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Effect of continuous white light illumination on glucosinolate metabolism during postharvest storage of broccoli

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Highlights

- Light treatment delays yellowing and chlorophyll degradation during broccoli storage.
- White light diminishes glucosinolate losses during postharvest storage of broccoli
- Light enhances the expression of glucosinolates pathway genes after five days

Abstract

Broccoli is a vegetable consumed globally due to its important nutritional properties, including high concentrations of glucosinolates. Light treatment can be an important tool to delay postharvest senescence. In this work it was evaluated the effect of postharvest continuous white light illumination on glucosinolate metabolism of broccoli heads. Five glucosinolates were identified, one aliphatic (glucoraphanin) and four indolics (glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin). Level of total glucosinolates decreased from 10.1 µmol / g dry tissue to 1.4 µmol / g dry tissue in control samples after five days of storage, while the decrement was only until 3.0 µmol / g dry tissue in treated samples. The expression of genes associated with glucosinolate metabolism decreased during the first three days but this decrease was greater in illuminated samples. After five days, treated samples showed a higher expression (more than twice) in most of these genes with respect to the controls, coinciding with the higher glucosinolate content. Storage of broccoli heads under continuous white light allows to keep higher values of glucosinolate contents while maintaining at the same time the visual quality.

Keywords: *Brassica oleracea*, nutraceuticals, senescence, gene expression

1. Introduction

Broccoli (*Brassica oleracea L. var. Italica*) is a vegetable that belongs to the Brassicaceae family. Broccoli has low calories content, but is a rich source of proteins and fibre. It also contains all essential amino acids, and significant amounts of vitamin A, riboflavin, thiamine, ascorbic acid, phenols, flavonoids and folates (Jeffery et al., 2003). Additionally, broccoli, like other crucifers, has high content of glucosinolates (Razis & Noor, 2013). These are secondary metabolites and are synthesized from amino acids through a series of reactions that involve oxidative decarboxylation, side chain elongations and secondary modifications (Yan & Chen, 2007). Glucosinolates have been classified into three groups on the basis of the nature of their precursor amino acid: aliphatic, indolic and aromatic glucosinolates (Fahey, Zalcmann, & Talalay, 2001). Biosynthetic pathways of aliphatic and indolic glucosinolates are shown in Figure 1.

A major physiological role of glucosinolates in plants is associated with defence against herbivores. Glucosinolates are substrates of the enzyme myrosinase, which is stored in a different cell compartment with respect to glucosinolates. If tissue damage occurs, then the enzyme comes into contact with its substrates and catalyses the loss of sugar producing an unstable aglycon. Aglycons decompose rapidly releasing volatile isothiocyanates and nitriles, compounds that possess anti-insect activity. From the point of view of human health, it has been shown that isothiocyanates produced by the breakdown of glucosinolates have a protective effect against cancer of colon, bladder, lung, etc. (Mandrich & Caputo, 2020). Continuous consumption of crucifers with adequate content of glucosinolates decreases the risk of contracting these pathologies (Jeffery & Araya, 2009).

Broccoli heads are harvested when they are still in development. The harvest causes significant mechanical damage, which in turn leads to a rapid loss of nutrients

and hormonal changes (Cai et al., 2019; Fang et al., 2020). This stress is particularly severe in organs that are rapidly growing and, as a consequence, there is no normal development with an accelerated senescence during postharvest storage (Downs, Somerfield, & Davey, 1997). During postharvest storage, broccoli flower buds undergo rapid yellowing and loss of green color caused by the degradation of chlorophylls. Additionally, there is a significant loss of sugars, proteins, and a decrease in glucosinolate content which decrease the nutritional value of the product (Coupe, Sinclair, Somerfield, & Hurst, 2002; Pogson & Morris, 1997; Xu et al., 2016, Jones & Dangl, 2006).

Several works have demonstrated that interaction between electromagnetic radiation and broccoli heads has a positive effect on postharvest shelf life. Different postharvest treatments had been utilized to delay senescence in broccoli florets, as the use of visible light with fluorescent lamps (Büchert, Gómez Lobato, Villarreal, Civello, & Martínez, 2011) or LEDs (Favre, Bárcena, Bahima, Martínez, & Costa, 2018; Hasperué, Guardianelli, Rodoni, Chaves, & Martínez, 2016; Jin, Yao, Xu, Wang, & Zheng, 2015b), UV-C (Costa, Vicente, Civello, Chaves, & Martínez, 2006; Khalili, Shekarchi, Razavi, & Rastegar, 2017) and UV-B (Aiamla-or, Kaewsuksaeng, Shigyo, & Yamauchi, 2010). The aim of this study was to evaluate the effect of postharvest storage of broccoli heads under continuous white light on glucosinolate content and on the expression of genes involved in their biosynthesis and degradation.

