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Riboflavin improves postharvest cold tolerance in zucchini fruit inducing non-enzymatic antioxidant response and phenolic metabolism

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ABSTRACT

The storage of zucchini fruit at low temperatures during postharvest induces a physiological disorder called chilling injury that drastically reduces fruit quality and shelf life. The phytohormone abscisic acid (ABA) is involved in the acquisition of cold tolerance in zucchini fruit, being the riboflavin pathway one of the most differentially induced with ABA treatment. Thus, the aim of this work was to elucidate the involvement of riboflavin in quality maintenance of zucchini fruit during postharvest cold storage. After testing different concentrations of exogenous riboflavin, 0.5 mM showed the best results. Riboflavin treatment reduced H_2O_2 content, but the enzymatic antioxidant defense did not change significantly. This response is due to a rise of non-enzymatic antioxidant defense, by accumulating metabolites like ascorbate and carotenoids, as well as inducing phenolic metabolism. The application of this vitamin enhanced the phenolic and flavonoid content in fruit, concomitant with an induction of PAL and C4H activities and an inhibition of PPO activity. This enhancement of the phenylpropanoid pathway resulted in high vanillic acid and quercetin levels at first day of cold storage. The induction of numerous antioxidant compounds and abscisic acid by riboflavin treatment at short-term postharvest period could be responsible for the lack of chilling injuries in zucchini fruit. Therefore, riboflavin could be successfully implemented in the food industry as an alternative to physical or chemical treatments, due to it is an innocuous additive with good water solubility and low cost, as it prolongs the shelf-life of zucchini fruit and increases its nutraceutical properties.

1. Introduction

One of the main challenges of fruit and vegetable companies is maintaining postharvest fruit quality during transport and commercialization period, being cold storage the most common used procedure to prevent fruit decay. However, when zucchini fruit is stored at low-temperature a physiological disorder named chilling injury (CI) may appear, due to its subtropical origin and that this fruit is commercially harvested immature (Carvajal et al., 2011). The cold stress affects membrane stability, cell wall degradation, epicuticular wax composition and impairs the redox balance of the plant cell (Carvajal et al., 2015a, 2021), resulting in an important weight loss (WL), softening and development of exocarp pitting. Different approaches have been investigated as possible techniques to avoid these damages in fruit, like the application of physical treatments like temperature preconditioning or edible coatings (Carvajal et al., 2015b; Castro-Cegrí et al., 2023b), or the chemicals like putrescine or GABA, that activate GABA shunt pathway (Palma et al., 2015, 2019), nitric oxide to induce antioxidant defense system (Jiménez-Muñoz et al., 2021) or abscisic acid (ABA), which appears to be a key phytohormone in the regulation of postharvest cold tolerance of zucchini fruit (Benítez et al., 2022; Carvajal et al., 2017). In fact, in zucchini fruit under chilling stress, the application of ABA increases antioxidant defense (Castro-Cegrí et al., 2023c), modulates the primary metabolism of the fruit, with changes in several amino acids, organic acids or sugars, and induces the biosynthesis of other phytohormones like cytokinin or methyl jasmonate, as well as vitamins such as riboflavin (Castro-Cegrí et al., 2023a, 2024). Riboflavin is an important metabolite, known as vitamin B2, widely distributed in plants (Fischer and Bacher, 2006), which is involved in metabolic homeostasis, and is found beneficial for human health. It has been described that a riboflavin deficiency alters important biological processes such as iron absorption, tryptophan metabolism or mitochondrial function in humans (Thakur et al., 2017). Thus, riboflavin is used in food industry, since is a safe additive for the consumer and the environment (Bampidis

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et al., 2022). This vitamin is also the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), hence, the riboflavin metabolism affects the growth and development of plants, as well as the defense responses against biotic and abiotic stress (Jiadkong et al., 2024; Li et al., 2012). In fact, riboflavin has been applied in fresh-cut apples and cherry tomatoes to avoid microbial contamination (Hyun et al., 2022). Furthermore, this vitamin has been used to delay the senescence in grape (Wang et al., 2019), and in pear it alters the phenylpropanoid pathway and increases the antioxidant defense (Li et al., 2012). Besides, FAD is a cofactor of several antioxidant enzymes, including glutathione reductase and monodehydroascorbate reductase (Deng et al., 2014). The previous facts make riboflavin a chemical of high interest in the field of postharvest fruit preservation. Thus, this work investigates the effect of exogenous application of riboflavin to prolong the postharvest shelf-life of zucchini fruit under chilling stress.

2. Material and methods

2.1. Fruit material and experimental design

Freshly-harvested zucchini fruits (*Cucurbita pepo* L. morphotype Zucchini) from the cultivar 'Logos' (Syngenta), were supplied by Fruits & Vegetables La ÑECA S.A.T. Fruits of this cultivar were harvested in a range of weight between 250 and 350 g, with a size of about 5 cm diameter and 20 cm length and at an immature stage. Uniform and healthy fruits were divided into three replicates per treatment and storage period, of 6 fruits each one. Two experiments were carried out in this study, a first on to set up the optimal riboflavin concentration, an a second one to study its implication in fruit cold postharvest.

2.1.1. Experiment 1

A concentration range of riboflavin of 0.1, 0.25, 0.5 and 1 mM was tested by submerging the fruits in each solution, using distilled water as the control, at room temperature for 20 min. After treatment, fruits were then dried during 2 h and placed in a temperature-controlled chamber at 4 °C, 85–90 % RH and permanent darkness. Weight loss (%) and chilling-injury index parameters were determined at 1, 5 and 14 days of cold storage, and based on these results a second experiment was carried out.

2.1.2. Experiment 2

According to the results obtained in experiment 1, and following the same methodology, three replicates were prepared per treatment (control and 0.5 mM riboflavin), and storage period (0, 1, 5 and 14 days) each consisting of 6 fruits (126 fruits in total). In each biological replicate the exocarp was completely removed, frozen, powdered in liquid nitrogen and stored at −80 °C, being a part lyophilized and stored at room temperature in permanent darkness.

2.2. Weight loss and chilling injury index

Changes on percentage of weight loss along cold storage per each fruit were calculated following the formula:

$$\%\ Weight\ loss = \frac{(W_i - W_f)}{W_i} x\ 100$$

Being Wi the initial fruit weight and Wf the final fruit weight.

