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Modified atmosphere affects glucosinolate metabolism during postharvest storage of broccoli

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Abstract

Broccoli is a vegetable with a growing consumer demand worldwide due to its important nutritional properties, including high amounts of glucosinolates. However, this vegetable has a short postharvest life and quickly loses its organoleptic and nutritional quality. Modified atmosphere is one of the numerous methodologies used to delay deterioration during postharvest storage of broccoli heads. In this study, the effect of a modified atmosphere during postharvest storage of broccoli on glucosinolate metabolism was evaluated. Five glucosinolates were identified by using a UPLC system coupled to a mass spectrometer. We detected one aliphatic glucosinolate and four indolic glucosinolates, and their concentration was found to decrease during storage. The decrease in content was less marked, and in some cases, an increased level was observed in the treated samples. Moreover, the treatment made it possible to maintain higher expression (analyzed by real-time quantitative PCR) of genes linked to glucosinolate biosynthesis. We also detected an increased expression of some genes related to indolic glucosinolate biosynthesis. Overall, storage of broccoli in modified atmospheres allowed for maintaining better visual quality and higher levels of glucosinolates.

Keywords: *Brassica oleracea*; nutraceuticals; postharvest; modified atmosphere; gene expression.

Practical Application: The study of the effect of a modified atmosphere on the metabolism of glucosinolates during postharvest storage of broccoli provides important information for consumers and researchers about one of the most important bioactive compounds in brassicas.

1 INTRODUCTION

Vegetables belonging to the Brassicaceae family have numerous nutraceutical compounds, including all the essential amino acids and some vitamins and antioxidants such as ascorbic acid, phenols, and flavonoids (Moreira-Rodríguez et al., 2017). These vegetables also have high concentrations of glucosinolates, a group of secondary metabolites, which are synthesized from amino acids and classified as aliphatic, indolic, and aromatic depending on the precursor amino acid (Blažević et al., 2020).

The biosynthetic pathways of aliphatic and indole glucosinolates are shown in Figure 1. The route comprises various stages that could be classified into three: the first consists of the elongation of the side chains of the amino acids if the precursor amino acid is methionine or phenylalanine (Prieto et al., 2019). The second stage is the formation of the basic structure of the glucosinolate, which includes the oxidative decarboxylation of the amino acid to its corresponding aldoxime and conversion of the oxime to the glucosinolate backbone. Finally, secondary modifications occur that give rise to the great variety of glucosinolates that are known, such as hydroxylation, methoxylation, oxidation, desaturation, or benzoylation, mainly in its side chains (Blažević et al., 2020).

In the case of aliphatic glucosinolates, if the amino acid precursor is methionine, the elongation stage is conducted by BCATs and MAM (methylthioalkylmalate synthase) genes. MAM1 enzyme catalyzes condensation reactions in the first three cycles of methionine elongation, whereas MAM2 catalyzes only in the first cycle (Sønderby et al., 2010). In the second phase, the CYP79F1, SUR1, and UGT74B1 genes are responsible for the oxidative decarboxylations and formation of desulfo-glucosinolates that are subsequently converted to aliphatic glucosinolates by the enzyme sulfotransferase, ST5b,c. Finally, at the stage of