2. Materials and methods

2.1 Plant material

Broccoli heads (*Brassica oleracea L.* var. *Italica* cv Legacy) were obtained from a local producer (La Plata, Buenos Aires, Argentina). Heads were harvested at 8:00 am and immediately transported to the laboratory to be processed. Selection of heads was

done according to the usual cultivation practices. The diameter of the heads ranged from 18-20 cm, with dark green colour without senescent sepals, and without mechanical damage or development of pathogens.

2.2 Visible light treatment

Visible light treatment was performed according to Büchert et al. (2011). Broccoli heads were put in plastic cups containing distilled water to avoid dehydration and separated in control and treated groups. Heads destined to light treatments were stored in a well ventilated chamber at 20 ºC, isolated from external light and exposed to a continuous dose of 12 µmol m^{-2} s^{-1} using 40 W white light fluorescent tubes and the same time, control group was kept in dark (< 1 µmol m^{-2} s^{-1}).

Five broccoli heads from control and treated samples were analysed at time 0, 3 and 5 days of storage. Superficial color was evaluated and then florets were separated from heads, pooled and frozen with liquid nitrogen and subsequently kept at −80°C for posterior analysis.

2.3 Superficial color measurement

Superficial color was measured on broccoli heads with a Minolta CR-300 chromameter (Minolta, Osaka, Japan). Five measurements were performed in five different points of the head. One point was the central area of the head and in the other four positions two centimetres from the centre in four directions, with angles of 90° between each direction. Hue angle (h^o) was calculated as: $h^o = \tan^{-1}(b/a)$ for a and b>0 or $h^o = \tan^{-1}(b/a) + 180°$ for a<0 and b>0.

2.4 Chlorophyll content

Approximately 10 g of frozen broccoli florets were ground in liquid nitrogen. Then, 0.1 g were taken and mixed with 1 mL acetone. The mix was stored in darkness for 4 h and then centrifuged at 10.000 x g for 10 min at 4 ºC. The resulting supernatant was utilized to measure concentration of chlorophylls spectrophotometrically according to Lichtenthaler (1987). Five biological replicas and three technical repetitions were performed. Results were expressed as mg per gram of fresh tissue.

2.5 RNA extraction and cDNA synthesis

A hot borate method with minor modifications was used to obtain the total RNA of the broccoli flower bud samples according to Wan & Wilkins (1994). RNA quality control was carried out by electrophoresis and quantified by UV spectrophotometry (ClarioStar, BMG LABTECH, Ortenberg, Germany). A sample of 6 µg of each RNA was purified using RQ1 DNAase (Promega, Madison, USA) according to the manufacturer´s protocol and with little modifications as described previously (Gomez Lobato, Mansilla, Civello, & Martínez, 2014).

To obtain cDNA, a reverse transcription reaction was carried out using 2 µg of purified RNA, an MML-V reverse transcriptase (Promega) and random primers (hexamers) following the manufacturer's protocol.

2.6 Real time quantitative PCR analysis

A total of nine genes related to glucosinolate metabolism were analysed. They included seven genes related to biosynthesis (*BolMAM1*, *BolMAM2*, *BolCYP79F*1, *BolCYP83B1*, *BolSUR1*, *BolST5a*, and *BolCYP81F4* and two genes associated with glucosinolates degradation (*BolMyr* and *BolESP*). Specific primers were designed based on Brassica Database (Cheng et al., 2011) (Supplementary Table 1). Gene expression was evaluated 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 gene normalizer. Each measurement was performed by using three biological replicates.

2.7 Analysis, identification and determination of glucosinolate content

Extraction and analysis of glucosinolates were carried out following the protocol described by Ramos, Yuan, Faquin, Guilherme, & Li (2011). Approximately 10 g of frozen broccoli florets were freeze dried in a Freeze Dryer, Scientz-10N (Ningbo Scientz Biotechnology Co., LTD, Zhejiang, China). Then, 25 mg of freeze-dried tissues was mixed in 1.2 mL of 800 mL L^{-1} methanol preheated to 75−80 °C and vortexed for 10 s. The mixtures were incubated in a water bath for 15 min at 80 °C and then centrifuged at 12000 x g for 12 min. The supernatants (0.8 mL) were transferred to 1 cm^3 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 then 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 tryptophan was added to the sample diluent so that its concentration was identical in all samples. Thus, the intensity of the Tryptophan peak was used to normalize the intensities of the DS-glucosinolates between runs. Sinigrin was utilized as standard to calculate glucosinolate concentration. Identification and quantification of individual glucosinolates was 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 a 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, analysing the *m/z* range from 50 to 1200. 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 carried out following the methods as reported (Mellon, Bennett, Holst, & Williamson, 2002; Zimmermann, Gerendás, & Krumbein, 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 $(M + H - C_6H_{10}O_5)^+$ and the observed metal ion adducts: $(M + Na)^+$ and $(M + K)^+$.