Chilling injury index (CI) was evaluated by using the subjective scale of visual symptoms proposed by Carvajal et al. (2011).

2.3. Firmness and electrolyte leakage

Both parameters were measured in a randomly set of fruit whose exocarp was not powered in liquid nitrogen. Changes on fruit firmness were measured as in Castro-Cegrí et al. (2023b), the results were expressed in Newtons (N). The electrolyte leakage measurements were done in fresh zucchini exocarp, obtaining 10 disc per replicate with an 11-mm diameter stainless-steel cork borer, according to Mao et al. (2007), with the modifications proposed by Carvajal et al. (2015b).

2.4. Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) content was analyzed as in Alexieva et al. (2001) with the modifications proposed by Palma et al. (2015). 0.5 g of fresh exocarp grounded in liquid nitrogen was homogenized with 0.1% (w/v) trichloroacetic acid (1:4, w/v) and centrifuged for 15 min at 4 °C and 12000×*g*. Then, 0.25 mL of supernatant was mixed with 0.25 mL of 0.1 M potassium phosphate buffer (pH 7) and 1 mL of 1 M KI. Samples were incubated in darkness at room temperature for 1 h, and their absorbance was measured at 390 nm. Results were indicated as mg of H_2O_2 per Kg of fresh weight.

2.5. Enzymatic activities

Enzymatic activities were performed in fresh exocarp grounded in liquid nitrogen. The hydrogen peroxide scavengers enzymes catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11) were determined as in Castro-Cegrí et al. (2023b). The catalase activity was expressed as g H_2O_2 per Kg of protein and s, and the ascorbate peroxidase activity was expressed as g ascorbate per Kg of protein and s.

Phenylalanine ammonia lyase activity (PAL; EC 4.3.1.24) was determined as Civello et al., (1997). Samples were measured at 290 nm of wavelength and PAL activity was calculated by increasement of absorbance expressed as U mg^{-1} protein, determining the protein content of each sample; 1 unit was expressed as an increase of 0.001 of absorbance per hour.

The cinnamate 4-hydroxylase activity (C4H; EC 1.14.13.11) was extracted as in Li et al. (2014). The enzymatic activity was measured as in Bi et al. (2007). Samples were measured at 290 nm and the results indicated as μg t-cinnamic acid per Kg of protein and s.

Polyphenol oxidase (PPO; EC 1.14.18.1) activity was determined as in Castro-Cegrí et al. (2023b). The increase of absorbance after reaction was measured at 410 nm for 1 min and the results were indicated as μg catechol per Kg of protein and s.

2.6. Protein determination

The protein content of each enzymatical extract was analyzed following the method proposed by Lowry et al., (1951).

2.7. Ascorbate content

Ascorbate was extracted following the method proposed by Carvajal et al., (2015b) using fresh exocarp grounded in liquid nitrogen, and quantified by HPLC as in the method described by Castro-Cegrí et al., (2023c). Results were expressed as mg ascorbate per Kg of fresh weight.

2.8. Carotenoids quantification

In order to perform the quantification of lutein, zeaxanthin, criptoxanthin, α-carotene and β-carotene the lyophilized samples were extracted and analyzed by HPLC following the method proposed by Castro-Cegrí et al., (2023c). Results were expressed as mg ascorbate per Kg of dry weight.

2.9. Phenolic compounds content

Phenolic compounds were extracted following the method proposed by Castro-Cegrí et al. (2023c). 0.3 g of lyophilized exocarp sample was extracted with 1 mL of methanol 80 % in water (v/v), shaken in darkness

Table 1
Changes in percentage of weight loss (WL) and chilling injury index in zucchini fruit treated with different concentrations of riboflavin (mM) and stored during 0, 1, 5 and 14 days at 4 °C.

	Days at 4 °C	C	0.1	0.25	0.5	1	LSD $p < 0.05$
WL (%)	1	1.88 ± 0.07^{a}	1.66 ± 0.04^{a}	1.68 ± 0.06^{a}	1.68 ± 0.06^{a}	1.72 ± 0.06^{a}	0.51
	5	4.79 ± 0.15^{a}	4.67 ± 0.18^{ab}	4.73 ± 0.15^{ab}	4.01 ± 0.09^{c}	4.61 ± 0.13^{ab}	0.39
	14	10.98 ± 0.41^{a}	10.49 ± 0.38^{ab}	10.44 ± 0.39^{ab}	8.38 ± 0.21^{c}	10.01 ± 0.38^{ab}	1.06
CI (0–3)	1	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0
	5	1.5 ± 0.15^{a}	1.39 ± 0.14^{ab}	1.43 ± 0.11^{ab}	0.75 ± 0.13^{c}	1.4 ± 0.13^{ab}	0.56
	14	2.36 ± 0.21^{ab}	2.5 ± 0.25^{ab}	2.64 ± 0.19^{a}	1.31 ± 0.33^{c}	2.24 ± 0.2^{ab}	0.69

Data presented are means of 18 fruits. Different letters reveal significant differences between treatments for each storage time, according to Duncan's test ($p < 0.05$).

for 1 h at 4 °C and centrifuged at 5000×*g* for 10 min at 4 °C. The supernatant obtained was hydrolyzed with 2 M HCl at 96 °C in a sealed vial for 1 h. When the mixture was at room temperature, anhydrous diethyl ether was added, vortexed for 30 s and centrifuged at 5000×*g* for 5 min to extract the soluble phenolic compounds. The upper phase was collected and evaporated to dryness under a stream of air, and redissolved in methanol 50 % in water (v/v). The total soluble phenolic content was determined with the Folin–Ciocalteu method according to Singleton et al., (1999). The content of phenolic compounds was expressed as g of gallic acid per Kg of dry weight.

Finally, to quantify individually quercetin and vanillic acid, the samples were also injected in an UPLC-MS/MS system coupled to a Xevo TQ-S triple quadrupole mass spectrometer, with the transitions: *vanillic acid, m/z* 151.14 → 135.59 and *quercetin, m/z* 301.07 → 150.63 and 178.67.

