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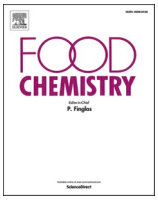

The riboflavin-mediated reprogramming of specialized metabolites enhances postharvest cold tolerance and functional traits of zucchini fruits

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ABSTRACT

Shelf-life extension has a pivotal role in postharvest since fruits are cold-stored to slow down respiration and metabolic processes associated with degradation. When stored at low temperatures, zucchini fruits (*Cucurbita pepo* L.) are prone to chilling injury, which causes surface damage and reduces quality and nutraceutical value, leading to substantial economic losses. Recent studies featured riboflavin as an enhancer of postharvest cold tolerance in fruits inducing antioxidant defense mechanisms. This work aimed to elucidate the metabolic changes triggered by riboflavin in zucchini fruits during cold storage to extend postharvest shelf life and increase nutraceutical properties. A broad metabolic reprogramming was revealed, with terpenoids and phenolic compounds being the most differentially accumulated metabolites during cold storage. Additionally, riboflavin was found to influence the biosynthesis of alkanes, diacylglycerols, triacylglycerols, phytohormones, and vitamins. This metabolomic shaping supports shelf-life extension and the increase in antioxidant properties of zucchini fruits.

1. Introduction

Nowadays, the worldwide increasing consumption of fresh fruit has raised the need for finding new techniques to prolong shelf-life and maintain fruit quality throughout the postharvest period. Cold storage is the most commonly used technique to avoid the rapid deterioration of these commodities. However, when fruits of subtropical origin, such as zucchini (*Cucurbita pepo* L.), are exposed to temperatures below 10 °C, a physiological disorder called the chilling injury (CI) appears (Carvajal et al., 2011; Valenzuela et al., 2017). This disorder is characterized by an increase in weight loss (WL), softening, and the appearance of pitting on the fruit surface, negatively impacting fruit appreciation by consumers and thus leading to significant economic losses. To avoid this significant depreciation of commodities, two chilling contrasting cultivars were investigated, revealing that the induction of the phytohormone abscisic acid (ABA) is a key factor for the cold tolerance of zucchini fruit (Carvajal et al., 2017). The ABA application induced the non-enzymatical antioxidant defense of fruits (Castro-Cegrí, Sierra, et al., 2023), and it has been described that ABA modulates several metabolic pathways of zucchini fruit during the cold storage period, featuring a significant induction of riboflavin metabolism (Castro-Cegrí, Carvajal, et al., 2023).

Riboflavin (vitamin B2) is an important metabolite for human health that is widely distributed in fruits and vegetables (Fischer & Bacher, 2006). Riboflavin is essential for important processes, such as iron absorption, tryptophan metabolism, or mitochondrial function (Thakur et al., 2017). Thus, this vitamin is used as a food additive in the food industry to obtain greater nutritional value in these commodities with a safe outcome for the consumer and the environment (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al., 2022). On the other hand, riboflavin has been tested as an effective treatment to extend the postharvest shelf-life of fruits exposed to cold stress, such as strawberry (Zhang, Wang, et al., 2023) and zucchini (Castro-Cegrí, Carvajal, et al., 2023). These studies have demonstrated

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the effectiveness of this vitamin in enhancing the antioxidant defense system, which mitigates the damage caused by reactive oxygen species (ROS) in fruits exposed to low temperatures, thus prolonging the shelf-life and maintaining fruit quality. However, to the best of our knowledge, the impact of postharvest riboflavin application at a metabolome-wide level remains unknown.

Metabolomics is a powerful tool for conducting studies on compositional changes during the postharvest period in fruits (Yun et al., 2022), as demonstrated for Cucurbitaceae species, such as *Cucumis melo* L. (Zainal et al., 2019). These approaches shed light on the biochemical changes involved in fruit physiology during cold storage periods by revealing the properties of fruits as sources of bioactive compounds, such as lipids, phenolics, or vitamins (Monribot-Villanueva et al., 2019; Vega et al., 2023), with many potential benefits for human health, influencing antioxidant, anti-inflammatory and cardio-protective properties (Pap et al., 2021). This work investigate the effect of riboflavin application on zucchini fruit metabolism and its relationship to cold tolerance and nutraceutical properties. With this aim, an untargeted metabolic approach based on ultra-high-performance liquid chromatography coupled to quadrupole-time-of-flight high-resolution mass spectrometry (UHPLC/QTOF-HRMS) was applied to comprehensively investigate the modulation of specialized metabolites following riboflavin application in zucchini fruits during cold storage.

2. Material and methods

2.1. Fruit material and experimental design

Freshly harvested zucchini fruit (*Cucurbita pepo* L. morphotype Zucchini) from the cultivar 'Logos' (Syngenta) were supplied by Fruits & Vegetables La ÑECA S.A.T. Three replicates of 6 healthy and uniform fruits were prepared per treatment and cold storage period, as proposed in previous studies performed in zucchini fruit under cold storage (Megías et al., 2016; Palma et al., 2019; Zuo et al., 2022). As result, 18 fruits were sampled for the freshly harvested group (T0), while 54 fruits were dipped at room temperature for 20 min in 0.5 mM riboflavin, and the same amount was dipped in distilled water as the control. This riboflavin dose was selected based on findings from a previous study in which various concentrations were evaluated. Among them, 0.5 mM was identified as the most effective in enhancing the postharvest cold tolerance of zucchini fruit (Castro-Cegrí, Carvajal, et al., 2023). All fruits were then dried at room temperature for 2 h and then stored in permanent darkness in a temperature-controlled chamber at 4 °C and 85–90 % relative humidity for 1, 5, and 14 days. After each timepoint, the exocarp of fruits was removed entirely, pooled, frozen and powdered in liquid nitrogen, lyophilized, and stored at room temperature in permanent darkness.

2.2. Weight loss and chilling injury index

Changes in the percentage of weight loss along cold storing per each fruit were calculated following the Eq. 1, being Wi the initial fruit weight and Wf the final fruit weight.

:

$$\%Weight\ loss = \frac{(Wi - Wf)}{Wi} x\ 100 \quad (1)$$

In order to evaluate the chilling injury index (CI), the subjective scale of visual symptoms proposed by Carvajal et al. (2011) was used, according to the following scale: 0, no pitting; 1, mild pitting (≤ 10 % of pitting in fruit surface); 2, medium pitting (10–20 % of pitting in fruit surface); and 3, severe pitting (> 20 % of pitting in fruit surface).

2.3. Firmness and electrolyte leakage

Changes in fruit firmness were measured as in Castro-Cegrí, Ortega-Muñoz, et al., 2023 using a fruit hardness tester (TR TURONI-Italy model 53,205) on the equatorial zone of fruits at 90° fitted with an 8-mm diameter plunger. Firmness was expressed in Newtons (N). The electrolyte leakage measurements were done according to Mao et al. (2007), with the modifications proposed by Carvajal et al. (2015) using a conductometer Consort C860 provided with a conductivity electrode Consort SK10T (Consort nv, Belgium).

2.4. TEAC and DPPH antioxidant assays

Lyophilized material was homogenized (1:100, *w/v*) in acetone 80 % (*v/v*) and shaken for 2 h at 4 °C. The samples were then centrifuged at 10,000 ×*g* for 15 min at 4 °C, and the supernatant was collected. These extracts were used for both assays.