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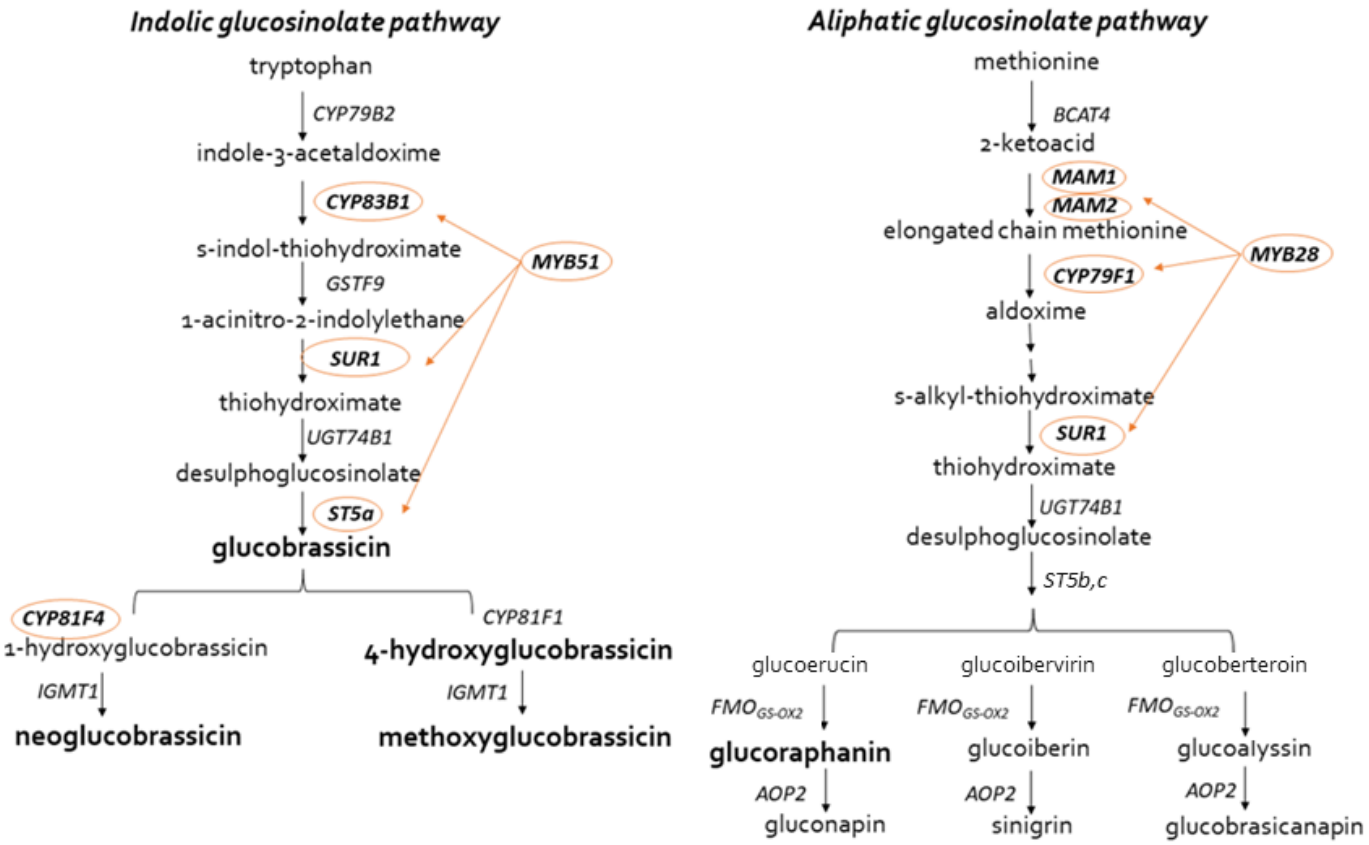


Figure 1. Biosynthetic pathways of glucosinolates (indolic glucosinolates at left and aliphatic glucosinolate at right) with the enzymes and genes involved in each step. The glucosinolates detected and the genes analyzed are highlighted.

secondary modifications, FMOGS-OX1 catalyzes the conversion of glucoerucin to glucoraphanin, while AOP2 is responsible for the conversion of glucoraphanin to gluconapin. MYB28 is a main transcription factor involved in the biosynthesis pathway of aliphatic glucosinolates (Blažević et al., 2020).

The indole glucosinolate biosynthesis pathway begins when tryptophan is converted to indole-3-acetaldoxime by the CYP79B2/B3, and indole-3-acetaldoxime is converted to indole-3-acetonitrile in a reaction catalyzed by the CYP83B1 enzyme (Xu et al., 2017). A number of genes including GSTF, SUR1, UGT74B1, and ST5a are involved in the formation of the core structure 3-indolemethylglucosinolate (glucobrassicin). The enzyme ST5a sulfotransferase is involved in the last reaction to transform desulfo-glucosinolate into glucosinolate. Secondary modifications are catalyzed by the enzymes CYP81F and IGMT: CYP81F1 catalyzes the conversion of glucobrassicin to 4-hydroxyglucobrassicin, and CYP81F4 catalyzes the passage from glucobrassicin to 1-hydroxyglucobrassicin. Finally, MYB51 codifies one of the major transcription factors regulating the pathway of biosynthesis of indole glucosinolates (Frerigmann & Gigolashvili, 2014).

The physiological function of glucosinolates in plants is linked to defense against insect herbivores (Martínez-Ballesta et al., 2013). When tissue damage occurs, glucosinolates come into contact with the enzyme myrosinase, which is stored in different cellular compartments from glucosinolates. Myrosinase catalyzes the loss of a single sugar, producing an unstable aglycone that decomposes to generate isothiocyanates and nitriles. From a human health point of view, these isothiocyanates have a protective effect against various types of cancers (Mandrich & Caputo, 2020). Prolonged consumption of cruciferous vegetables with an adequate content of glucosinolates decreases the risk of contracting these pathologies (Jeffery & Araya, 2009).