2.8 Statistical analysis

The statistical analysis was performed by 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 t- test ($p <0.05$). The whole experiment was repeated three times on three different harvested times and similar results were obtained.

3. Results and discussion

3.1 Visible light treatment reduces glucosinolate turnover at extended postharvest storage

Post-harvest treatments carried out on broccoli generally endeavour to delay senescence, preserving the appearance of the product and emphasizing the maintenance of greenness and sensory quality. In this sense, numerous works have shown that visible light, applied with fluorescent lamps (Büchert et al., 2011; Charles, Nilprapruck, Roux, & Sallanon, 2018; Jin, Jin, Chen, Cen, & Yuan, 2015a; Jin et al., 2015b) or LED light of different wavelengths (Favre et al., 2018; Hasperué et al., 2016; Jiang et al., 2019; Ma et al., 2014), both in a continuous way or in pulses, can delay the progress of senescence.

In previous studies, it had been determined that broccoli heads stored under continuous low intensity white light show delayed senescence (Büchert et al., 2011). In this work, it was determined the effect of this treatment on glucosinolate metabolism. To verify the effectiveness of the treatment, visual appearance (Supplementary Figure 1), surface color and chlorophyll content (Table 1) were determined. The samples kept under the white light showed both a reduced color change and lower rate of chlorophyll degradation.

Five glucosinolates were identified, one aliphatic (glucoraphanin) and four indolic (glucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin), which are highlighted in Figure 1, from the broccoli samples. The broccoli cultivar used in this work has higher contents of indolic glucosinolates, with glucobrassicin being the highest. At harvest, the contents of glucoraphanin, glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin varied between 450 and 4500 nmol / g dry tissue, while the content of 4-hydroxyglucobrassicin was very low. The glucosinolate profile may vary according to the cultivar studied; some varieties present high contents of glucoraphanin while other have greater amounts of indolic glucosinolates such as glucobrassicin or neoglucobrassicin (Ávila et al., 2013; Ku, Choi, Kushad, Jeffery, & Juvik, 2013). A description of this high variability was made by

Wang et al. (2012), who analyzed up to 143 broccoli lines and found significant variations in the concentration of individual glucosinolates.

Changes in the content of the individual glucosinolates are shown in Table 2. A dramatic decrease in the content of glucoraphanin, glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin was detected after three days of storage in both control and treated heads. In contrast, the content of 4-hydroxyglucobrassicin increased after three days, and the increment in illuminated heads was even greater (Table 2). The content of 4-hydroxyglucobrassicin may be directly linked to the expression of genes codifying CYP81F1-3. Yi et al. (2016) suggested that the expression of these genes increases with MeJa treatment. Considering that harvesting causes mechanical damage and that this in turn usually results in increased MeJa biosynthesis, harvesting could indirectly increase the expression of CYP81F1-3, and consequently the biosynthesis of 4-hydroxyglucobrassicin.

After five days of postharvest storage, the content of all detected glucosinolates decreased in control samples. However, only 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin decreased significantly in treated samples, while glucoraphanin, glucobrassicin and neoglucobrassicin remained unchanged ($p < 0.05$) (Table 2). Thus, light-treated samples contained significantly higher values of glucoraphanin, glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin than controls after five days of storage.

The decrease in glucosinolate content during post-harvest storage of broccoli has been long documented (Jones, Faragher, & Winkler, 2006; Rodrigues & Rosa, 1999). The flower buds of broccoli are an immature organ that needs a continuous supply of nutrients, water and phytohormones. Moreover, the biosynthesis of glucosinolates is strongly dependent on the supply of N and S (Yan & Chen, 2007). Harvesting causes

an abrupt cessation of this nutrient flow and could be the main cause of the decline in glucosinolate content.

The impact of visible radiation on the content and metabolism of glucosinolates has been extensively studied during growth. The review by Neugart et al. (2018) describes an increase in the biosynthesis of glucosinolates in those crops subjected to medium and high photosynthetically active radiation, although in some cases low radiation may also induce an increase in indolic glucosinolates as described in *Brassica rap*a (Fallovo, Schreiner, Schwarz, Colla, & Krumbein, 2011) and broccoli (Schonhof, Kläring, Krumbein, Claußen, & Schreiner, 2007).