The Trolox equivalent antioxidant capacity (TEAC) was evaluated by the $ABTS^{+}$ (2,2′-azinobis (3-ethylbenz-thiazoline-6-sulphonic acid) decolorization assay proposed by Re et al. (1999) with some modifications. Briefly, 0.1 mL of supernatant was mixed with 0.9 mL 20 mM potassium acetate buffer pH = 4.5 supplemented with 0.07 mM ABTS and 0.03 mM potassium persulfate. The mixture was incubated at room temperature for 1 h, and the absorbance was measured at 734 nm. Results were expressed as μg Trolox per mg dry weight (DW).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was determined following the method by Brand-Williams et al. (1995) with some modifications. Briefly, 0.1 mL of extract was mixed with 1 mL of 1.5 % (*w/v*) DPPH in methanol. The samples were incubated for 1 h at room temperature and permanent darkness, and the decrement of absorbance was measured at 517 nm. The results were expressed as percentage of DPPH inhibition against a blank.

2.5. Lipid peroxidation by TBARS assay

The content in malondialdehyde (MDA) was determined by the thiobarbituric acid reactive species (TBARS) assay (Heath & Packer, 1968), following the modifications detailed by Castro-Cegrí, Ortega-Muñoz, et al. (2023). Briefly, 20 mg of lyophilized material were homogenized with 1 mL 20 % trichloroacetic acid (TCA) (*w/v*) and 0.2 mL 4 % butylated hydroxytoluene (BHT) (*w/v*). The extract was centrifuged for 15 min at 10,000 ×*g* and 4 °C. The reaction mixture was composed by 0.25 mL of supernatant and 0.75 mL of 0.5 % thiobarbituric acid (TBA) (*w/v*) and the samples were incubated for 30 min at 94 °C, then the reaction was stopped in ice for 10 min and the samples were centrifuged again at 10,000 ×*g* during 10 min at 4 °C. Lipid peroxidation rate was calculated using a calibration curve obtained with MDA as positive control. The samples were measured at 532 nm, and 600 nm for non-specific absorbance. Results were expressed as ng MDA per mg DW.

2.6. Sample extraction and metabolomics analysis

For metabolomic analysis, the lyophilized samples were homogenized (1:40, *w/v*) with 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 15 min. Samples were then centrifuged at 8000 ×*g* for 10 min at 4 °C, and the supernatants were collected and syringe-filtered (cellulose membrane, 0.22 μm pore size) into analytical vials. The extraction was carried out in triplicate. In parallel, quality controls (QC) were obtained by pooling 20 μL of all samples into the same vial.

The metabolic profiling of samples was performed as proposed by Rivera-Pérez et al. (2023) 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). Briefly, 6 μL of each sample were injected into an Agilent® ZORBAX RRHD Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm), the solvent flow rate was set at 0.2 mL/min with gradient elution (6–94 % of eluent B in

32 min), prepared with eluent A (water) and eluent B (acetonitrile), both containing 0.1 % (*v/v*) formic acid. The mass spectrometer was operated in SCAN and positive ionization mode (ESI+). Additional parameters were set as follows: nitrogen was used as both sheath gas (12 L/min and 315 °C) and drying gas (14 L/min and 250 °C), nebulizer pressure was 45 psi, nozzle voltage was 350 V, capillary voltage 4 kV. The mass acquisition of samples was performed in MS-only mode set in the *m/z* 100–1200 range (1 spectra/s) with a mass resolution of 30,000 full width at half maximum (FWHM) at 200 *m/z*. The data-dependent mode was performed for precursor fragmentation (10, 20, and 40 eV) and acquisition of MS/MS data from QC samples, with a mass resolution of 30,000 (FWHM), selecting 8 precursors per cycle (1 Hz, m/z 80–1200, and active exclusion after 2 spectra). The processing of chromatograms was performed using the MassHunter Qualitative Analysis software (version B.06.00, Agilent Technologies®).

2.7. Data processing

Once the detection of features was completed, the acquired raw data were processed by MS-DIAL software (v. 4.90), including peak finding, feature identification by library matching, gap filling, and alignment steps. Firstly, the raw '.d' format files were converted into '.abf' files by the Reifycs Abf Converter. These '.abf' data files were imported into MS-DIAL, searching the features in the retention time range of 1–32 min, *m/z* range 100–1200 for MS-only data and 80–1200 for MS/MS data, setting a minimum of 3000 counts for peak detection. The FooDB database (https://foodb.ca/) was chosen to identify features with tolerances of 0.01 Da and 0.05 Da for MS and MS/MS, respectively, and with an identification score cut-off >60 %. Feature identification was based on mass accuracy data, isotopic patterns and spectral matching. For isotope recognition, only the maximum number of isotopes was set to 1, and the maximum charged number was set to 2, as the mass range for metabolite annotation was <2000 Da. The adducts considered for annotation were selected based on the experimental matrix and the analytical procedure, i.e. $[M + H]^+$, $[M + H_2O]^+$, $[M + H\text{-}H2O]^+$ (neutral loss), $[M + ACN]^+$, $[M + Na]^+$, $[M + K]^+$, and all the possible combinations among them (Risoli et al., 2025; Tsugawa et al., 2015). All features that were not detected in at least 83.33 % of replications per group were removed. According to the Metabolomics Standards Initiative (MSI) (Salek et al., 2013), a level 2 of confidence in features' annotation was achieved, conferring the assessment of putatively annotated compounds further involved in multivariate statistical analysis.

2.8. Statistical analysis

The experiments were randomized. The statistical analysis of the results from quality parameters on zucchini fruits was performed by one-way analysis of variance (ANOVA) using the SPSS 28.0 program (SPSS Inc.). Means were compared with Duncan's least significant differences test, assuming significant differences at $p < 0.05$. To ensure that the fruit evaluation was sufficiently representative for statistical analysis using ANOVA, homoscedasticity was assessed using Levene's test ($p < 0.01$).

Raw metabolomics data were first subjected to $\log_2$ transformation and normalization at the 75th percentile by the software Mass Profiler Professional 15.1 (Agilent Technologies®), before applying multivariate statistical analysis. Next, an unsupervised hierarchical cluster analysis (HCA; Euclidean distance, Ward's linkage method) was performed to provide a metabolome-wide overview of sample clustering due to riboflavin treatment and cold storage period. In parallel, supervised multivariate data analysis was carried out in terms of orthogonal projection to latent structures discriminant analysis (OPLS-DA) by SIMCA® 16 software (Sartorius®, Umeå, Sweden), building independent models to discriminate the effect of riboflavin against the control for each day of cold storage. The goodness-of-fit (R^2Y) and the goodness-of-prediction (Q^2) parameters were considered to evaluate the quality of the models, setting a Q^2 predictive ability >0.5 as a threshold for good predictability. OPLS-DA models were validated by cross-validation analysis of variance (CV-ANOVA, *p*-value <0.05), and the overfitting was excluded by permutation test ($n = 100$). Finally, the models were combined with the variable importance in the projection (VIP) approach to identify the compounds showing the highest contribution to the discriminating effect of riboflavin. Common VIP markers showing the highest discrimination power (VIP score > 1.4), as in (Rivera-Pérez et al., 2022), over time were investigated through a Venn diagram by jvenn tool ().

