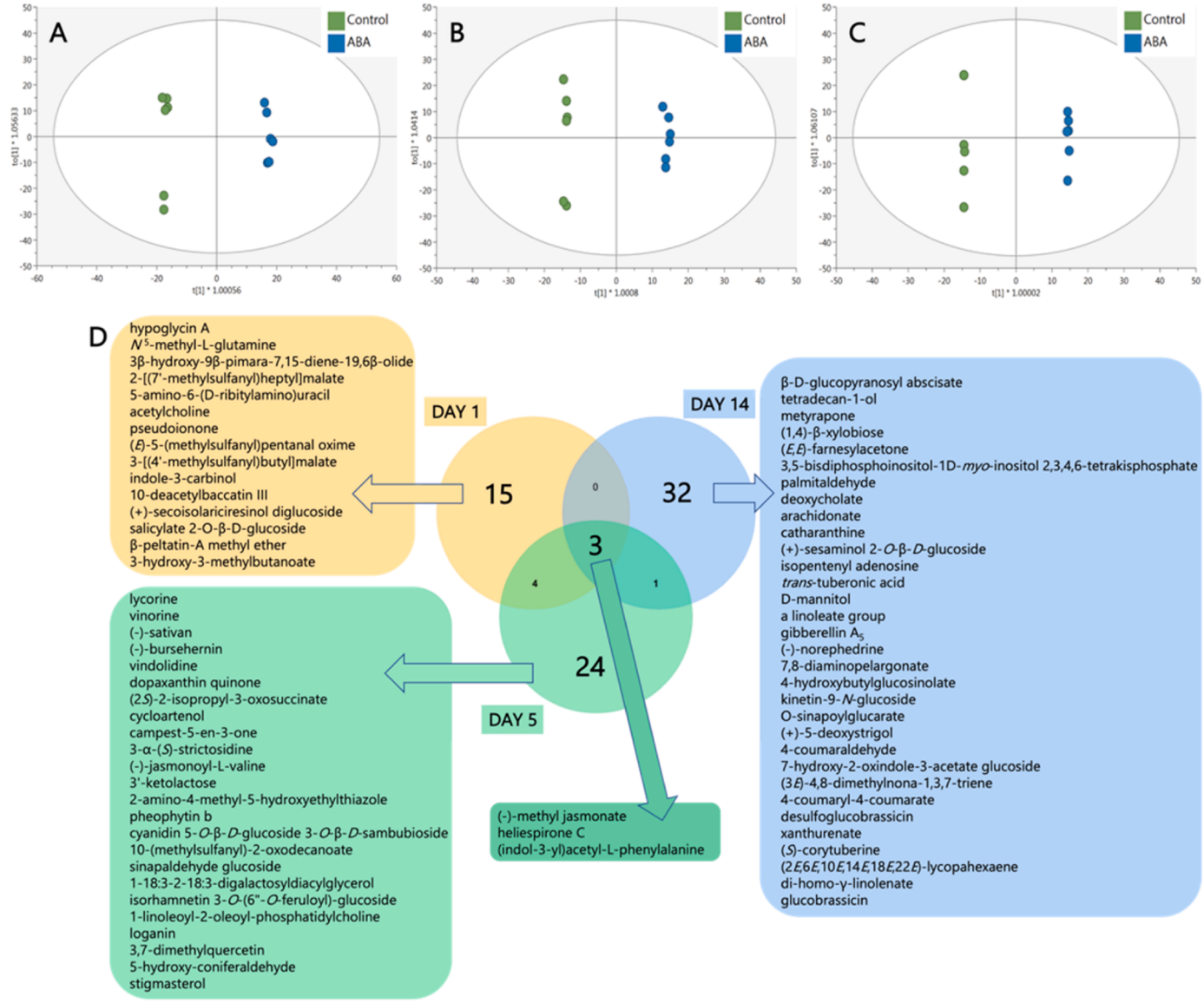

Fig. 3. Multivariate OPLS-DA models for the metabolic profile of exocarps from zucchini fruits under cold storage untreated (control) or treated with ABA (ABA). A. OPLS model for day 1 of cold storage. B. OPLS model for day 5 of cold storage. C. OPLS model for day 14 of cold storage. D. Venn diagram for the VIP markers found as discriminant for each model (VIP score > 1.9).