2.10. Total flavonoids compounds content

Total flavonoid content was measured in lyophilized exocarp by the colorimetric method described by Chang et al., (2002). Results were expressed as g quercetin per Kg of dry weight.

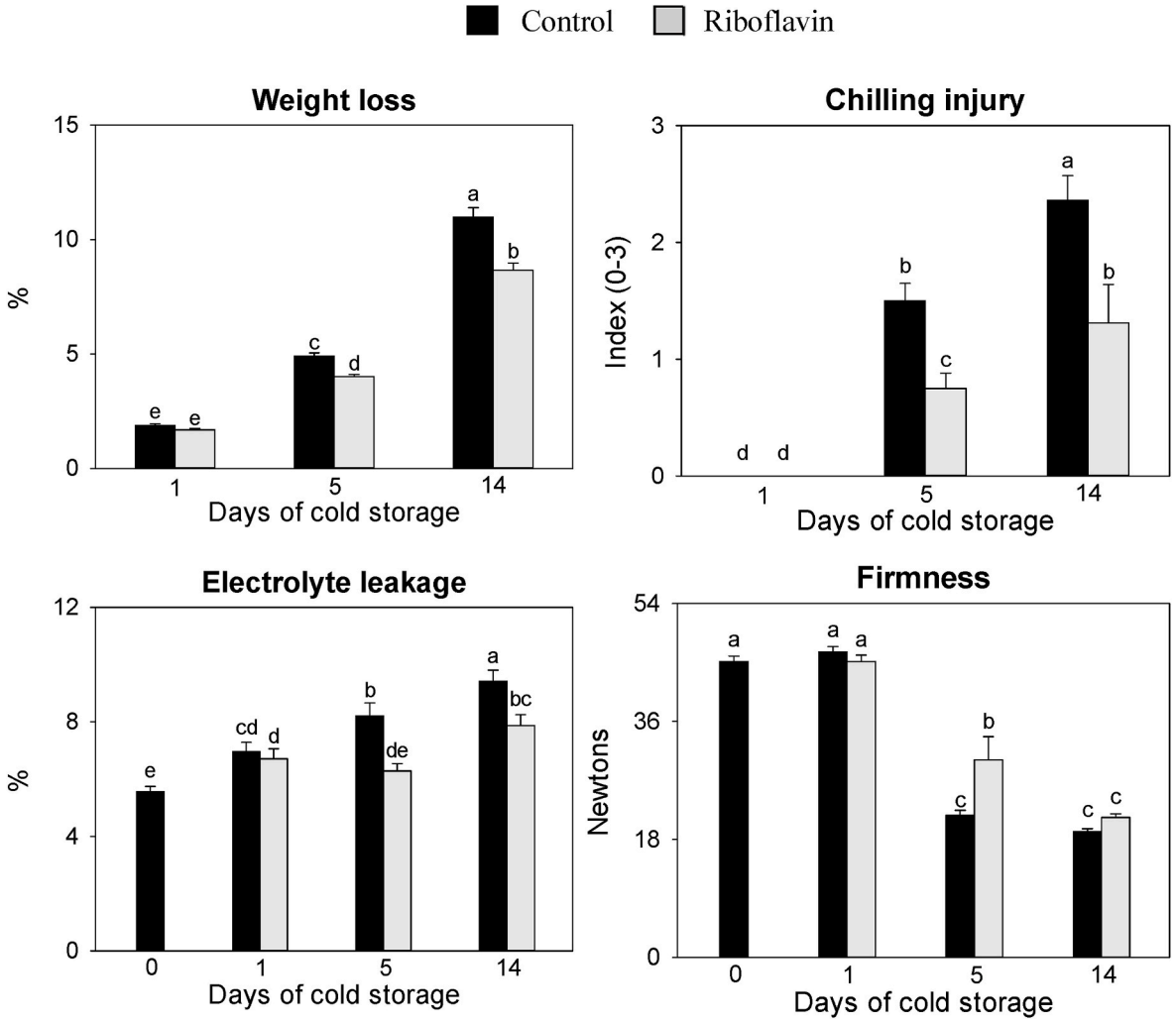

Fig. 1. Changes of weight loss (WL), chilling injury index (CI), electrolyte leakage (EL) and firmness (F) in zucchini fruit treated with 0 and 0.5 mM of riboflavin after 0, 1, 5 and 14 days at 4 °C. Data presented are means of 18 fruits ± SE. Different letters reveal significant differences according to Duncan's test ($p < 0.05$).

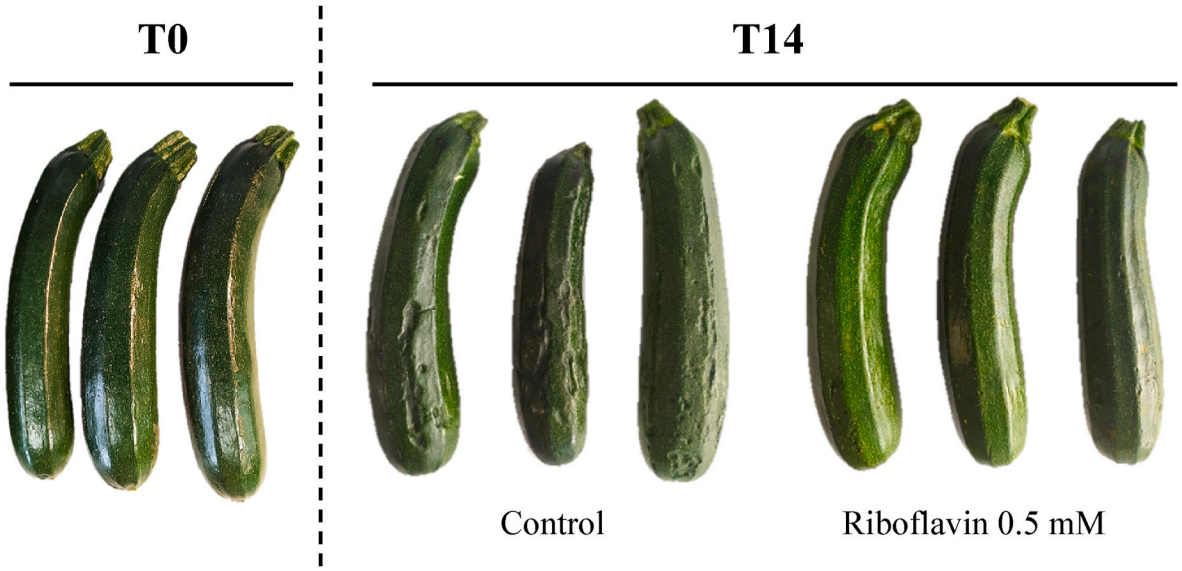

Fig. 2. Zucchini fruits freshly harvested (T0), control fruits with distilled water, and fruits treated with 0.5 mM riboflavin after 14 days of cold storage at 4 °C (T14).