Volcano analysis, by means of fold change analysis (FC), setting cut-off = ±2, combined with one-way ANOVA followed by Tukey's post hoc test ($p < 0.01$) was performed by Mass Profiler Professional 15.1 software to identify the differentially accumulated metabolites (DAMs) between riboflavin treatment and the control for each cold storage timepoint. Furthermore, DAMs were subjected to a chemical enrichment approach (ChemRICH) (Barupal & Fiehn, 2017) to provide a detailed overview of significantly impacted metabolite clusters during cold storage because of riboflavin treatment.

Pearson's correlation analysis was conducted using the online platform MetaboAnalyst 5.0 (www.metaboanalyst.ca). Prior to analysis, the increase ratio data for each metabolic class identified through ChemRICH analysis at 1, 5, and 14 days of cold storage, along with the phenotypic data, were normalized by the median and auto-scaled to ensure comparison across variables.

3. Results

3.1. Effect of riboflavin treatment on quality maintenance of zucchini fruit during cold storage

Phenotypic parameters were evaluated for homogeneity of variance using Levene's test prior to performing ANOVA. All models yielded statistically significant results ($p < 0.01$), confirming the assumption of variance homogeneity across fruits within each biological replicate. Cold storage caused a significant reduction in the quality of zucchini fruits, resulting in an overall increase in weight loss (WL) and chilling injury index (CI) over time (Table 1), reaching the maximum differences between control and riboflavin-treated fruits at 14 days of cold storage, by increasing both parameters and affecting the appearance of fruits (Table 1 and Fig. 1).

These quality changes may be linked to cellular damage in the cuticle and membrane, leading to important water losses. In fact, there was an increase in electrolyte leakage throughout the cold storage period coupled with an abrupt change in fruit firmness, suggesting a water loss leading to softening, one of the main symptoms of chilling injury. Riboflavin treatment significantly reduced WL by 18.4 % and 20.9 % compared to control at 5 and 14 days of cold storage, respectively, following the same trend reported for changes in electrolyte leakage (Table 1). On the other hand, riboflavin treatment was found to reduce fruit softening during cold storage, showing remarkably higher firmness values after 5 days, which is related to the changes observed for CI, by reducing 50 % and 44.5 % at 5 and 14 days of cold storage, respectively.

The main effects in antioxidant activity were detected at the first day of cold storage, when DPPH levels decreased in control fruits, suggesting a short-term oxidative damage, while riboflavin-treated fruits maintained the same antioxidant capacity as freshly harvested fruit up to 5 days (Table 1). A similar response was detected by TEAC assay, with higher antioxidant capacity after 1 day of cold storage in riboflavin-treated fruits compared to control. In parallel, cold stress caused an increase in lipid peroxidation in zucchini fruit over time. However, riboflavin softened this response, showing significantly reduced levels of MDA after 5 and 14 days compared to control fruits, showing a 12.1 % and 9.8 % decrease, respectively (Table 1).

Table 1
Effect of cold storage and riboflavin treatment on quality parameters of zucchini fruits, measured by changes in weight loss (%), chilling injury index, electrolyte leakage (%) and firmness (N), antioxidant capacity by DPPH and TEAC, and lipid peroxidation by MDA content, at 0, 1, 5, and 14 days of cold storage, in control and riboflavin-treated fruits.

Days of storing at 4 °C	Treatment	Weight loss (%)	Chilling injury	Electrolyte leakage (%)	Firmness (N)	DPPH (% inhibition)	TEAC (μg Trolox/g DW)	Lipid peroxidation (ng MDA/g DW)
0	At harvest	–	–	5.6 ± 0.2^{e}	45.1 ± 0.8^{a}	86.6 ± 0.7^{a}	10.1 ± 0.2^{c}	12.4 ± 0.4^{e}
1	Control	1.9 ± 0.1^{e}	0^{d}	7 ± 0.3^{cd}	46.6 ± 0.9^{a}	82.8 ± 1^{bc}	11.1 ± 0.2^{b}	14.3 ± 0.7^{de}
	Riboflavin	1.7 ± 0.1^{e}	0^{d}	6.7 ± 0.4^{d}	45.2 ± 1^{a}	86.1 ± 0.4^{a}	12.3 ± 0.2^{a}	13.6 ± 1.1^{de}
5	Control	4.9 ± 0.1^{c}	1.5 ± 0.2^{b}	8.2 ± 0.5^{b}	21.7 ± 0.8^{c}	83.1 ± 0.5^{bc}	12.2 ± 0.3^{a}	17.3 ± 0.3^{ab}
	Riboflavin	4.0 ± 0.1^{d}	0.8 ± 0.1^{c}	6.3 ± 0.3^{de}	30.1 ± 3.6^{b}	84.7 ± 0.3^{ab}	12.5 ± 0.2^{a}	15.2 ± 0.4^{cd}
14	Control	11 ± 0.4^{b}	2.4 ± 0.2^{a}	9.4 ± 0.4^{a}	19.2 ± 0.4^{c}	82.2 ± 1^{c}	10.4 ± 0.2^{bc}	18.3 ± 0.1^{a}
	Riboflavin	8.7 ± 0.3^{a}	1.3 ± 0.3^{b}	7.9 ± 0.4^{bc}	21.4 ± 0.5^{c}	82.1 ± 0.4^{c}	11.1 ± 0.2^{b}	16.5 ± 0.6^{bc}

The data presented as the mean ± standard deviation of 18 fruits for weight loss, chilling injury index, and triplicate samples of six fruits for the other measurements. Different letters within each column indicate statistically significant differences according to Duncan's test ($p < 0.05$).

3.2. Unravelling the hierarchical effects of cold storage and riboflavin application on the metabolic profile of zucchini exocarps by multivariate statistics

Exocarp samples from control and riboflavin-treated fruits at different stages of cold storage were subjected to an untargeted metabolomic approach, resulting in 7749 chemical entities detected on the analyzed extracts, from which 1404 were putatively annotated against FooDB database: 151 entities were annotated with MS^2 fidelity, whereas 1253 entities were reported at the MS-only level. Comprehensive information for each feature, including metabolite name, retention time (Rt, min), mass (*m/z*), molecular formula, ontology, MS and MS/MS spectra, annotation, and total score, is provided in Supplementary Table S1. To investigate the influence of both factors on the metabolic profile of zucchini exocarps, an unsupervised hierarchical cluster analysis (HCA) was performed (Fig. 2). As observed, cold storage time had the most important influence on the metabolome of exocarps, following time-dependent segregation: 0 and 1 days apart from 5 and 14 days. Subsequently, samples were clustered by treatment, reflecting a clear separation of riboflavin-treated fruits from the control at each time point (Fig. 2). These results suggested that the chilling injuries suffered in zucchini fruits exposed to cold stress had a key impact on the accumulation profile of a wide range of metabolites, and this impact was partly mitigated by riboflavin-mediated elicitation.

Subsequently, a supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed to gain further insight into the impact of riboflavin application throughout the postharvest period under cold storage on the metabolome of zucchini fruits. The results demonstrated that riboflavin affects the composition of the exocarp at all timepoints since all models presented high-quality parameters in terms of goodness-of-fit ($R^2 > 0.997$) and goodness-of-prediction ($Q^2 = 0.8–0.888$), being all of them validated by CV-ANOVA showing *p*-value <0.05 (Fig. 3). Each OPLS-DA model was further investigated through variable importance in projection (VIP) analysis, identifying the metabolites with the highest discrimination power (VIP score > 1.40), revealing 163, 151, and 183 VIP markers for 1, 5, and 14 days of cold storage, respectively (Supplementary Table Error! Reference source not found.).