and phytohormone biosynthesis (Fig. 4A). In all cases, a time-dependent positive modulation was observed, increasing the effect of cold storage over time, differently affected by ABA. Thus, a subtle modulation was observed in the short term, showing a slight down-accumulation of secondary metabolism and lipid metabolism at day 1 (Fig. 4A). In contrast, a mid-term elicitation was observed, reaching higher rates due to ABA, as observed on day 5. However, the stimulation of biosynthetic metabolism was maximal in the long term, reaching the highest modulation rates in the case of control fruit (Fig. 4A).

Due to the noticeable increase reported for the secondary metabolism, a deeper insight revealed that the biosynthesis of three major families was elicited by cold storage and ABA, following the trend: phenylpropanoids, nitrogen-containing compounds (NCCs), and terpenoids, and reporting the same time- and ABA-dependent influence described earlier (Fig. 4B). In the case of phenylpropanoids, polyphenol biosynthesis was predominantly triggered over time for both control and ABA treatments, being represented by the flavonoids 4'-hydroxy-rot-2'-enonate, ternatin C5 (logFC = 8.10 and 9.20, respectively for both compounds), and 3,7,3',4'-tetramethylquercetin (logFC = 5.00 and 5.27, respectively), and the lignans (+)-sesaminol and diphyllin (logFC = 8.10 and 9.20, respectively for both compounds; Table S3). However, the highest induction was observed for the control exocarp at day 14 due to the wider biosynthetic coverage that affects other compounds, such as the lignan (-)-deoxypodophyllotoxin, and the isoflavonoids dalcochinin-8'-*O*-β-glucoside and (*S*)-dihydrodaidzein (logFC = 8.10 for all compounds; Table S3).

Concerning NCCs biosynthesis, the same trend as for phenylpropanoids was observed, reaching the highest elicitation rates at day 14 on either control or ABA-treated exocarps (Fig. 4B). In general, the NCCs showing the strongest biosynthetic up-regulation at day 14 were alkaloids, i.e., berbamunine, deoxypumiloside, β-chaconine, and 3-α(*S*)-strictosidine, all of them showing logFC = 8.10 for control and logFC = 9.20 for ABA (Table S3). Interestingly, *N*-hydroxytetrahomomethionine, an amino acid derivative recognized as a precursor of glucosinolates, exhibited an enhanced long-term biosynthesis (logFC = 8.10 for control and logFC = 9.20 for ABA at day 14; Table S3).

Concerning terpenoids biosynthesis, increasing up-regulation rates were found accordingly throughout the cold storage period, reaching the