Broccoli (*Brassica oleracea* L. var. *Italica*) has all the nutritional properties of cruciferous vegetables but has a very short postharvest life when stored at room temperature. Broccoli heads are harvested while they are still developing, which causes stern stress and an accelerated senescence during postharvest storage (Ahlawat et al., 2022). Under these conditions, broccoli heads undergo rapid degreening due to chlorophyll degradation and significant loss of sugars and proteins. An important decrease in glucosinolate content has also been detected, which determines a loss in the nutritional value of the product (Jones & Dangl, 2006; Xu et al., 2016).

Several methodologies have been employed to delay senescence and nutraceutical loss during postharvest storage of broccoli, including refrigeration, heat treatments, UV, visible light, 1-MCP, and modified atmospheres (Aiamla-or et al., 2010; Duarte-Sierra et al., 2017; Fernández-León et al., 2013; Ma et al., 2010; Pintos et al., 2021). Modified atmosphere packaging (MAP) is the generation and stabilization of an equilibrium

atmosphere when a fruit or vegetable product is hermetically packaged in a plastic film of selective gas permeability. The equilibrium atmosphere has a composition lower in oxygen and richer in carbon dioxide than the outside atmosphere (Oliveira et al., 2015). Low oxygen concentrations decrease the respiratory activity of the product and high carbon dioxide concentrations decrease ethylene synthesis, thus delaying the appearance of senescence symptoms (Oliveira et al., 2015). MAP of broccoli inflorescences allows for the retention of vitamin C and antioxidant content, as well as the maintenance of green color, corresponding to higher chlorophyll content (Jia et al., 2009; Singh et al., 2018).

This study aimed to provide new insights into the effect of modified atmospheres on glucosinolate metabolism during postharvest storage of broccoli heads, by analyzing the glucosinolate content and the expression of genes involved in the biosynthesis and degradation of these compounds.

2 MATERIALS AND METHODS

2.1 Plant material

Broccoli heads (*B. oleracea* L. var. *Italica* cv Legacy) were harvested from a local farmer (La Plata, Buenos Aires, Argentina, 34°59'07.5"S 58°02'47.3"W). Broccoli heads were obtained early in the morning and immediately carried out to the laboratory to be processed. Broccoli heads were selected according to the usual cultivation practices. The diameter of the heads varied from 18 to 20 cm, with a dark green color and without senescent inflorescences, mechanical damage, or development of pathogens.

2.2 Modified atmosphere treatment

A total of 45 broccoli heads were individually covered with a low-density polyethylene (LDPE) bag (40 µm thick, 20 cm × 30 cm; permeability to O_2, CO_2, and water; 6.168×10^{-7} mL m^{-2} s^{-1} Pa^{-1}, 7.32×10^{-7} mL m^{-2} s^{-1} Pa^{-1}, and 4.989×10^{-4} g m^{-2} s^{-1} Pa^{-1}, respectively), sealed, and stored in darkness at 20°C for 5 days (Gómez Lobato et al., 2012). The O_2 and CO_2 concentration in the package's headspace was monitored at initials and during storage using a gas analyzer (Servomex, Series 5200, UK) through a self-adhesive septum. The same number of heads was loosely covered with PVC film and utilized as controls. The sample storage chamber was sufficiently ventilated to avoid possible ethylene accumulation. For each time point (0, 3, and 5 days), superficial color was measured, and then florets were separated from heads, pooled and frozen with liquid nitrogen, and subsequently kept at -80°C for subsequent analysis.

2.3 Superficial color measurement

Superficial color was measured on broccoli heads with a Minolta CR-300 chromameter (Minolta, Osaka, Japan). Five different points of the head were selected to perform the measurements. Hue angle (h^o) was calculated as follows: $h^o = \tan^{-1}$ (b/a) for a and $b > 0$ or $h^o = \tan^{-1}$ (b/a) + 180° for $a < 0$ and $b > 0$.

2.4 Chlorophyll content determination

The determination of chlorophyll content was carried out according to Casajús et al. (2020). Five biological replicas and three technical repetitions were performed. Results were expressed as grams per kilogram of fresh tissue.