2.11. Abscisic acid quantification

Abscisic acid was determined as in Castro-Cegrí et al. (2023a). ABA was extracted from fresh exocarp grounded in liquid nitrogen and measured by UPLC-MS/MS system coupled to a Xevo TQ-S triple quadrupole mass spectrometer, with the transitions: *abscisic acid, m/z* 263.10 → 153.00. Results were expressed as μg abscisic acid per Kg of fresh weight.

2.12. Riboflavin content

Riboflavin was quantified in zucchini exocarp following the method proposed by Castro-Cegrí et al. (2023a). Briefly, fresh exocarp grounded in liquid nitrogen was used to perform the extraction and the samples were analyzed by HPLC using an Agilent 1260 Infinity system. Results were expressed as μg ascorbate per Kg of fresh weight.

2.13. Statistical analysis

The experiments were completely randomized. The statistical analysis was performed with an ANOVA using the SPSS 28.0 program (SPSS Inc.). Means were compared with Duncan's least significant differences test ($p < 0.05$).

3. Results and discussion

Abscisic acid (ABA) is one of the main phytohormones that regulate postharvest cold tolerance in zucchini fruit (Carvajal et al., 2017). Postharvest treatment with ABA alleviates chilling injury in this fruit by regulating phenolic metabolism and non-enzymatic antioxidant system (Castro-Cegrí et al., 2023c). Furthermore, the exogenous application of this phytohormone modulates the primary metabolism of zucchini fruit and activates the t-zeatin and riboflavin biosynthesis during low-temperature storage conditions (Castro-Cegrí et al., 2023a). Recently, it has been proved the importance of riboflavin in extending the shelf life in several fruits such as pepper or strawberry (Pintos et al., 2022; Zhang et al., 2023). Thus, to study the direct effect of riboflavin in the quality maintenance of zucchini fruit under cold stress could shed light into the mechanisms to acquire fruit cold resistance in *C.pepo* during postharvest.

3.1. Selection of riboflavin concentration to maintain postharvest quality of zucchini fruit

A postharvest treatment with ABA augmented the cold tolerance in zucchini fruit and increased the riboflavin content in exocarp (Castro-Cegrí et al., 2023a). Therefore, in a first experiment we applied different concentrations of this vitamin (0, 0.1, 0.25, 0.5 and 1 mM) in order to select the riboflavin concentration that could be the most adequate to maintain the postharvest quality of zucchini fruit. Weight loss and CI index were evaluated over the storage period at low temperature, and the results are shown in Table 1.

Both quality parameters showed no significant changes between control and riboflavin treatment during cold stress, except for the 0.5 mM concentration at 5 and 14 days of storage. The weight loss and CI decreased significantly with this dose, being the only concentration that increased the cold tolerance of the zucchini fruit. These preliminary results suggest that 0.5 mM of riboflavin increases the defense against cold stress in zucchini fruit, however when higher concentrations were applied a negative effect was detected. This adverse result could be due to the prooxidant effect of riboflavin, since it has been proved that elevated concentrations of this vitamin can induce ROS production provoking oxidative damage in vegetal tissues (Deng et al., 2014). Treatments with 0.1 and 0.02 mM riboflavin were selected to extend the shelf-life in fresh-cut sweet pepper and strawberry, respectively (Pintos et al., 2022; Zhang et al., 2023). Therefore, the riboflavin concentration must be adjusted for each type of fruit. According to the obtained results, a second experiment was carried out to study the riboflavin-mediated regulation of chilling tolerance in zucchini fruit.

3.2. Effect of 0.5 mM riboflavin treatment on zucchini fruit during cold storage

3.2.1. Changes in quality parameters of zucchini fruit by exogenous riboflavin application

The weight loss and CI showed similar results to those obtained in the first experiment. Fig. 1 shows that riboflavin treatment reduced both weight loss and CI at 5 and 14 days of storage at low temperature compared to non-treated fruit. Zucchini fruit is sensitive to cold storage, suffering a firmness loss and softening (Castro-Cegrí et al., 2023b). In fact, the low temperature reduced firmness around 60 % after 5 and 14 days in control fruit compared to freshly harvested fruit, while the application of riboflavin showed a lower softening after 5 days at 4 °C