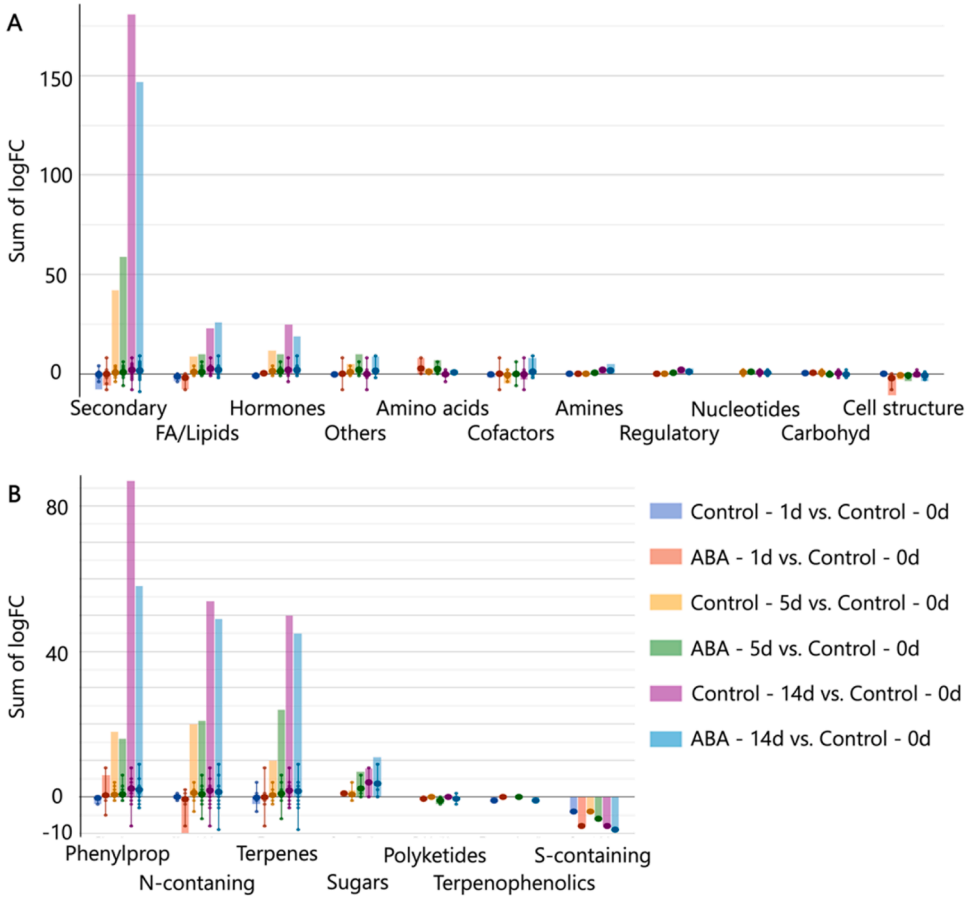

Fig. 4. Pathway analysis on the metabolic profile of exocarps from zucchini fruits under cold storage (at 1, 5, and 14 days) untreated (control) or treated with ABA (ABA). A. Modulation of biosynthetic pathways. B. Modulation of secondary metabolism. Results are expressed as the sum of the logarithm of fold change (logFC) values of each category with respect to day 0. Only the compounds showing a differential abundance (FC > 2 or FC < −2) and statistically significant differences according to one-way through one-way ANOVA and Tukey HSD *post hoc* test ($p < 0.05$) with respect to harvest conditions at day 0 were considered. The interpretation was achieved by PlantCyc Pathway Tool (https://www.plantcyc.org). Large dots represent the average logFC values of each category, whereas small dots represent the logFC values of individual compounds. Secondary, secondary metabolism biosynthesis; FA/Lipids, fatty acid and lipid biosynthesis; regulatory, regulatory metabolites biosynthesis; carbohyd, carbohydrate biosynthesis; cell structure, cell structure-related metabolites biosynthesis; phenylprop, phenylpropanoids biosynthesis.

highest values at day 14 for either control or ABA-treated exocarps (Fig. 4B). The compounds showing the highest accumulation at day 14 were represented essentially by apocarotenoids, such as 3β-hydroxy-β-cyclocitral and bixin dimethyl ester, the diterpenoid stevioside, and the sesquiterpenoid desoxyhemigossypol-6-methyl ether (all of them showing logFC = 8.10 for control and logFC = 9.20 for ABA; Table S3). Interestingly, regarding sugar metabolism, a slight up-regulation was observed over time, mostly represented by the polyol galactopinitol B (logFC = 8.10 for control and logFC = 9.20 for ABA at day 14; Table S3).