2.5 RNA extraction and cDNA synthesis

Frozen broccoli florets were ground with liquid nitrogen, and total RNA was obtained using the hot borate method according to Wan and Wilkins (1994). Total RNA was quantified by UV spectrophotometry (ClarioStar, BMG LABTECH), and 6 µg was treated with RQ1 DNAase (Promega) according to the manufacturer protocol, with little modifications as described previously (Gómez Lobato et al., 2014). Purified RNA was then quantified again, and 4 µg was used for cDNA synthesis using MML-V reverse transcriptase (Promega) and random primers hexamers. Five biological replicas were obtained.

2.6 Real-time quantitative PCR analysis

The expression of 11 genes related to glucosinolate metabolism was analyzed: nine genes related to glucosinolate biosynthesis (*BolMYB51*, *BolMYB28*, *BolMAM1*, *BolMAM2*, *BolCYP79F1*, *BolCYP83B1*, *BolSUR1*, *BolST5a*, and *BolCYP81F4*) and two genes involved in glucosinolates degradation (*BolMyr* and *BolESP*). Specific primers were designed based on the Brassica Database (Cheng et al., 2011) (Supplementary Table 1). Gene expression was evaluated following the methodology of Casajús et al. (2020), by using a StepOnePlus™ Real-Time PCR System (Applied Biosystems; San Francisco, USA) and FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) with the following program: one cycle at 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min. Actin (AF044573) was used as a gene normalizer. Each measurement was performed by using five biological replicates and three technical replicates.

Table 1. Changes in color and total chlorophyll contents (expressed as g kg^{-1} on the basis of fresh tissue) in broccoli heads stored for 5 days at 20°C*.

	Hue		Total Chlorophylls	
	Control	MA	Control	MA
Day 0	121.9 ± 10.8 a	121.90 ± 10.9 a	0.112 ± 0.009 a	0.112 ± 0.009 a
Day 3	98.9 ± 3.4 a	115.0 ± 6.1 b	0.016 ± 0.005 a	0.075 ± 0.005 b
Day 5	89.2 ± 1.9 a	114.8 ± 6.6 b	0.009 ± 0.001 a	0.050 ± 0.002 b

*Data represent a mean ± standard deviation. Different letters indicate significant differences at the same storage time ($p < 0.05$).

2.7 Analysis, identification, and determination of glucosinolate content

Glucosinolates were extracted and analyzed by following the protocol described by Casajús et al. (2020). Approximately 10 g of frozen broccoli florets were freeze-dried and an aliquot of 25 mg was mixed in 1.2 mL of 800 mL·L^{-1} methanol preheated and vortexed for 10 s. The mixtures were incubated in a water bath for 15 min at 80°C and then centrifuged at 12,000×*g* for 12 min. The supernatants (0.8 mL) were transferred to columns loaded with 600 µL of wetted DEAE (diethyaminoethanol) Sephadex A-25 resin (1:1, resin:water, v/v). To each column, 140 µL (0.25 µkat) of purified sulfatase enzyme (Sigma, St. Louis, USA) was added, and the mixtures were incubated at room temperature for 18 h in the dark. Desulfated glucosinolates were then eluted by vacuum through the columns, with the addition of 0.2 mL of 800 mL L^{-1} methanol followed by 0.2 mL of water. The eluents were combined, dried in a Centrivac Concentrator, and dissolved in 250 µL of 0.01 g L^{-1} formic acid, with L-tryptophan and sinigrin added as internal standards, for analysis. The intensity of the tryptophan peak was used to normalize the intensities of the DS-glucosinolates between runs, and sinigrin was used as a standard to calculate glucosinolate concentration. Identification and quantification of individual glucosinolates were carried out by LC–MS/MS on an Acquity UPLC system coupled to a Xevo G2 QToF mass spectrometer with a LockSpray source (Waters Corp., Milford, USA) using a mobile phase program described previously (Tian et al., 2018). The desulfo-glucosinolates were separated on an HSS T3 column (2.5 µm, 2.1 mm × 150 mm, Waters) and then detected by UV absorbance of 229 nm and the Xevo G2 QToF using an ESI ion source. The Xevo G2 QToF was operated in positive ion mode, analyzing the *m/z* range from 50 to 1,200. The MS data were locked mass corrected using the monoisotopic mass at *m/z* 566.2771 of the singly-charged ion of leucine enkephalin. Identification of individual glucosinolates was done by following the methods as reported (Mellon et al., 2002; Zimmermann et al., 2007). Each desulfo-glucosinolate was identified on the basis of the protonated precursor ion masses (M + H)+ and its group-specific fragment ions generated via in-source decay including the ion generated by the loss of a sugar group (M + H - C6H10O5)+ and the observed metal ion adducts: (M + Na)+ and (M + K)+.