Lipids, mostly composed of terpenoids, fatty acids, and other related compounds, constituted the most representative family of VIP markers. Among them, terpenoids exhibited the greatest proportion of discriminating compounds, followed by amino acids and peptides (11–16 %), nitrogen-containing compounds (NCCs, 9–15 %), and phenolic compounds (11–15 %). Interestingly, only 9 metabolites were found as discriminants at all stages of cold storage (Fig. 3D), of which seven were strongly accumulated by riboflavin treatment: *S*-acetyldihydrolipoamide ($log_2FC = 9.08–9.15$), phosphoribosylformylglycineamidine ($log_2FC = 5.83–6.41$), for protoporphyrin ($log_2FC = 4.80–5.66$), cincassiol B ($log_2FC = 5.52–6.28$), dukunolide E ($log_2FC = 1.52–5.69$), garcinone C ($log_2FC = 5.52–6.41$), and prunasin ($log_2FC = 0.29–0.64$) (Supplementary Table S2). In turn, only one metabolite was commonly down-regulated by riboflavin-treated: 3-hydroxy-N6,N6,N6-trimethyl-L-lysine (logFC = −1.23 – -0.15). Therefore, these metabolites may be recognized as signature markers of riboflavin treatment in the exocarp of zucchini fruit exposed to low temperatures.

3.3. Chemical enrichment analysis provides the functional signature of riboflavin on zucchini fruit exocarps during cold storage

ChemRICH enrichment analysis was performed considering the differentially accumulated metabolites provided by Volcano analysis (DAM; $p < 0.01$ and FC cut-off = ±2) to discriminate between riboflavin-treated fruits for 1, 5, and 14 days of cold storage against freshly harvested fruit (Supplementary Fig. S1). The full list of DAMs is reported in Supplementary Table S3, together with the corresponding clusters and their key metabolites. The induction at a wide metabolomic level was represented in Supplementary Fig. S1, observing a huge heterogeneity of compounds classes elicited by the riboflavin treatment during cold storage. The results reveal a remarkable metabolic induction over time, accounting for 103, 253, and 357 DAMs for 1, 5, and 14 days, respectively (Supplementary Table S3). Furthermore, a gradual accumulating trend of metabolite classes was detected in riboflavin-treated fruits over time, as observed at either 5 or 14 days, whereas contrasting results were obtained among metabolite classes at a short term with up- and down-accumulated outcomes, according to their increased ratio (IR). Following the ChemRICH results, metabolite classes were further examined through linear correlation analysis using these IR together with phenotypic data, shown in a heatmap (Fig. 4). Vitamins and phenolic compounds, including phenylpropanoids, phenolic acids, and flavonoids, were among the strongest negative correlations with weight loss and chilling injury ($r = −0.50$ to $−0.82$), and positive correlations with fruit firmness, with phenolic acids showing the highest ($r = 0.95$). In contrast, lignans, hydrocarbons, and sugars and polyols were positively correlated with increased weight loss and chilling injury ($r = 0.73$ to 0.96) (Supplementary Table S4). Subsequently, the induction of each metabolic class due to riboflavin treatment during cold storage was further assessed. Riboflavin treatment induced a significant reprogramming of zucchini fruit metabolism, initially reducing the accumulation of stress-related compounds such as sugars and polyols, amino acids, and dipeptides after one day of cold storage. Concurrently, it upregulated the levels of flavonoids, saturated fatty acids (SFAs), diacylglycerols (DAGs), and triacylglycerols (TAGs). Throughout cold storage this metabolic modulation shifted, resulting in a general upregulation of certain metabolite classes, including sugars and polyols, while others, such as glucosinolates, were downregulated (Supplementary Fig. S1). Considering polyphenols, a marked short-term accumulation of phenolic acids and flavonoids was observed at 1 d (IR = 1 in both cases). However, when cold stress is prolonged, a shift in phenolic compound accumulation was observed, following a gradual accumulation of long-chain alkylphenols at a long term in riboflavin-treated fruits

Fig. 1. Biological replicate of 6 control (A) and riboflavin-treated (B) fruits after 14 days of cold storage at 4 °C.

(IR = 0.7, 0.8, and 0.9, at 1, 5, and 14 days, respectively) (Fig. 5A).

In the case of lipids, a similar response ascribed to riboflavin was observed for acylglycerols, peaking at day 5 and decreasing harshly at day 14, while diacylglycerols (DAGs) and triacylglycerols (TAGs) were absent (Fig. 5B). On the other hand, major cuticle components, represented by hydrocarbons (alkanes and alkenes) exhibited an increasing accumulation induced by riboflavin treatment at mid and long-term cold storage.

Concerning plant growth regulators and primary metabolites, an increased accumulation in phytohormones was observed on riboflavin-treated fruits after 5 days and maintained at long-term cold storage, being the only class at this stage with IR = 1 (Fig. 5C). Among these riboflavin-elicited phytohormones, the derivative of jasmonic acid 12-Oxo-2,3-dinor-10,15-phytodienoic acid was key in the discrimination besides the jasmonate jasmonolone glucoside, along with some gibberellins, such as GA_{60}, GA_{59}, or GA_9, together with some indoleacetate derivatives, i.e., indole-3-acetyl-phenylalanine and indole-3-acetyl-tyrosine (Supplementary Table S3). In contrast, it is noteworthy that riboflavin-treated fruits showed a significant reduction in glucosinolate levels compared to control fruits through the whole cold storage period, indicating that this treatment effectively mitigates the deleterious effect of cold storage on zucchini exocarps by minimizing the accumulation of these stress-related specialized metabolites.

Interestingly, a wide range of vitamins was found elicited by riboflavin (vitamin B2), reaching a maximal accumulation at long term (Fig. 5C). These included tocotrienols (vitamin E derivatives), folic acid (vitamin B9), and the riboflavin derivative riboflavin cyclic-4′,5′-phosphate (Supplementary Table S3), the latter suggesting the efficient