Beyond secondary metabolism, lipid biosynthetic metabolism was also induced according to the cold storage period, reaching the highest rates at day 14 for ABA-treated fruit (Fig. 4A), with sterol biosynthesis playing the highest role. In fact, cycloartenol was highly up-regulated in ABA-treated samples from day 5 (logFC = 4.17 and 4.75 at days 5 and 14, respectively; Table S3), as well as campest-5-en-3-one (logFC = 3.64 and 4.06 at days 5 and 14, respectively; Table S3). In the same way, phytohormone biosynthesis was induced over time, reaching the highest up-regulation at day 14 (Fig. 4A). The results show that the biosynthesis of *trans*-zeatin derivatives was up-regulated in the presence of ABA, being maximal at day 14 for *trans*-zeatin-7-*N*-glucoside (logFC = 1.15) and *trans*-zeatin riboside diphosphate (logFC = 9.20; Table S3). In contrast, the up-regulation of kinetin biosynthesis was only observed in the absence of ABA, as indicated by kinetin-9-*N*-glucoside (logFC = 4.00 and 8.10 reported at day 5 and day 14, respectively, in control samples; Table S3).

3.4. The effect of cold storage and ABA treatment on the phenolic profile of zucchini exocarp

Following the results involving the biosynthetic modulation of zucchini exocarp under cold storage in response to ABA, phenylpropanoid derivatives' biosynthesis was strongly up-regulated. Therefore, the untargeted UHPLC/QTOF-HRMS phenolic profiling of zucchini was carried out to determine the importance of both factors on the accumulation of polyphenols by zucchini fruit.

The phenolic profiling of zucchini samples provided a total of 265 chemical entities that were putatively annotated through the Phenol Explorer database. The full list of annotated features, including their phenolic class and subclass, abundance values, retention time (min), mass (u), and molecular formula, is provided in Table S4. A wide variety of phenolic compounds was found within the annotated features, flavonoids being the most represented group with 139 annotated compounds- mainly divided into anthocyanins (44) and flavonols (29), 49 phenolic acids, from which 38 were hydroxycinnamic acids, 15 lignans and 8 stilbenes. In addition, 53 compounds were grouped into other phenolic subclasses, being collectively classified as low-molecular-weight phenolic compounds (LMW), such as tyrosols or alkylphenols (Table S4).

As previously described, a combination of multivariate statistical analyses combining HCA and OPLS-DA allowed the influence of ABA in cold-stored zucchini fruit to be deciphered based on the phenolic profile of exocarp. The results for HCA indicated that subtle differences were found in the exocarp of zucchini fruit regarding their phenolic profile, as only the samples from day 14 were independently grouped in an independent subcluster (Figure S1). In the same way, the ABA treatment did not have a marked effect on the phenolic profile of zucchini exocarp.

To better discriminate the effect of ABA treatment over time, a set of supervised OPLS-DA models were performed for each time point (1, 5, and 14 days), and results are displayed in Fig. 5. Additionally, each OPLS-DA model was combined with VIP analysis to determine the metabolite markers most involved in the discrimination between treatments, and the complete list of VIP markers (VIP score > 1.0) is found in Table S5. The results from multivariate supervised analysis indicate that ABA plays a discriminant role at all time points, as reflected by the OPLS models, which show high-quality parameters in terms of goodness-of-fit and goodness-of-prediction ($R^2 > 0.99$, $0.5 < Q^2 < 0.8$ for all models; Fig. 5A – 5 C). Moreover, a Venn diagram was provided to indicate the VIP markers exclusively associated at all time points (VIP score > 1.4; Fig. 5D), revealing that the proportion of discriminating phenolic classes evolved throughout time, showing a decrease in the proportion of phenolic acids combined with an increase of LMW and other phenolic compounds, whereas the proportion of flavonoids remained stable over time, with lignans and stilbenes being poorly represented (the latter being even absent after day 1; Fig. 5D).