2.8 Statistical analysis

The statistical analysis was performed using the SYSTAT software package. Data for superficial color (Hue) and chlorophyll content were analyzed by ANOVA, and means were compared by Tukey test ($p < 0.05$). The results for gene expression were analyzed by Student's *t*-test ($p < 0.05$).

3 RESULTS AND DISCUSSION

3.1 Modified atmosphere treatment reduces glucosinolate loss during postharvest storage

The use of selectively permeable films to achieve a modified atmosphere is widely used as a postharvest technology. Among some of the advantages reviewed by Oliveira et al. (2015), low oxygen concentrations decrease the respiratory activity of the product, and high concentrations of carbon dioxide decrease ethylene synthesis and therefore the symptoms of senescence. Likewise, these envelopes maintain high relative humidity and reduce weight loss. The MAP of the broccoli inflorescences allows retention in the content of vitamin C and antioxidants, in addition to maintenance of the green color, corresponding to a higher content of chlorophylls (Fernández-León et al., 2013). In our case, the use of polyethylene bags allowed reaching equilibrium concentrations of approximately 10% O_2 and 5% CO_2. Treatment reduced yellowing and chlorophyll degradation as shown in Supplementary Figure 1 and Table 1. The chlorophyll content decreased by about 92% in the control samples, while it decreased by only 55% in the samples stored in MAP.

We identified and quantified five glucosinolates: glucoraphanin (aliphatic) and glucobrassicin, neoglucobrassicin, 4-hydroxiglucobrassicin, and 4-methoxyglucobrassicin (indolics) (Table 2). In this broccoli cultivar, the highest content was detected to be glucoraphanin and glucobrassicin, reaching values around 1.40–1.80 mmol kg^{-1} dry tissue on the day of harvest. The majority of glucosinolate may vary between different broccoli varieties as described by Wang et al. (2012), who looked at up to 143 broccoli lines and discovered that the content of individual glucosinolates varied significantly. During storage, the content of individual glucosinolates was different in heads stored under MAP conditions in contrast to samples kept in the air. In general, the storage under MAP conditions increased the content of individual glucosinolates as detected in the case of glucoraphanin, glucobrassicin, and neoglucobrassicin at days 3 and 5 of postharvest in comparison with air storage (control condition). In the case of 4-methoxyglucobrassicin, no differences were detected at day 3, but a fivefold increment was observed at the final postharvest storage (day 5). In the case of 4-hydroxyglucobrassicin, the glucosinolate that was found in the least amount, no significant changes were observed in any time-point analyzed (Table 2).

Table 2. Changes in the content of glucosinolates (expressed as mmol kg^{-1} on the basis of dry tissue) of broccoli heads stored for 5 days at 20°C[1].

	Day 0	Day 3		Day 5	
		Control	MA	Control	MA
Glucoraphanin (aliphatic)	1.40 ± 0.13	1.34 ± 0.06	1.98 ± 0.14 *	0.34 ± 0.02	1.71 ± 0.17 *
Glucobrassicin (indolic)	1.80 ± 0.24	0.97 ± 0.12	1.51 ± 0.24 *	0.22 ± 0.01	1.59 ± 0.29 *
Neoglucobrassicin (indolic)	1.15 ± 0.09	1.06 ± 0.19	1.51 ± 0.16 *	0.20 ± 0.01	1.59 ± 0.17 *
4-Hydroxyglucobrassicin (indolic)	0.015 ± 0.001	0.019 ± 0.006	0.012 ± 0.001	0.003 ± 0.001	0.002 ± 0.001
4-Methoxyglucobrassicin (indolic)	0.11 ± 0.02	0.14 ± 0.01	0.16 ± 0.02	0.03 ± 0.01	0.15 ± 0.02 *