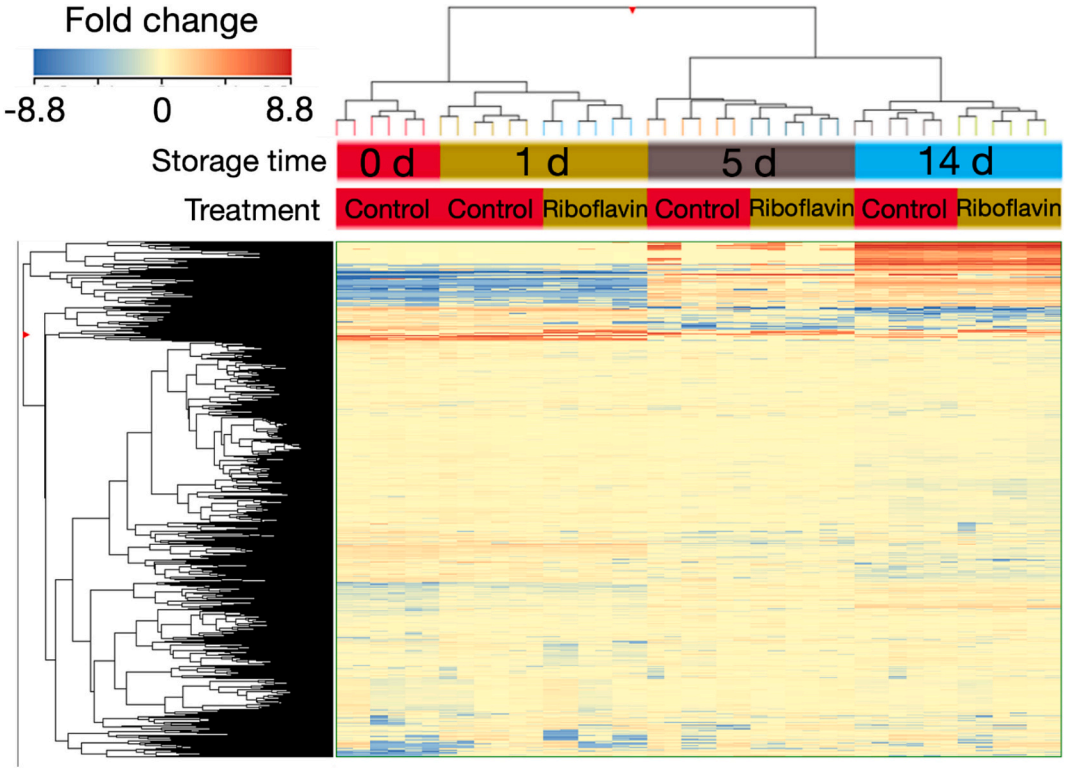


Fig. 2. Hierarchical cluster analysis (HCA) of the untargeted metabolic profile of exocarps from zucchini fruits during cold storage (at 0, 1, 5, and 14 days) untreated (control) or treated with riboflavin. Clustering was performed according to the fold change-based heatmap, baselined to the median values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

assimilation of riboflavin even under sustained cold stress. Overall, these results support a deep metabolic reprogramming on zucchini fruits elicited by riboflavin throughout cold storage, modulating a wide range of both specialized and primary metabolites, which is shown to be more efficient along cold storage.

Concerning terpenoids, a similar trend attributed to riboflavin was found for most classes, i.e., monoterpenoids, sesquiterpenoids, and steroids (the latter including the cucurbitacin kuguacin C, and the saponins collettiside I and torvonin A), peaking at day 5, and slightly decreasing at day 14, although diterpenoids showed a gradual down-accumulation over time (from IR = 1 to IR = 0.6 in the period 1–14 days) (Fig. 5D).

Finally, regarding nitrogen-containing compounds, the highest impact due to riboflavin treatment during cold storage was noted in glucosinolates, down-regulating these compounds leading to IRs (0, 0.2, and 0.1; for 1, 5, and 14 days) (Fig. 5E).

4. Discussion

The exogenous application of riboflavin to zucchini fruit exposed to cold storage has been shown to induce deep metabolic reprogramming, mediated by the hierarchical effect of the cold storage period. In this study, HCA mainly clustered samples from injured fruits (5d and 14d) apart from non-injured fruits (0 and 1d). The role of cold exposure in modulating the metabolic profile during the postharvest period has been widely discussed in other fruits, like peach or tomato (Bai et al., 2023; Li et al., 2023), as well as in ABA-treated zucchini fruits (Castro-Cegrí, García-Pérez, et al., 2024). Interestingly, ABA was shown to be a critical agent in improving zucchini shelf-life in a cold-sensitive cultivar by modulating primary metabolism and inducing riboflavin accumulation (Castro-Cegrí, Carvajal, et al., 2023). In this regard, riboflavin application played a discriminant role in zucchini metabolome along cold storage, requiring further insight to decipher the mechanism of action of this vitamin as an enhancer of cold tolerance.

Regarding the VIP markers from OPLS-DA models, terpenoids and phenolic acids were the main representatives reflecting a pivotal role in the discrimination at each stage of cold storage induced by riboflavin. These groups feature specialized metabolites responsible for fruits' aroma and nutritional properties (Pott et al., 2019). Comparing all the VIP markers modulated by riboflavin treatment, 7 compounds were up-regulated during the entire period (*S*-acetyldihydrolipoamide, phosphoribosylformylglycineamidine, protoporphyrin, cincassiol B, dukunolide E, garcinone C and prunasin), while just one was down-regulated (3-hydroxy-N6,N6,N6-trimethyl-L-lysine). Interestingly, riboflavin elicited the accumulation of important signaling-associated mediators, since *S*-acetyldihydrolipoamide is involved in acetyl-coenzyme A biosynthesis (Broz et al., 2014), while phosphoribosylformylglycineamidine is involved in purine biosynthesis, which can be supported by the role of riboflavin as a precursor of flavin nucleotide cofactors (Henriques et al., 2010). These findings support a mechanistic explanation of riboflavin as an effective postharvest cold protectant, driven by nucleotide-mediated signaling. In parallel, the riboflavin-mediated accumulation of protoporphyrin, considered a chlorophyll precursor, suggests an active elicitation of the fruit exocarp, as this intermediate metabolite may participate in preventing oxidative stress (Martínez et al., 2023), thus assessing the improvement in antioxidant activity of the exocarp discussed earlier. In contrast, (3-hydroxy-N6,N6,N6-trimethyl-L-lysine), an intermediate of carnitine biosynthesis (Rippa et al., 2012), was found negatively associated with riboflavin, indicating an impairment of lipid metabolism in zucchini exocarps. This observation may justify the accumulation of alkanes and alkenes over time in riboflavin-treated fruits during cold storage while decreasing the implication of carnitines and acylglycerols, as reported by ChemRICH analysis. Overall, these compounds reflect the comprehensive mechanism of action attributed to riboflavin on the defense response against cold storage in zucchini fruits.