Once the effect of cold storage and ABA was defined on the phenolic profile of zucchini exocarp, a semi-quantitative approach was performed to determine the content of each phenolic subclass. The results are displayed in Table 1. In general terms, lignans were the most abundant compounds in samples, followed by anthocyanins. In particular, anthocyanin content was not significantly altered by any of the testing factors, ranging 20.4 – 24.5 mg CyE g^{-1} DW among different treatments. In the case of flavanols and flavones, the highest contents were observed at day 14, which did not differ significantly in response to ABA, suggesting that cold storage promoted the accumulation of those subfamilies (1.49 – 1.57 mg CaE g^{-1} DW and 4.71 – 5.04 mg LE g^{-1} DW, respectively, at day 14; Table 1). Concerning flavonols, the highest content was also recorded at day 14 (≈ 3 mg QE g^{-1} DW), although it was not significantly different from the content at harvest time (day 0). In contrast, lignan content decreased significantly over time, reaching the lowest content at day 14 in control samples, 34.8 mg SE g^{-1} DW, representing a decrease of 16.7% with respect to day 0 (Table 1). The content of LMW and other phenolics, as well as that of phenolic acids, also increased over time, being significant in the long term, reaching the highest rates at day 14 for either control and ABA-treated samples (18.8 and 18.6 mg TE g^{-1} DW, respectively, for LMW phenolics and 18.7 and 17.2 mg FE g^{-1} DW, respectively, for phenolic acids; Table 1). Finally, stilbenes content did not vary significantly through the experimental course, being positively affected by ABA at all times. However, differences were not recognized statistically, showing all phenolic subfamilies' lowest content, ranging 1.36 – 2.08 mg RE g^{-1} DW (Table 1).

4. Discussion

The role of ABA inducing a tolerant response in plants against abiotic stresses, such as drought or low temperatures, has been widely discussed (Guajardo et al., 2016; Rizvi et al., 2022). Interestingly, ABA has been