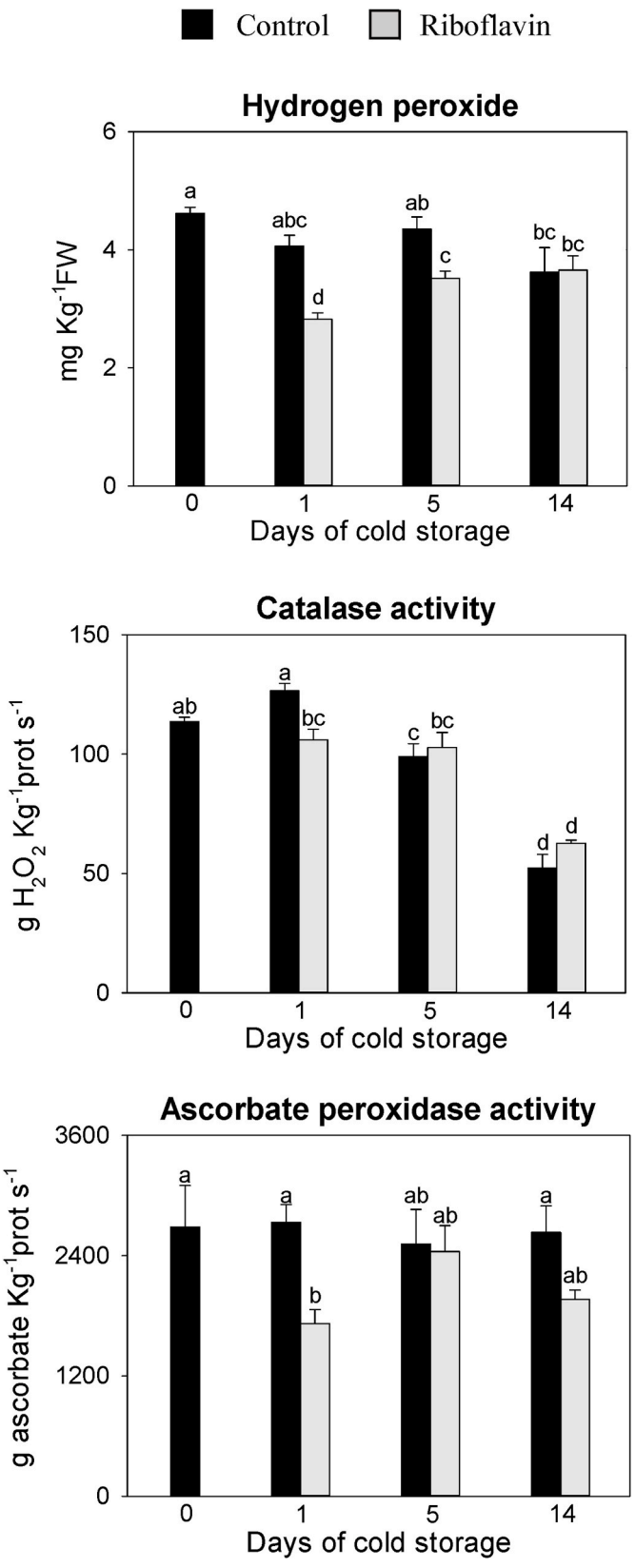

Fig. 3. Determination of hydrogen peroxide content, catalase and ascorbate peroxidase activities in zucchini fruit treated with 0 and 0.5 mM of riboflavin after 0, 1, 5 and 14 days at 4 °C. Data presented are means of three biological replicates consisting of 6 fruits each one ± SE. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

(Fig. 1).

Riboflavin applications also reduced the electrolyte leakage after 5 and 14 days of storage compared to control fruit, indicating that this treatment helps to maintain the integrity of the membrane, maintaining the zucchini fruit quality during cold storage (Fig. 1). The effects of riboflavin application can be observed in Fig. 2, where treated fruits exhibited fewer pitting symptoms on the exocarp compared to control fruits, maintaining a better appearance after 14 days of cold storage.

3.2.2. Changes in H_2O_2 content and antioxidant enzymes by riboflavin treatment

Preventing oxidative damage to fruit during postharvest period is one of the most important challenges in horticulture research. The production of reactive oxygen species (ROS) is a common outcome of low temperature (Dreyer and Dietz, 2018). Among the various ROS, H_2O_2 acts as a central player under stress conditions (Hossain et al., 2015), and if the production persist, lipid peroxidation and membrane damage occur. The content of H_2O_2 was determined in order to evaluate the effect of riboflavin in ROS accumulation, observing a significant reduction in the treated fruit compared to control fruit at the beginning of cold storage, being of about 30 and 20 % after 1 and 5 days at 4 °C, respectively (Fig. 3). However, exogenous riboflavin also reduced two of the main hydrogen peroxide scavenging activities (catalase and ascorbate peroxidase) after 1 day at 4 °C (Fig. 3). This response is contrary to the results obtained in strawberry, where it has been described that riboflavin treatment increased both activities in the first days of cold storage (Zhang et al., 2023). In zucchini, the results suggest that the H_2O_2 detoxification may be induced by other mechanisms. Zucchini fruit treated with, abscisic acid during postharvest alleviated chilling injury by regulating phenolic metabolism and non-enzymatic antioxidant system (Castro-Cegrí et al., 2023c).

Furthermore, this phytohormone induced the accumulation of riboflavin in zucchini fruit (Castro-Cegrí et al., 2023a), which would indicate that also in the case of riboflavin treatment the reduction would also be due to an increase in the non-enzymatic antioxidant system and in the phenolic metabolism.

3.2.3. Changes in non-enzymatic antioxidants by riboflavin treatment

Ascorbate, carotenoids and phenolic compounds are the main non-enzymatic antioxidants in zucchini fruit and play a significant role in scavenging ROS free radicals, maintaining fruit quality (Castro-Cegrí et al., 2023c; Palma et al., 2016). These non-enzymatic antioxidants were evaluated to study how riboflavin diminishes chilling damage (Table 2 and Fig. 4).

Cold stress induced ascorbate accumulation in both treated and untreated zucchini fruit. However, riboflavin-treated fruit showed higher content in ascorbate than control fruit at 1 day of cold storage, in this case around 30% (Table 2). The increase at the beginning of storage would be important to ameliorate the chilling injuries in the following days, as has been proved to occur in pear fruit treated with melatonin (Liu et al., 2024) or in riboflavin treated strawberries (Zhang et al., 2023). Other metabolites with an important antioxidant capacity are carotenoids, whose involvement in tolerance against cold stress has been widely studied in fruits like mandarin or zucchini (Castro-Cegrí et al., 2023c; Rey et al., 2020). To get a deeper insight into how riboflavin application affects these antioxidant metabolites, some of the most important carotenoids were individually quantified. Lutein, zeaxanthin and α-carotene did not show significant changes between control fruit and freshly harvested fruit (Table 2). On the contrary, riboflavin treatment increased α-carotene content at 1 day of cold storage, and lutein content at 5 and 14 days. Furthermore, cryptoxanthin content was higher in treated than in untreated fruit during the first days of storage at 4 °C. The β-carotene content showed a similar response, with significant differences at 5 and 14 days of storage at low temperature. Zeaxanthin was the only carotenoid that showed no statistical differences.

The induction of phenylpropanoid metabolism for the synthesis of

Table 2
Determination of ascorbate (mg Kg^{-1} FW) and carotenoids content (mg Kg^{-1} DW) in zucchini fruit treated with 0 and 0.5 mM of riboflavin after 0, 1, 5 and 14 days at 4 °C.