The untargeted metabolomic analysis conducted in this study

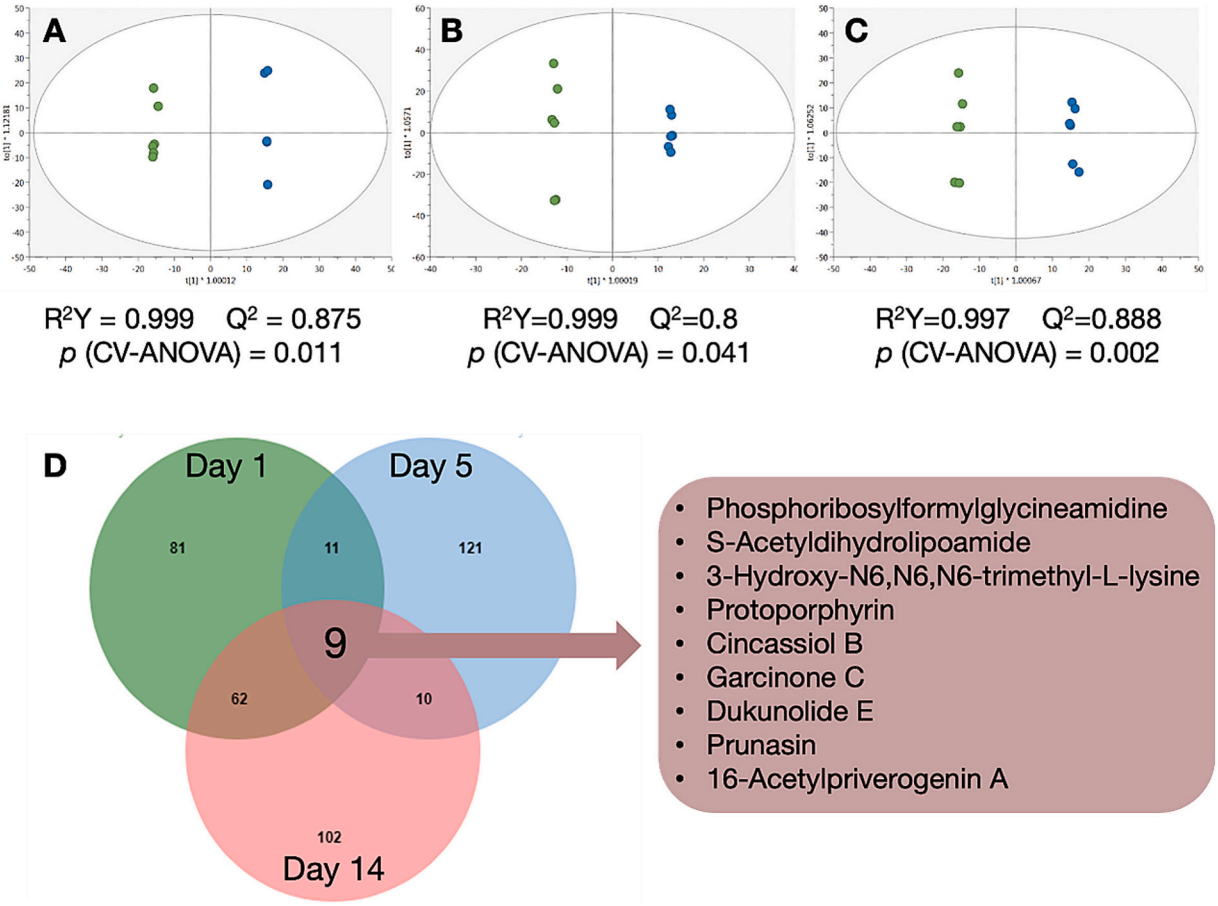


Fig. 3. Multivariate OPLS-DA models for the metabolomic profile of exocarps from zucchini fruits during cold storage untreated (control) and treated with riboflavin. A. OPLS model for day 1 of cold storage. B. OPLS model for day 5 of cold storage. C. OPLS model for day 14 of cold storage. D. Venn diagram for the VIP markers found as discriminant compounds for each model (VIP score > 1.4).

provided deeper insights not only into the major metabolites differentiating the samples in response to riboflavin treatment but also into the broader metabolic shifts involved. This approach enables the characterization of metabolic modulation on a wide scale, encompassing a highly heterogeneous array of compound classes, which is an essential aspect for understanding how the fruit activates its defense mechanisms during postharvest storage (Belay & James Caleb, 2022). In this context, ChemRICH analysis proved valuable in clearly visualizing the extensive dataset, highlighting how riboflavin treatment modulates the tissue metabolome. A similar approach was previously applied in onion plants to evaluate the beneficial impact of wood vinegar as a plant growth regulator, revealing an enrichment of phenolic compounds, terpenoids, and membrane-associated metabolites, among others (Zhang, García-Pérez, et al., 2023). Therefore, in the present study, this strategy was employed to elucidate the effect of riboflavin on the overall metabolism of zucchini fruits during postharvest cold storage. Integrating phenotypic data with metabolomic changes identified through ChemRICH analysis revealed that vitamins, diterpenoids, and phenolic compounds, including phenylpropanoids, alkylphenols, phenolic acids, and flavonoids, were among the most significantly impacted metabolite classes. These compounds exhibited strong negative correlations with weight loss and chilling injury, and positive correlations with fruit firmness. These results highlight the potential mechanistic role of riboflavin treatment in prolonging the postharvest shelf life of zucchini fruit by inducing the accumulation of these bioactive metabolites.

As assessed by ChemRICH analysis, a significant induction of the phenylpropanoid derivatives, represented mainly by phenolic acids and flavonoids, was noted on the first day of cold storage by riboflavin treatment, coupled with an increase in the antioxidant properties of zucchini fruit by TEAC and DPPH. These results over the rapid response of riboflavin treatment on postharvest cold defense are consistent with those observed in strawberries and zucchini (Castro-Cegrí, Carvajal, et al., 2023; Zhang, Wang, et al., 2023). Inducing in riboflavin-treated zucchini fruits a non-enzymatical antioxidant defense, not affecting to catalase or ascorbate peroxidase activities. Indeed, Niu et al. indicated that phenolic biosynthesis was enhanced as a tolerant response against cold stress in kiwifruit, leading to the accumulation of trans-cinnamic acid, considered as the major precursor of phenolic acids, agreeing with these results (Ilea et al., 2024; Niu et al., 2023). In the same way, cold tolerance in tomato fruits was mediated by the combined accumulation of both phenolic acids and flavonoids, as observed here for zucchini fruits at short- and mid-term. In parallel, the riboflavin induced the long-term accumulation of alkylphenols, especially alkylresorcinols. These phenols have been found in the outer layers of grains and fruits, which was also reported here, supporting the holistic reprogramming at a metabolic level hypothesized in this work for zucchini fruits as components of waxy tissues, because of their amphipathic nature consisting of a phenolic ring coupled to a long hydrophobic alkyl chain (Boulebd & Spiegel, 2023). The amphiphilic nature of alkylresorcinols underlies their biological activity, as their structural compatibility allows for efficient integration into phospholipid bilayers. This incorporation influences membrane fluidity and stability, while also facilitating interactions with membrane-associated proteins. Such interactions may modulate enzymatic functions, particularly those related to membrane-bound enzymes (Kozubek & Tyman, 2005; Sampietro et al., 2013). Consequently, the long-term accumulation of alkylresorcinols reported