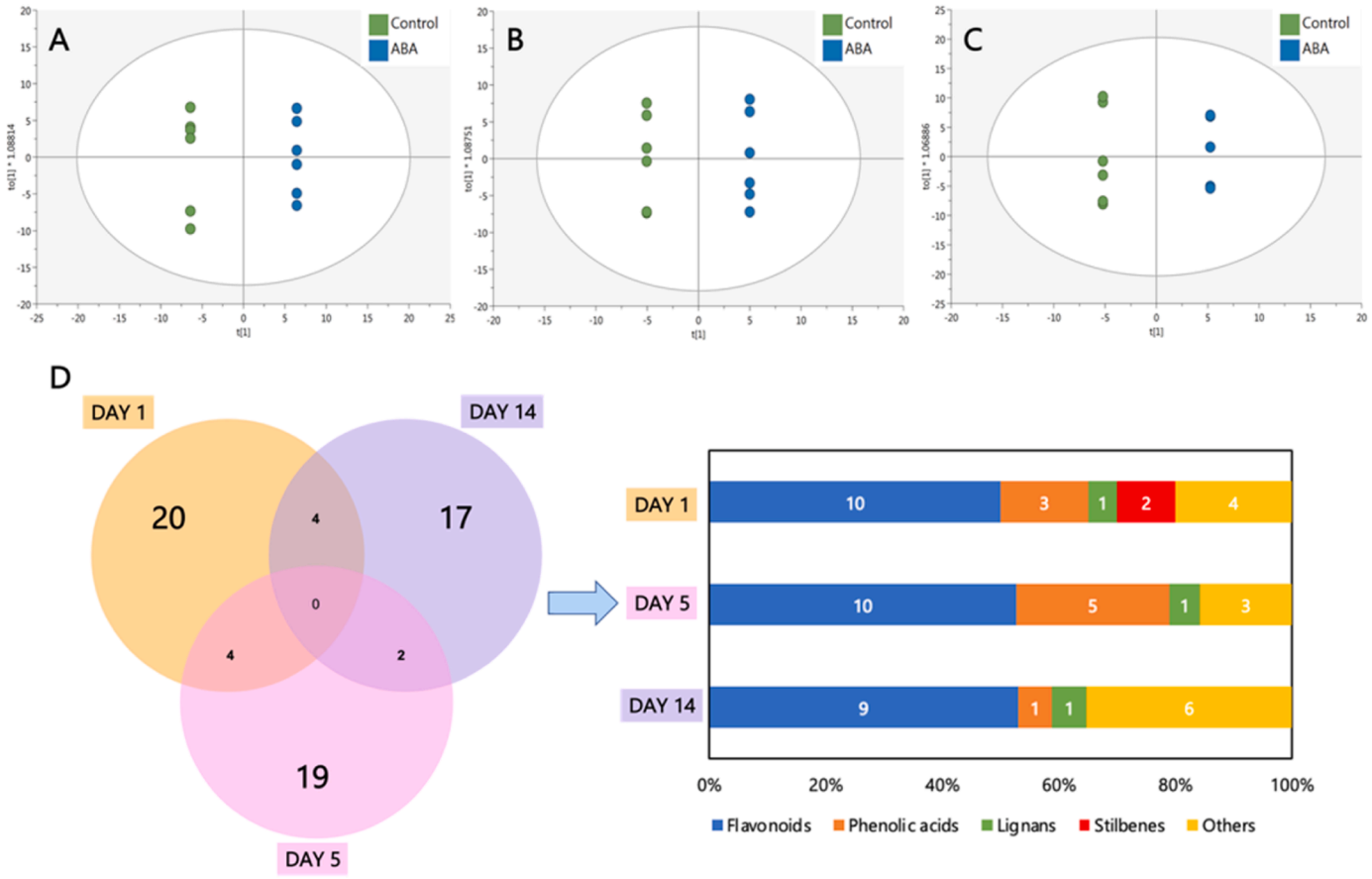

Fig. 5. Multivariate OPLS-DA models for the phenolic profile of exocarps from zucchini fruits under cold storage untreated (control) or treated with ABA (ABA). A. OPLS model for day 1 of cold storage. B. OPLS model for day 5 of cold storage. C. OPLS model for day 14 of cold storage. D. Venn diagram for the VIP markers found as discriminant for each model (VIP score > 1.4), together with their proportion at each time point.

Table 1
Semi-quantitative determination of phenolic content of exocarps from zucchini fruits under cold storage (at 1, 5, and 14 days) untreated (control) or treated with ABA (ABA).

Days of storage at 4 ºC	0	1		5		14	
	At harvest	Control	ABA	Control	ABA	Control	ABA
Anthocyanins	20.36±3.64a	21.07±5.37a	20.76±4.4a	24.49±2.47a	22.24±2.53a	21.6±1.61a	22.53±2.4a
Flavanols	1.13±0.33b	1.17±0.36b	1.23±0.35ab	1.35±0.31ab	1.37±0.27ab	1.57±0.24a	1.49±0.2ab
Flavones	2.88±0.29c	3.05±0.24bc	3.15±0.69bc	3.62±0.88b	3.59±0.44b	5.04±0.34a	4.71±0.44a
Flavonols	2.96±0.43a	2.29±0.35bc	1.77±0.29c	2.18±0.47bc	2.6±0.64a	3.06±0.7a	3.1±0.44a
Lignans	41.82±3.4a	37.73±1.7b	38.38±1b	38.66±0.4b	38.6±0.99b	34.82±4c	38.03±0.6b
LMW and others	15.2±2.86bc	13.26±2.23c	16.54±1.1ab	16.32±1.6ab	16.14±2.3ab	18.8±2.9a	18.6±2.17a
Phenolic acids	13.09±3.1c	14.69±3.5bc	15.02±1.8bc	14.55±3.6bc	15.7±1.3abc	18.7±3.5a	17.21±2.1ab
Stilbenes	2.08±0.22a	1.32±0.18b	1.66±0.3ab	1.36±0.13b	1.6±0.56b	1.54±0.5b	1.71±0.41ab

Results are expressed in mg of reference standards for each subfamily per gram of dry weight (mg g-1 of dry weight). Data are expressed as mean ± standard deviation (n = 6). Different letters indicate significant statistical differences according to Duncan's test ($p < 0.05$).