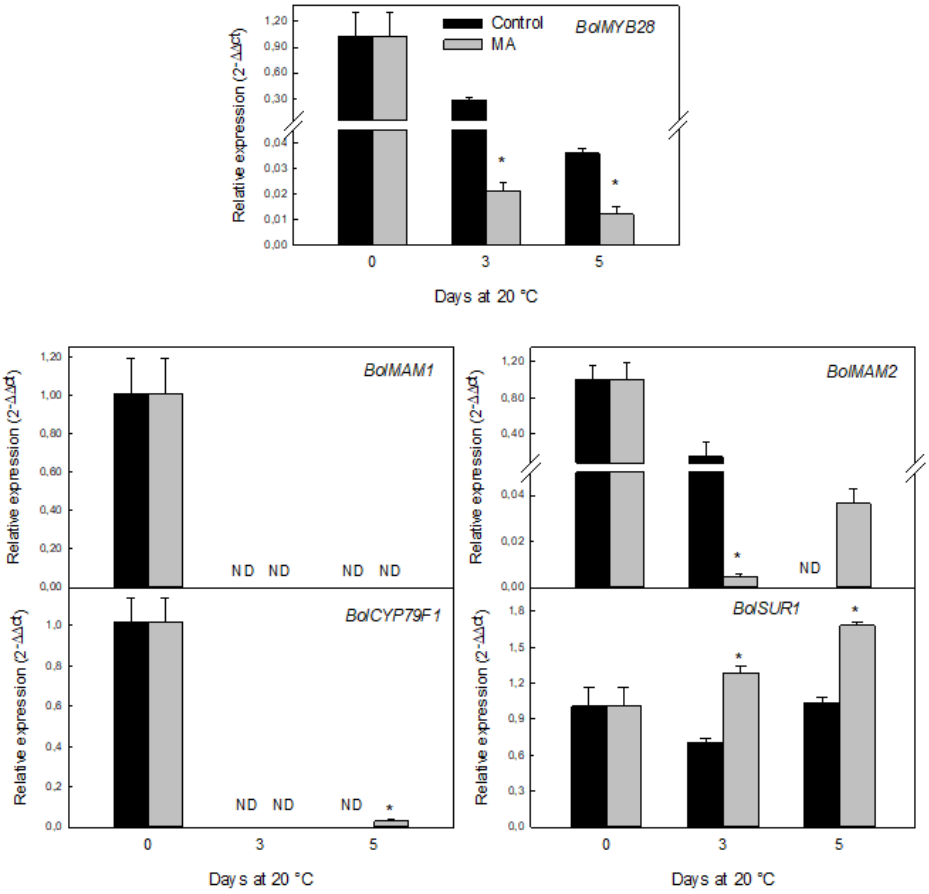
[1]Data represent a mean ± standard deviation ($n = 5$); *significant differences at the same storage time ($p < 0.05$).

Several studies have demonstrated the impact of abiotic stresses on plant secondary metabolism. The review by Martínez-Ballesta et al. (2013) showed that stresses as diverse as saline stress, drought stress, or nutrient deficiency can increase glucosinolate content in different brassica species. In this sense, postharvest storage in high CO_2 and/or low O_2 concentrations can cause anoxia or hypoxia stress. Cramer et al. (2011) proposed a *de novo* synthesis of glucosinolates in response to stress caused by these types of storage. Halkier and Du (1997) found an increase in glucoraphanin in mature broccoli stored in an atmosphere of 0.5% O_2 and 20% CO_2, while Xu et al. (2006) reported that an atmosphere rich in CO_2 can keep the content of glucoraphanin during 20 days at 5°C. Moreover, mini broccoli stored in a modified atmosphere of 8% O_2 and 14% CO_2 allows the content of indole and aliphatic glucosinolates to be maintained for 7 days at 8°C (Schreiner et al., 2006). Rangkadilok et al. (2002) recommended MAP and refrigeration as the best storage conditions to keep glucoraphanin content and visual quality of the broccoli heads for 10 days. Also, the storage of florets (minimally processed broccoli) in microperforated polypropylene bags reduces glucosinolate losses during storage at 5°C (Fernández-León et al., 2013).

In this study, we observed that the use of atmosphere-modified packaging in broccoli heads at room temperature not only allowed maintaining the visual quality but also keeping the nutritional qualities with a higher content of some glucosinolates.

3.2 Expression of genes involved in glucosinolate metabolism is higher following modified atmosphere treatment

Stress generated by low O_2 or high CO_2 concentrations can alter the expression of a large number of genes (Eason et al., 2007). For example, storage in modified atmospheres can modulate the expression of genes related to chlorophyll catabolism (Gómez Lobato et al., 2014; Reyes Jara et al., 2019). Furthermore, the expression of genes associated with glucosinolate biosynthesis can be induced by various stresses such as drought (Eom et al., 2018), gamma radiation (Banerjee et al., 2016), or short-term high temperature (Rao et al., 2021). However, so far, there are no reports of the expression of genes involved in the biosynthesis of aliphatic and indolic glucosinolates under modified atmosphere treatment on broccoli. In this study, the relative expression of some of these genes (highlighted in Figure 1) was evaluated. In control samples, the relative expression of genes involved in aliphatic glucosinolate biosynthesis presented a marked decrease (*BolMAM2* and *BolMYB28*) or it was not detected (*BolMAM1* and *BolCYP79F1*) during postharvest storage (Figure 2).