Days of storing at 4 °C	Treatment	Ascorbate	Lutein	Zeaxanthin	Criptoxanthin	α-carotene	β-carotene
0	At harvest	28.4 ± 1.2^{e}	354.4 ± 17.9^{b}	14.8 ± 2.8^{a}	0.84 ± 0.04^{b}	7.9 ± 0.7^{bcd}	520.1 ± 35.1^{a}
1	Control	38.8 ± 0.8^{d}	358.6 ± 16.7^{b}	16.2 ± 0.9^{a}	0.77 ± 0.09^{b}	7.5 ± 0.4^{bcd}	490.0 ± 25.8^{ab}
	Riboflavin	50.6 ± 2.1^{bc}	320.6 ± 31.3^{b}	16.6 ± 1^{a}	1.08 ± 0.02^{a}	11.4 ± 0.9^{a}	512.9 ± 21^{a}
5	Control	47.4 ± 2.9^{cd}	351.2 ± 3.2^{b}	10.8 ± 3.7^{a}	0.34 ± 0.02^{d}	6.7 ± 0.2^{d}	431.7 ± 16.6^{bc}
	Riboflavin	47.2 ± 2.8^{cd}	460.0 ± 23.5^{a}	10.2 ± 5.4^{a}	0.55 ± 0.06^{c}	8.6 ± 0.2^{bc}	505.6 ± 9.1^{a}
14	Control	60.9 ± 5.1^{ab}	368.6 ± 9.8^{b}	19.2 ± 2.2^{a}	0.31 ± 0.05^{d}	6.8 ± 0.4^{cd}	403.9 ± 17.7^{c}
	Riboflavin	64.5 ± 5.7^{a}	451.2 ± 4.6^{a}	16.2 ± 3.9^{a}	0.36 ± 0.02^{d}	8.5 ± 0.5^{b}	502.3 ± 18^{a}
LSD (p<0.05)		10.1	65.1	9.4	0.14	1.6	66.2

Data presented are means of triplicate samples of six fruit each. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

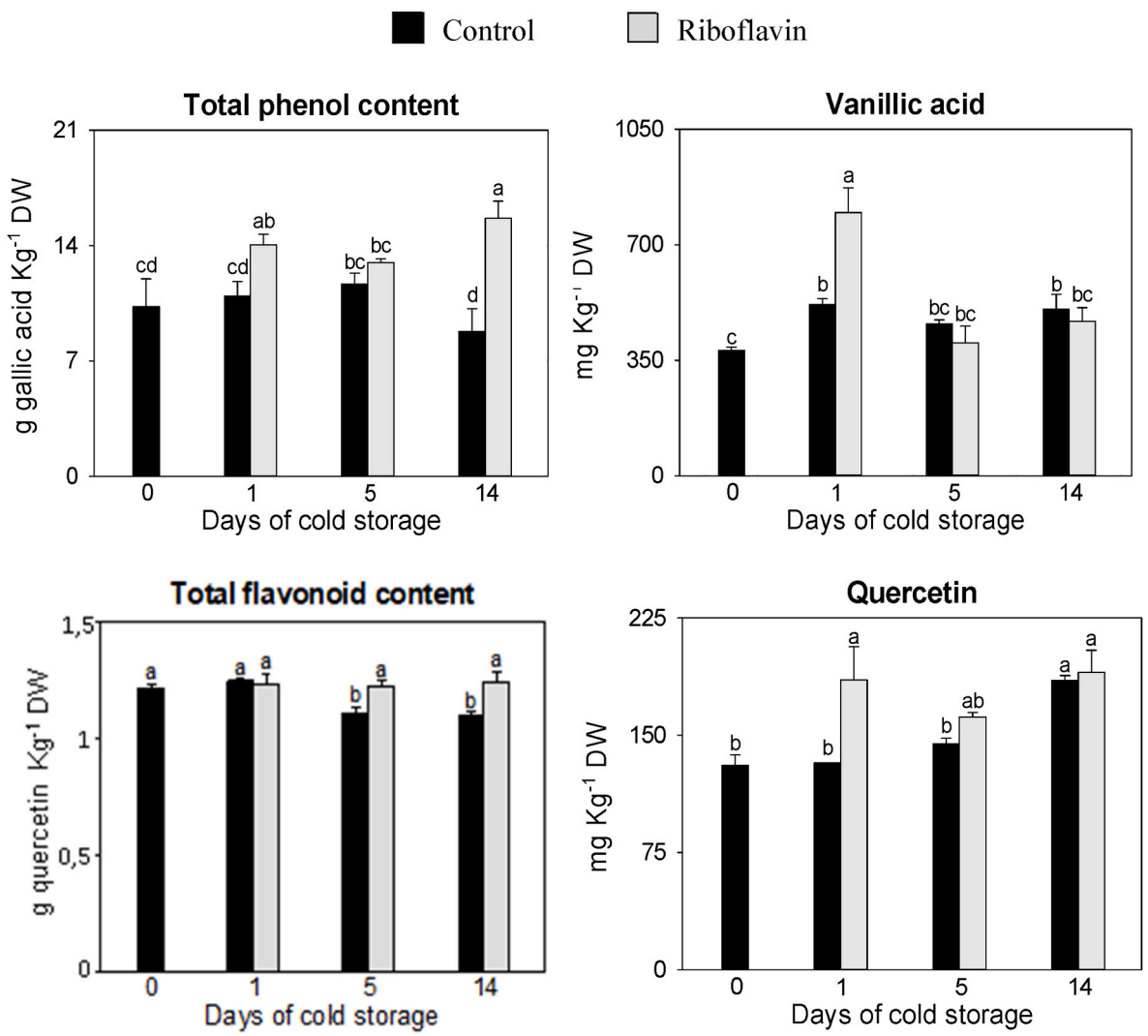

Fig. 4. Determination of total phenol, total flavonoid, vanillic acid and quercetin content in zucchini fruit treated with 0 and 0.5 mM of riboflavin after 0, 1, 5 and 14 days at 4 °C. Data presented are means of three biological replicates consisting of 6 fruits each one ± SE. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

phenolic compounds is another important pathway in fruit chilling tolerance (Wang et al., 2021a). For this reason, several postharvest treatments have maintained the quality of the fruit by increasing these antioxidant compounds, such as a melatonin treatment in sweet cherry (Michailidis et al., 2021) or the application of nitric oxide to zucchini (Jiménez-Muñoz et al., 2021). Riboflavin application in zucchini fruit enhanced the total phenol content at 1 and 14 days of cold storage respect to freshly harvested fruit (Fig. 4), whereas control fruit maintained the same phenol levels that were measured for freshly harvested fruit. In previous experiments with ABA treatment the phenolic compounds vanillic acid and quercetin showed bigger changes at 1 day of treatment (Castro-Cegrí et al., 2023c). The riboflavin treatment increased the content in vanillic acid after 1 day of cold storage about 54 % compared to the control (Fig. 4). Vanillic acid is one of the major hydroxybenzoic compounds in peel of mango fruit, being its concentration strongly increased during postharvest period (Vithana et al., 2018). Methyl-jasmonate treatment also enhanced the accumulation of vanillic acid increasing the quality maintenance in olive fruits during