reported to play an important role in maintaining the quality of zucchini fruit during the cold storage period (Carvajal et al., 2017). Such effect has been partly associated with transcriptomic changes (Benítez et al., 2022), as well as with biochemical and phytochemical events, represented by the enhancement of the chemical and enzymatic antioxidant capacities and the accumulation of bioactive compounds, such as carotenoids or ascorbate (Castro-Cegrí et al., 2023c). The effect of ABA on primary metabolism and some specific pathways was proved (Castro-Cegrí et al., 2023a). Nevertheless, to the best of our knowledge, little is known about the impact of long-term cold storage on zucchini fruits at a metabolome-wide level, as well as its ABA-mediated modulation.

Firstly, the modulation of the metabolic profile by the cold storage period was found to be of great importance in the clustering of samples as reported for other fruits like peach or plum (Bustamante et al., 2016; Xu et al., 2022), while ABA played a secondary role in this modulation. However, in this work, ABA-treated fruits exhibited a different metabolic profile than control fruits over time. In the short term, ABA-treated fruits showed an accumulation of glucosinolates and derivatives, whose biosynthesis is well-known to be induced by ABA (Yan & Chen, 2007). Glucosinolates are specialized metabolites with dual properties, as they are known to be involved in plant stress tolerance response and reported as nutraceutical compounds (Ilahy et al., 2020; Variyar et al., 2014). Furthermore, among long-term VIP markers, decreased levels of polyphenols and glucosinolates were attributed to ABA, thus suggesting an early implication of glucosinolates in the ABA-mediated response against cold stress in zucchini fruits. In contrast, a long-term accumulation of lipids was reported in ABA-treated fruits, which may indicate their contribution to the late response of this phytohormone against cold stress. The accumulation of lipids was mostly represented by fatty acid derivatives, including tetradecanoic (14:0), palmitic (16:0), arachidonic (20:4), and linoleic (18:2) acid derivatives. The ABA-mediated accumulation of these metabolites suggests that it could be involved in the reinforcement of cuticular wax during cold storage, as a slight decrease in C14 to C32 fatty acids was reported in the exocarp of zucchini fruits under cold storage, thus partially countering this effect (Carvajal et al., 2021).

Three VIP markers were positively associated with ABA treatment during the cold storage period. Methyl-jasmonate is one of the most important phytohormones in controlling postharvest events in fruit, regulating crucial processes such as the phenolic and membrane lipid metabolism or ripening. Indeed, methyl jasmonate has been successfully employed to enhance the deleterious effects of chilling injury during the post-harvest stage in peach and pepper (Duan et al., 2022; Ma et al., 2020). In zucchini fruit, the application of methyl jasmonate reduced chilling injury through an accumulation of abscisic acid and polyamines (Wang & Buta, 1994). Heliespirone C belongs to the terpenoids family, previously reported to contribute to abiotic stress relieving in plants (Lucini et al., 2015). As well, terpenoids have been found to play a mitigating role against water stress in response to the ABA-mediated metabolic regulation of epicuticular wax in different fruits, like citrus and zucchini (Carvajal et al., 2021; Romero & Lafuente, 2020). Finally, (indol-3-yl)acetyl-L-phenylalanine could be pointed as a marker of phenylalanine metabolism, of great importance for the phenylpropanoid pathway, modulating antioxidant response against chilling stress in fruit (Castro-Cegrí, Sierra, et al., 2023; Kumar Patel et al., 2023).