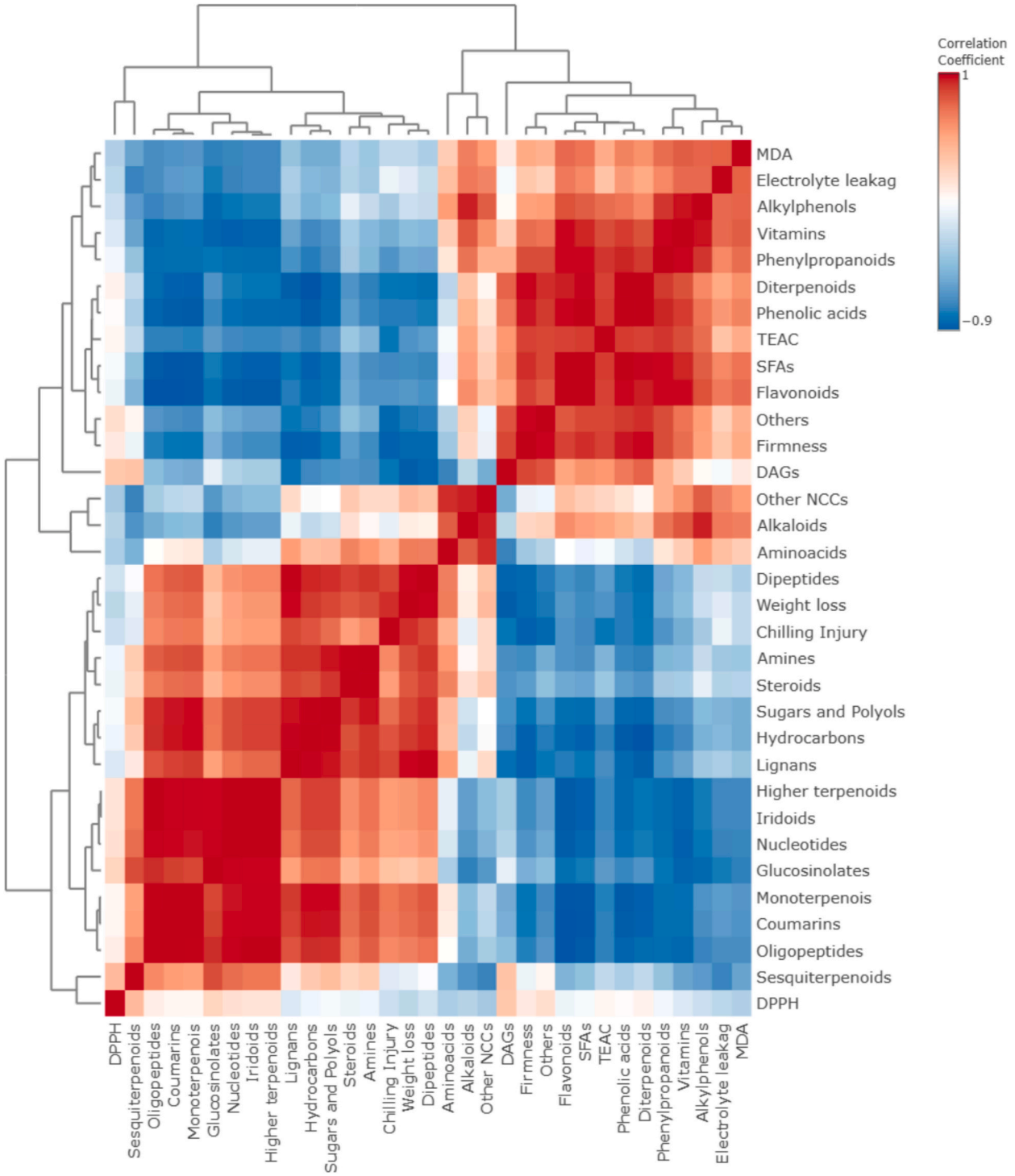
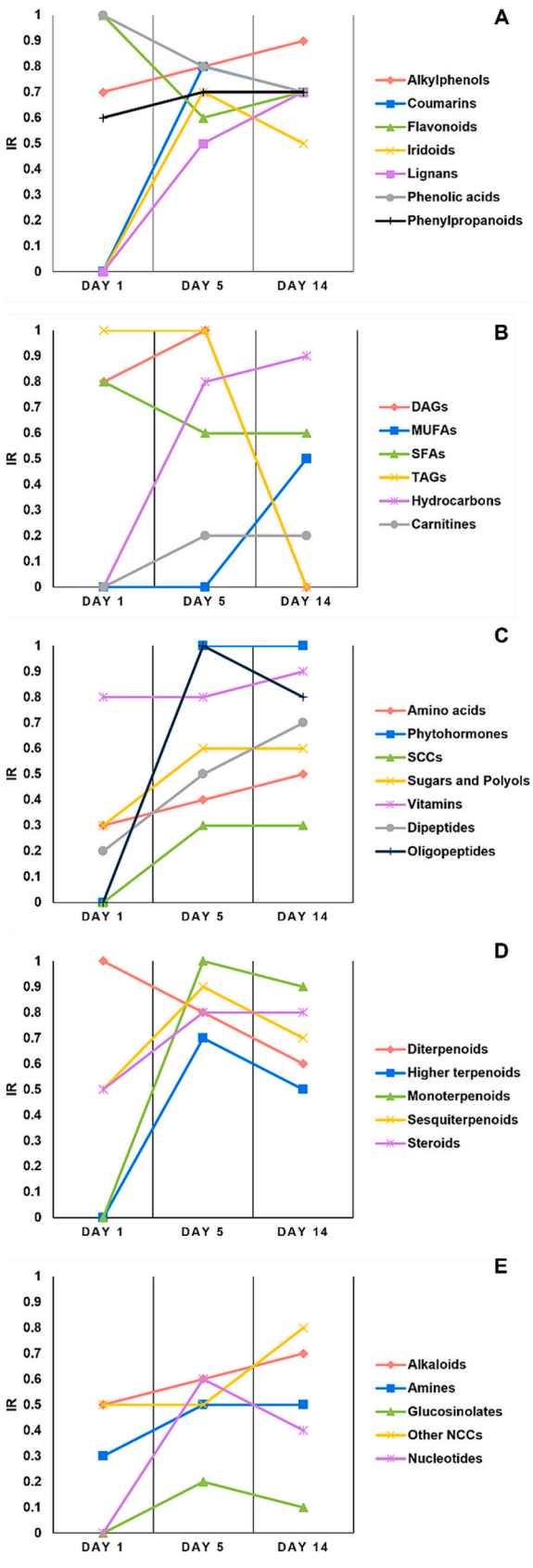


Fig. 4. Correlation heatmap performed using the phenotypic data by calculating the change against T0 (logFC) due to cold storage (1, 5, and 14 days) and riboflavin treatment for weight loss, chilling injury, electrolyte leakage, antioxidant capacity (TEAC and DPPH), lipid peroxidation (MDA), and metabolic classes evaluated in ChemRICH analysis.

here due to riboflavin treatment may contribute to the stabilization of the exocarp, revealing a novel mechanism to induce cold tolerance in zucchini fruits that deserves further attention.

Regarding the terpenoid group, cucurbitacin kuguacin C was identified as a marker metabolite of riboflavin treatment. Cucurbitacins have been associated to defense mechanisms against abiotic stress, acting as osmoprotectants under drought conditions (Mashilo et al., 2018). This finding suggests a novel research direction exploring the modulation of cucurbitacins by riboflavin treatment and their potential role in mitigating cold stress. Moreover, it is well established that steroids contribute significantly to membrane fluidity and stability, thereby regulating responses to abiotic stress (Hartmann, 1998), also inducing metabolic processes associated with membranes by forming special structures with sphingolipids (Rogowska & Szakiel, 2020).

Membrane stability is directly dependent on the composition and regulation of membrane steroids and lipids, including phospholipids, diacylglycerols (DAGs), and triacylglycerols (TAGs), among others. Lipid degradation increases phosphatidic acid levels, causing fruit



(caption on next column)

Fig. 5. Evolution of increase ratio (IR – accumulation of metabolites) throughout zucchini cold storage shelf life, following riboflavin application. Data are presented per different biochemical classes, being 1.0 in the cases where all metabolites showed an increase and 0.0 in the cases where all metabolites showed a decrease. Values are generated by comparing riboflavin-treated fruits against freshly harvested fruits against after 1, 5 and 14 days of cold storage. The IR was obtained from chemical similarity enrichment analysis (ChemRICH) of the differentially accumulated metabolites (DAMs). The DAMs were clustered by metabolite class grouping phenolic compounds and phenylpropanoids (A), lipids and related metabolites (B), functional and primary metabolites (C), terpenoids and derivatives (D) and nitrogen-containing compounds (NCCs, E).