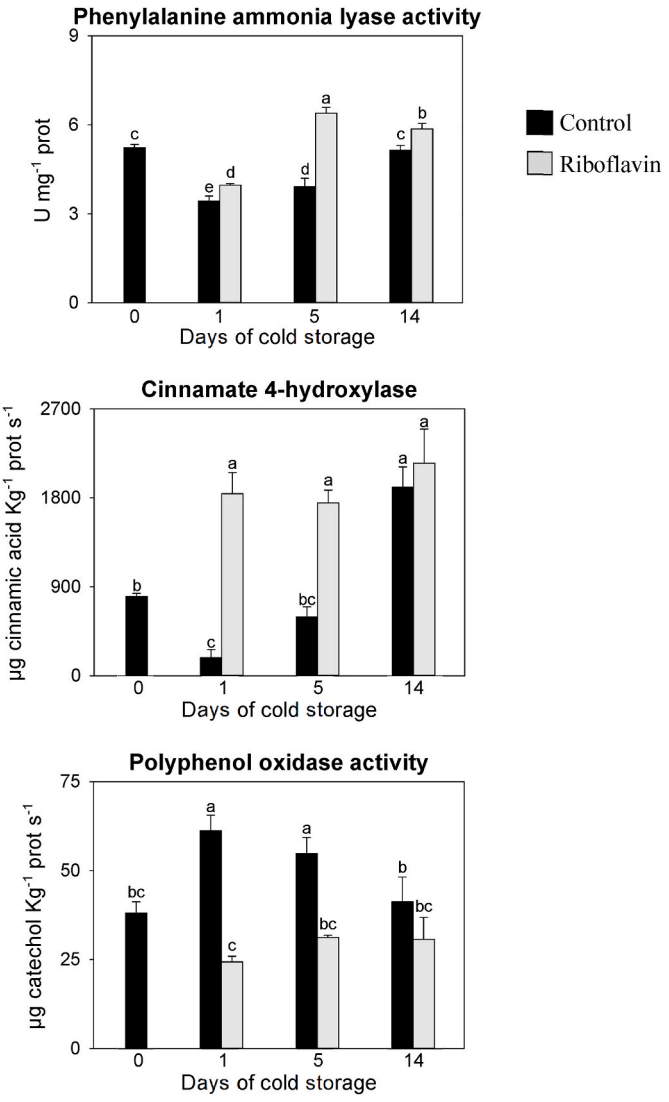

Fig. 5. Phenylalanine ammonia lyase, cinnamate 4-hydroxylase and polyphenol oxidase activities in zucchini fruit treated with 0 and 0.5 mM of riboflavin after 0, 1, 5 and 14 days at 4 °C. Data presented are means of three biological replicates consisting of 6 fruits each one ± SE. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

process period (Flores et al., 2017). Due to its antioxidant properties vanillic acid has been used as a functional compound to enrich chitosan coatings (Osondu et al., 2022). With respect to flavonoids, a diminution in their content was observed during cold storage period in control fruit, while in treated fruit the level of flavonoids remained similar to freshly harvested fruit (Fig. 4). Flavonoids constitute an important secondary ROS scavenging system in plants exposed to different stresses (Fini et al., 2011). Several treatments that increase the flavonoid accumulation also improve the capacity to resist chilling stress of fruit, like in sweet cherries exposed to UV-C irradiation (Zhang et al., 2021) or in persimmon by curing (37 °C for 3 days) treatment (Carmona et al., 2021). From the flavonoid group, quercetin is one of the most studied flavonoids since its accumulation is related to the acquisition of the chilling tolerance in mango and peach (Kumar Patel et al., 2023; Wang et al., 2021b). In this work, an increment of 40 % in quercetin content was detected in zucchini fruit after 1 day of cold storage and riboflavin treatment, compared to control fruit (Fig. 4).

Exogenous application of riboflavin in zucchini fruit increased the content of non-enzymatic antioxidants such as ascorbate, carotenoids and phenolic compounds, that could be sufficient to reduce ROS production inducing cold tolerance of zucchini fruit, without the need to increase the enzymatic antioxidant activity.

3.2.4. *Effect of riboflavin treatment on phenylpropanoid pathway enzymes*

The accumulation of phenolic compounds observed after riboflavin treatment could be the results of an induction in the activity of the main enzymes of the phenylpropanoid pathway. The first enzymatic step is carried out by phenylalanine ammonia lyase activity (PAL), which catalyzes the synthesis of trans-cinnamic acid through non-oxidative deamination of phenylalanine.