ND: not detected.

Figure 2. Relative expression of genes associated with aliphatic glucosinolate biosynthesis in broccoli heads treated with modified atmosphere (gray) and control (black) and stored for 5 days at 20°C. Error bars indicate standard deviation. Asterisks indicate significant differences for the same storage day ($p < 0.05$).

Similarly, samples stored under a controlled atmosphere showed undetected (*BolMAM1* and *BolCYP79F1*) or very low expression (*BolMAM2* and the transcript factor *BolMYB28*) after three days. However, on day 5, some genes (*BolCYP79F1* and *BolMAM2*) presented higher expression in comparison with controls. The behavior of *BolSUR1* expression was quite different (see below), probably because this gene also participates in indolic glucosinolate biosynthesis.

The expression of genes involved in the biosynthesis of indolic glucosinolates decreased (*BolCYP83B1* and *BolCYP81F4*) or showed no changes (*BolSUR1* and *BolST5a*) during the postharvest period (days 3 and 5) in control samples (Figure 3).

Treatment with a modified atmosphere caused an increment in the expression of *BolCYP83B1*, *BolST5a,* and *BolSUR1*, and a lower decrement in the expression of *BolCYP81F4*. The transcription factor *BolMYB51* increased its expression during the postharvest storage of control samples. However, the increase in *BolMYB51* expression was much higher in treated samples by day 3. After 5 days of storage, *BolMYB51* expression decreased in the treated samples, causing its expression to be slightly lower than that detected in the controls.

Finally, expression of genes associated with the degradation of glucosinolates (*BolMYR* and *BolESP*) presented a drastic decrease during the postharvest period in control samples but a lower decrement in treated heads. As a consequence, the expression of these genes was higher in modified atmosphere samples (Figure 4).

As previously mentioned, we believe this is the first report on the effect of modified atmospheres on the expression of genes related to glucosinolate metabolism. In general, there are few studies analyzing changes in the expression of these genes during broccoli postharvest. Casajús et al. (2020, 2021) showed a decrease in the expression of genes associated with glucosinolate biosynthesis and degradation during broccoli postharvest. However, this decrease was much less marked in genes linked to the indole glucosinolate metabolic pathway, as observed in this study. Similarly, treatments aimed at delaying senescence: harvest in the evening (Casajús et al., 2020) or visible light treatments (Casajús et al., 2021) mainly affect the expression of genes linked to indolic glucosinolate biosynthesis.

We found that heads stored in a modified atmosphere contained higher amounts of glucosinolates. This may be due to