damage when exposed to low temperatures, as observed in peach (Song et al., 2022). The dynamic balance of converting TAG and DAG to PA is crucial in determining tolerance against chilling stress in plants (Tan et al., 2018), as reported by ChemRICH analysis. Furthermore, the downregulation of the phospholipase gene *PLA1* prevented membrane damage due to ABA treatment in zucchini fruit (Benítez et al., 2022). Thus, the short and mid-term riboflavin-induced reprogramming on DAGs and TAGs could indicate an enhancement of membrane stabilization, preventing lipid peroxidation and cellular damage, as revealed by TBARS assay. The preservation of membrane fluidity-related compounds may contribute to mitigating chilling injury and reducing weight loss, thereby extending the shelf life of zucchini fruit. This effect was observed in zucchini fruits stored under near-saturated humidity conditions (NSH), which delayed the accumulation of saturated fatty acids and the reduction of unsaturated fatty acids. These changes were associated with the regulation of key lipid-metabolizing enzymes, including lipoxygenase, phospholipase C, phospholipase D, and lipase, suggesting a modulation of membrane lipid composition and integrity under NSH storage (Zuo et al., 2021). Thus, riboflavin-mediated lipidic response could be key for increasing tolerance against cold stress in zucchini fruit, as was also noted in strawberry by inhibiting polyphenol oxidase and peroxidase activities (X. Zhang et al., 2023). Interestingly, the combined membrane lipids and phenolic biosynthesis guided the cold tolerance of Chinese olives (Chen et al., 2024), which was also reported in this study. This supports the hypothesis of a holistic metabolic reprogramming underlying cold stress adaptation in zucchini fruits.

Riboflavin-treated fruit showed a remarkable accumulation of hydrocarbons at mid and long-term cold storage, which may increase the cold tolerance of zucchini fruit. Very-long chain alkanes are the predominant components of the fruit cuticle, positively correlated with water impermeability in pepper fruit (Parsons et al., 2012). To protect zucchini fruit from cold stress during the postharvest period, the cuticle composition plays a crucial role, as it serves as the primary protective barrier against environmental stressors. The cuticle is synthesized by the epidermal cell layer, with intra- and epicuticular waxes being key components of this tissue (Buschhaus & Jetter, 2011), whose biosynthesis can be regulated by abscisic acid (Gutiérrez et al., 2021; Romero & Lafuente, 2020). In fact, when comparing 'Natura', a cold-tolerant zucchini variety, with 'Sinatra', a sensitive one, a differential accumulation of alkanes was detected after the cold storage period, increasing their load almost twice in Natura fruit (Carvajal et al., 2021). This response could be due to the increase in the relative expression of some genes, such as *CpCER3-like* (Carvajal et al., 2021), which was also identified as a putative candidate to control the postharvest cold tolerance of Natura in a bulk segregant analysis performed comparing both varieties (Castro-Cegrí et al., 2025).

On the other side, a strong response was detected in the induction of some phytohormones after 5 and 14 days of cold storage. Among them, some of the most important were the jasmonic acid derivative 12-Oxo-2,3-dinor-10,15-phytodienoic acid and an indole-acetic acid derivative (indole-3-acetyl-phenylalanine). Interestingly, this response concerning an increase of jasmonate derivatives was also observed in zucchini fruit treated with ABA (Castro-Cegrí, García-Pérez, et al., 2024), highlighting

the importance of indole-3-acetyl-phenylalanine and jasmonates in postharvest cold tolerance. The effect of methyl-jasmonate applications increasing fruit quality and shelf-life has been widely investigated, with an activation of the phenylpropanoid pathway and accumulating phenolic and flavonoid compounds (Tao et al., 2022; Wang et al., 2021; K. Zhang et al., 2024). Therefore, these compounds could be used as signature markers of chilling tolerance throughout the postharvest period. Similarly, a direct effect of riboflavin-mediated mitigation of cold stress can be assessed by the reduction of glucosinolate levels, metabolites that are widely recognized as important stress markers in plants (Ilahy et al., 2020; Variyar et al., 2014), also playing a key role in zucchini fruit under cold stress (Castro-Cegrí, García, et al., 2024; Castro-Cegrí, García-Pérez, et al., 2024).

One of the main concerns in food research is to increase the nutritional value of the commodities by enhancing the content of bioactive compounds with antioxidant properties. In this way, riboflavin-treated fruit under cold stress improved significantly the vitamin content, inducing, among others, a riboflavin derivative and the tocopherols d-tocotrienol and 11'-Carboxy-gamma-tocotrienol. Riboflavin derivatives play a crucial role in plant biology, as their synthesis and transport impact a wide range of physiological processes. These derivatives serve as cofactors for FMN/FAD-dependent enzymes (Macheroux et al., 2011). While many of these enzymes catalyze redox reactions involved in primary metabolism, they also participate in various other biochemical pathways, including terpenoid biosynthesis (Fischer & Bacher, 2005; Miret & Munné-Bosch, 2014), which could explain the observed effects of riboflavin treatment on terpenoid accumulation described previously. Furthermore, enhancing the levels of riboflavin and its derivatives in zucchini fruit could significantly improve its nutraceutical value. As humans must obtain riboflavin through their daily diet, increasing its presence in food sources like zucchini can offer multiple health benefits. Riboflavin plays a crucial role in mitigating oxidative stress, reducing the risk of certain cancers, and providing neuroprotective effects (Lee et al., 2023). Tocopherols (vitamin E) are isoprenoids that play an important role in response to cold stress in plants (Maeda et al., 2006), having also been widely studied in fruits (Charoensiri et al., 2009) to discover natural sources of vitamin E. Tocopherols show a positive correlation with chilling tolerance in mandarin (Rey et al., 2021), and are also used as an additive to enrich alginate coating increasing the nutraceutical properties of the commodities (Gull et al., 2023). In parallel, riboflavin treatment led to folic acid (Vitamin B9) accumulation throughout the cold storage period. Folic acid applications have been shown to delay postharvest quality deterioration by increasing the antioxidant capacity and regulating cell wall metabolism (Pei et al., 2023; Xu et al., 2021). Increasing the amount of these vitamins would improve the nutritional quality of zucchini fruit, thereby enhancing the commercial value of the commodities.

5. Conclusions

Effective and safe solutions that improve the shelf-life of fruits in post-harvest are becoming essential nowadays. In this context, riboflavin application prolonged the shelf life of zucchini fruits exposed to cold storage, delaying lipid peroxidation and membrane damage by a time-dependent metabolic modulation. This deep metabolic reprogramming in the short and mid-term affected the antioxidant defense by shaping the phenolic signature and the membrane fluidity and stability, modulating DAGs and TAGs. Conversely, riboflavin-induced alkane biosynthesis enhances the cuticular wax properties and shapes the phytohormone profile, resulting in a differential accumulation of gibberellins, indole-acetate, and jasmonate derivatives.

Noteworthy, the treatment may induce a very interesting modulation of the commodities' nutritional value, involving a pronounced accumulation of vitamins throughout the period studied. Thus, the results obtained on fruits following the application of riboflavin reveal indole-3-acetyl-phenylalanine, jasmonates, tocopherols, and folates as marker metabolites in the development of new strategies to extend the shelf-life and increase the nutritional quality of zucchini fruit during the cold storage period. Overall, these findings highlight that riboflavin is a good candidate for developing new bio-based ecologically friendly strategies, using it as a GRAS additive that prolongs the shelf life and improves the antioxidant properties of the commodities. Furthermore, this research supports continued investigation into how riboflavin treatment modulates the nutraceutical properties of zucchini fruit, with the aim of elucidating the potential health benefits associated with the induction of these compounds, particularly in improving dietary outcomes for human health.

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CRediT authorship contribution statement

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

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.

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Data availability

Raw data were submitted alongside the manuscript.

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