The riboflavin application induced a significant increment in PAL activity during the entire cold storage period, especially after 5 days of cold storage when the activity was 163 % higher than in control fruit (Fig. 5). In the next step of the phenylpropanoid pathway, the cinnamate 4-hydroxylase (C4H) catalyzes the conversion of trans-cinnamic acid to coumaric acid. This enzyme drastically reduced its activity in control fruit in the first days of cold storage compared to the freshly harvested fruit. On the contrary, riboflavin treatment caused a 10-fold increment respect to control after 1 day of cold storage, and significant differences were also detected after 5 days (Fig. 5). PAL and C4H are crucial enzymes in the phenolic metabolism, therefore its up-regulation has been detected after methyl-jasmonate treatment during tomato ripening and in kiwifruit after exposure at low temperatures, both increasing the production of phenolic compounds (Tao et al., 2022; Xie et al., 2024). Thus, the results obtained by this treatment with riboflavin in zucchini fruit could open a new way to enhance the tolerance to abiotic stresses of other fruit and vegetables. Furthermore, increasing the production of phenolic compounds could be due to an increment in the enzymes of synthesis, such as PAL and C4H, but it could also be due to a decrease in their catabolism, being polyphenol oxidase (PPO) the enzyme that catalyzes the oxidation of phenolics to quinones, resulting in browning reactions (Yoruk and Marshall, 2003) and losses in quality. Storing at low temperatures provoked a significative increment of PPO activity in zucchini fruit at short-term cold period (1 and 5 days). By contrast, an important inhibition in the enzyme activity was detected by the application of riboflavin, being around 60 % and 45 % respect to control fruit after 1 and 5 days at 4 °C, respectively (Fig. 5). This improvement could help to maintain for a longer period the nutritional quality of fruit, preventing the flesh-browning and membrane damage as described previously. Similar results have been reported during postharvest period in pear fruit (Zheng et al., 2019) or in blood oranges (Habibi et al., 2023). The increase in PAL and C4H activity, and the decrease in PPO activity correlates with the higher amount of phenolic compounds detected after riboflavin treatment. Therefore, we hypothesized that the accumulation of phenolic compounds is a mechanism induced by riboflavin to increase the cold tolerance of zucchini fruit.

3.2.5. *Crosstalk between abscisic acid and riboflavin*

An enhancement of the phenolic metabolism has been also detected with ABA treatment (Castro-Cegrí et al., 2023c), that is involved in the postharvest cold tolerance of zucchini fruit (Benítez et al., 2022; Carvajal et al., 2017). Besides, after analyzing the differences between control and ABA-treated fruit using untargeted metabolomics, one of the most altered pathways was the riboflavin biosynthesis pathway, with an increase in the content of riboflavin after 1 day of cold storage (Castro-Cegrí et al., 2023a). To get a deeper insight into ABA and riboflavin feedback regulation, abscisic acid and riboflavin content has been measured in exocarp of control and riboflavin treated fruit during postharvest cold storage. Firstly, the treatment significantly enhanced the riboflavin content in zucchini exocarp, revealing an efficient absorption of this vitamin and also maintaining high levels throughout the cold storage period (Fig. 6). By other side, an important diminution in ABA content was detected just after exposure to cold stress in control fruit, reducing about 55 % the amount of this phytohormone at 1 day of cold storage, while in those fruits treated with riboflavin the ABA content remained without changes, both compared with freshly harvested

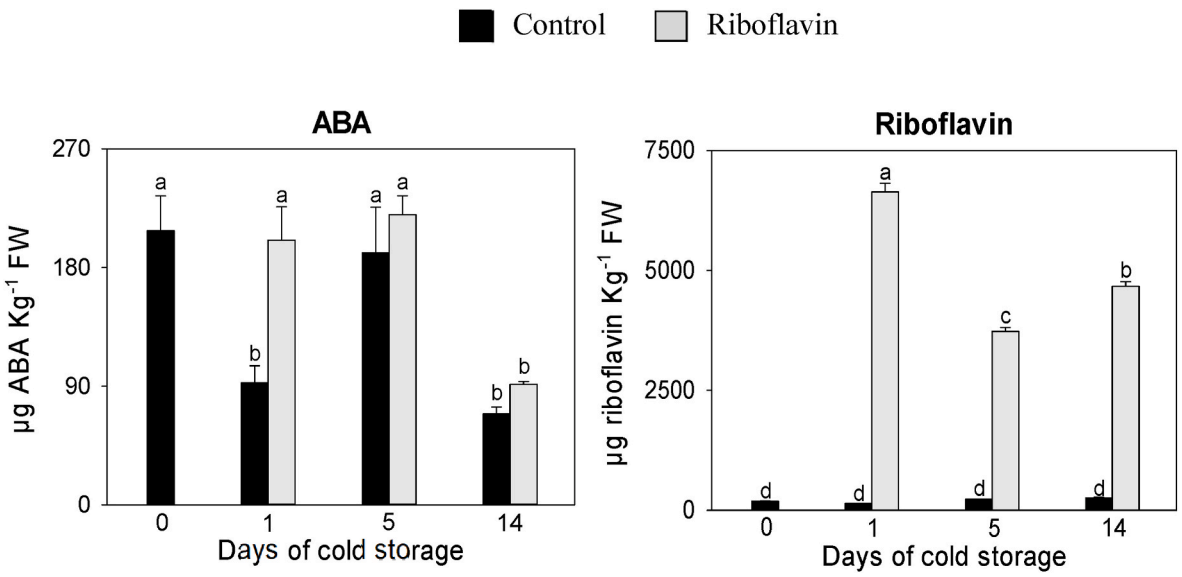

Fig. 6. Abscisic acid (ABA) and riboflavin content in zucchini fruit treated with 0 and 0.5 mM of riboflavin after 0, 1, 5 and 14 days at 4 °C. Data presented are means of three biological replicates consisting of 6 fruits each one ± SE. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

fruit (Fig. 6). These results indicate that exits a positive regulation between these compounds. Thus, a more in-depth study of the mechanisms underlying this crosstalk would shed light into the postharvest cold tolerance of zucchini fruit.

4. Conclusions

Riboflavin treatment enhanced non-enzymatic antioxidant defense by accumulating ascorbate and carotenoids, as well as by inducing phenolic metabolism. The accumulation of phenolic compounds and flavonoids at short-term cold storage is due to and increment in the activity of the main synthesis enzymes of the phenylpropanoid pathway (PAL and C4H), together with the inhibition of PPO activity. Vanillic acid and quercetin, two relevant compounds for postharvest cold tolerance in zucchini fruit, incremented their content with riboflavin at the first day of storage, likely preventing chilling injuries. Furthermore, riboflavin treatment maintained the abscisic acid content in exocarp during first days of cold storage, which could enhance the cold tolerance of zucchini fruit. Therefore, we propose riboflavin as a safe food additive of low cost to increase the nutritional value of zucchini fruit, as well as to prolong its shelf-life during postharvest cold storage.

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

Data will be made available on request.

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