Analyzing the metabolome-wide profile of exocarps of zucchini fruit with respect to initial storage conditions, a general induction of secondary metabolism biosynthesis was attributed to ABA under cold stress. Nitrogen-containing compounds (NCCs) and terpenoids, whose implication on chilling stress was explained before, and phenylpropanoids, due to an increased polyphenol biosynthesis, were the three families of compounds predominantly triggered in response to cold stress and ABA exogenous application. Besides secondary metabolism, lipid biosynthetic metabolism and phytohormone biosynthesis resulted up-regulated throughout the cold storage period to a lesser extent. Regarding lipids, cycloartenol, recognized as a major precursor of sterols and a campesterol derivative, was mainly accumulated in ABA-treated fruits. Sterols are essential structural components of the lipid membrane bilayer, contributing to the permeability and fluidity of cell membranes, being two crucial phenomena involved in mitigating CI-related damages in response to cold stress (Du et al., 2022). In addition, concerning phytohormone biosynthesis, ABA mainly promotes the long-term accumulation of *trans*-zeatin and derivatives, whose role in promoting cell division has been proved (Yang et al., 2021). Interestingly, the effectiveness of these phytohormones in the management of long-term storage has been widely reported, and they are commonly used in horticultural crops to cope with senescence-related phenomena (Aremu et al., 2020). Cytokinin treatments applied to summer squash delay cell wall degradation and softening, improving quality maintenance throughout cold storage (Massolo et al., 2014). Since phenylpropanoid biosynthesis reflected the strongest induction attributed to cold stress in zucchini fruits, a closer insight was provided by determining their untargeted phenolic profile. The importance of phenylpropanoids in maintaining fruit quality by preventing browning and inhibiting oxidative stress has been widely reported (Ge et al., 2018; Sun et al., 2022). Indeed, the role of phenolic compounds in non-enzymatic antioxidant defense is of great importance in fruits (Liu et al., 2019), even more in the exocarp, where cold stress shows its highest impact (de la Rosa et al., 2019). ABA treatment enhances the chilling tolerance of zucchini fruit, inducing high levels of polyphenols, such as vanillic and quercetin (Castro-Cegrí et al., 2023c). Moreover, as specialized metabolites, phenylpropanoids are widely recognized for their added-value properties, acting as multifaceted nutraceutical compounds, exerting a diversified beneficial outcome to human health, including antioxidant, anti-inflammatory, antitumor, and antimicrobial bioactivities, among others (Rodríguez-García et al., 2019). In absolute terms, lignans exhibited the highest content of polyphenolic families in zucchini exocarps. Indeed, zucchini was already recognized as a lignan-rich natural food (Rodríguez-García et al., 2019), regarded as a relevant source of polyphenols. Nevertheless, as indicated by multivariate statistics, the

proportion of discriminant phenolic markers over time showed a sustained implication of flavonoids in the response attributed to ABA. Specifically, flavanols and flavones were mostly found accumulated by semi-quantitative means in the long term, suggesting their major implication in managing cold stress.

5. Conclusions

A time-dependent metabolic modulation was found in zucchini exocarps treated with ABA in response to cold stress, showing an early accumulation of glucosinolates that switches over time towards a late accumulation of different lipids, such as fatty acid derivatives. In detail, three major metabolic markers were positively correlated with ABA throughout the cold storage period: methyl jasmonate, heliespirone C, and (indol-3-yl)acetyl-L-phenylalanine. The general contribution of the methyl jasmonate cascade in the management of plant stress through the induction of secondary metabolism and the universal presence of (indol-3-yl)acetyl-L-phenylalanine as a precursor of highly important biosynthetic pathways reflect the wide impact of ABA on the metabolome of zucchini exocarps during the elicitation of cold stress tolerance. Our metabolomic approach revealed the dual ABA-mediated response of zucchini during cold storage: from one side, the treatment induced secondary metabolite accumulation that resulted in cold stress mitigation; from the other side, the treatment enhanced the nutraceutical potential of zucchini fruits. These findings shed light on the mechanism underlying the ability of ABA to act as a protectant agent of zucchini fruits against chilling injuries during cold storage, supporting this novel approach as an efficient and sustainable alternative with promising industrial prospects.

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CRediT authorship contribution statement

Luigi Lucini: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition. **Francisco Palma:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition. **Dolores Garrido:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition. **Manuel Jamilena:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation. **Pascual García-Pérez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Investigation, Formal analysis, Data curation, Conceptualization, Methodology. **Alejandro Castro-Cegrí:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

I have shared the link to my data

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2024.112864.

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