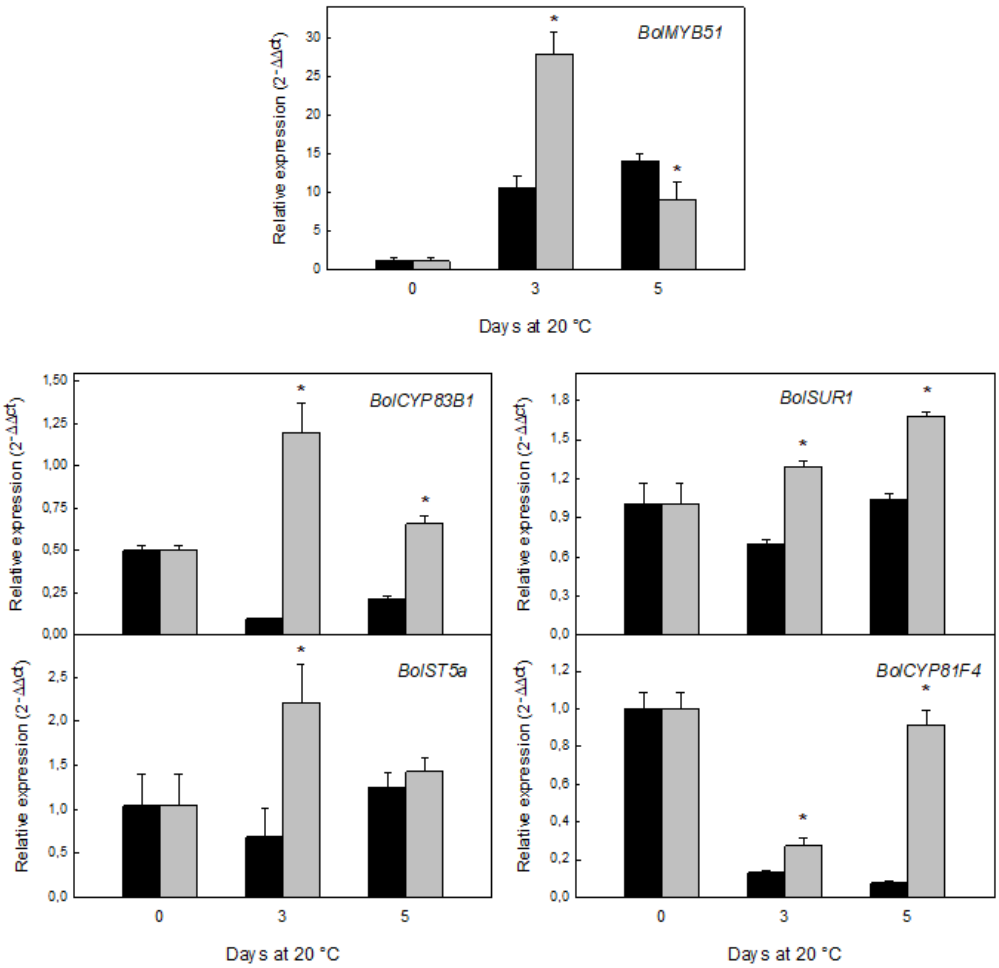


Figure 3. Relative expression of genes associated with indolic glucosinolate biosynthesis in broccoli heads treated with modified atmosphere (gray) and control (black) and stored for 5 days at 20°C. Error bars indicate standard deviation. Asterisks indicate significant differences for the same storage day ($p < 0.05$).

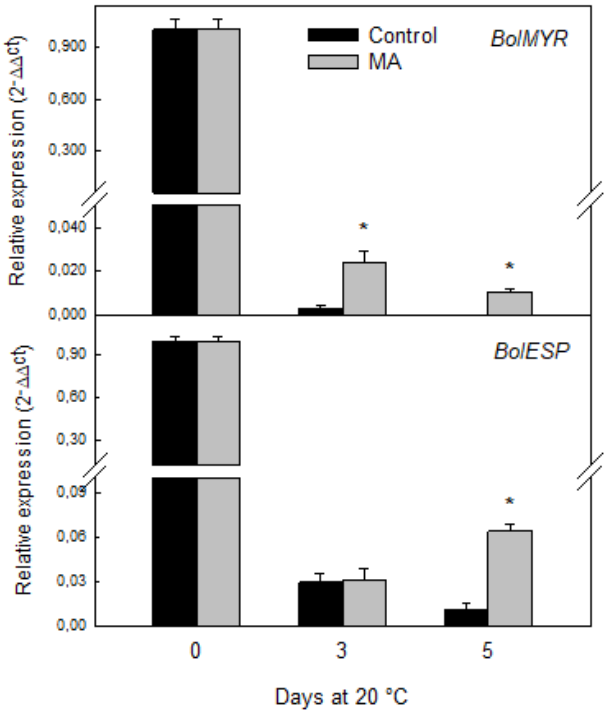


Figure 4. Relative expression of genes associated with glucosinolate degradation in broccoli heads treated with modified atmosphere (gray) and control (black) and stored for 5 days at 20°C. Error bars indicate standard deviation. Asterisks indicate significant differences for the same storage day ($p < 0.05$).

two factors: higher biosynthesis or lower degradation. Loss of membrane integrity and cellular compartmentalization are two of the main aspects of senescence. During this process, myrosinase can come into touch with its substrates when tissue integrity is compromised, and the rate of glucosinolate breakdown may increase as a result. It has been widely demonstrated that broccoli shows membrane injury and loss of cell compartmentalization during senescence (Guo et al., 2013). Moreover, it was shown that lipid peroxidation, an indicator of membrane damage, is lower in broccoli stored in controlled atmospheres (Guo et al., 2013). Consequently, less tissue deterioration of samples stored in modified atmospheres could indicate less loss of compartmentalization and lower degradation of glucosinolates. However, in the case of indole glucosinolates, the modified atmosphere caused a strong induction of genes associated with their biosynthesis, which could also contribute to an accumulation of these compounds.

4 CONCLUSIONS

The use of a modified atmosphere during postharvest storage of broccoli can delay senescence and degreening. The MAP was also useful to maintain a higher glucosinolate content after 5 days of storage at 20°C. The MAP made it possible to maintain higher expression of genes linked to glucosinolate biosynthesis and even to increase the expression of some genes related to indolic glucosinolate. Overall, storage of broccoli heads in a modified atmosphere allows for maintaining not only better visual quality but also higher glucosinolate content.

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