However, there are very few studies that analyse the effect of visible light treatment on the metabolism of glucosinolates during the post-harvest storage of vegetables. In this work it is showed that the reduction of glucosinolate content can be attenuated by using visible radiation. Rybarczyk-Plonska et al. (2016) showed that the application of visible light during storage at 10 or 18 °C did not influence total and individual glucosinolate levels of broccoli flower buds. However, Jin et al (2015b) found that LED generated green light could be a useful technique to prevent the decrease of glucosinolates in broccoli florets. On the other hand, Liu et al. (2015) showed that, in kale and cauliflower, the maintenance of a light-dark cycle allows better levels of glucosinolates in comparison to dark storage. These different responses would suggest that the effect of visible light on glucosinolate metabolism is likely to be dependent on factors such as the intensity of radiation, the wavelength used, and the storage temperature.

3.2 Expression of genes involved in glucosinolate metabolism is higher following visible light treatment at 5 days of postharvest storage

The expression of some of the genes involved in the biosynthesis of aliphatic and indolic glucosinolates was also measured (highlighted in Figure 1). A decrease in the expression of *BolMAM1* and *BolCYP79F1* genes was detected after three days and of all analysed genes after five days in the control samples kept in the dark ($p < 0.05$) (Figures 2 and 3). Differently, irradiated samples showed a dramatic decrease in the expression of all analysed genes, with values significantly lower than those of controls after three days of storage. This reduction stopped and in most cases (*BolMAM1*, *BolMAM2*, *BolCYP79F1*, *BolCYP83B1* and *BolCYP81F4*) reversed and the expression increased after five days ($p < 0.05$) (Figure 2 and 3). In the case of *BolSUR1* the expression did not change, while in the case of *BolST5a* continued to decline ($p < 0.05$). As a consequence, after five days most of analysed genes showed a higher expression in treated samples with respect to control ones. A similar behavior was observed in degradation genes of glucosinolates, *BolMyr and BolESP* (Figure 4).

Usually, the expression of genes involved in the biosynthesis of glucosinolates is positively regulated by light (Huseby et al., 2013; Schuster, Knill, Reichelt, Gershenzon, & Binder, 2006). However, Kim et al. (2014) working on Chinese cabbage seedlings showed that some of these genes are expressed more in darkness or exhibit variable behaviour in relation to light or darkness, depending on the stage of development of the seedling.

It should be noted that after three days, samples stored under light have greater or equal glucosinolate content than controls, despite the fact that the expression of the genes involved in biosynthesis is lower. Samples kept in darkness may have a higher glucosinolate biosynthesis as indicate gene expression, but degradation is likely also higher. One of the characteristics of senescence is the loss of membrane integrity and cell compartmentalization. When tissue integrity is loss, myrosinase enter in contact with its substrates and the rate of degradation of glucosinolates is increased. Jiang, et

al., (2019) showed that lipid peroxidation, an indicator of membrane damage, is lower in broccoli subjected to LED irradiation. Consequently, a lower tissue deterioration of samples held in light (Büchert et al., 2011) could result in a lower loss of compartmentalization and less degradation of glucosinolates.

4. Conclusions

The use of visible lights during postharvest storage of broccoli can delay senescence and degreening (Supplementary Figure 1). In this work, a postharvest treatment by storing broccoli heads under continuous white light, was performed (Büchert et al., 2011). This is a useful methodology to maintain organoleptic quality. Additionally, it was found a lower reduction of glucosinolate content after five days of storage. Genes involved in glucosinolate biosynthesis and degradation showed a lower expression after three days of storage, but a higher one after five days decreases. Taken together, storage of broccoli heads under continuous white light allows not only to maintain a better visual quality but also to keep higher values of glucosinolate contents.

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Figure Captions

Fig. 1. The biosynthetic pathways of glucosinolates (indolic glucosinolates pathway at left and aliphatic glucosinolate pathway at right) and the enzymes and genes involved in each step. The glucosinolates detected and the genes are highlighted.

Fig. 2. Relative expression of genes associated to aliphatic glucosinolate biosynthesis in broccoli heads under continuous visible light (grey) and controls (black) stored during five days at 20 °C. Error bars indicate standard deviation Different letters indicate significant differences for the same gene ($p < 0.05$).

Fig. 3. Relative expression of genes associated to indolic glucosinolate biosynthesis in broccoli heads under continuous visible light (grey) and controls (black) stored during five days at 20 °C. Error bars indicate standard deviation Different letters indicate significant differences for the same gene ($p < 0.05$).

Fig. 4. Relative expression of genes associated to glucosinolate degradation in broccoli heads under continuous visible light (grey) and controls (black) stored during five days at 20 °C. Error bars indicate standard deviation Different letters indicate significant differences for the same gene ($p < 0.05$).

Supplementary Figure 1: Visual appearance of broccoli heads, control at left and under visible light treatment at right at different days of postharvest senescence (day 0, 3 and 5).

Table 1

Changes in color and total chlorophylls contents (expressed as mg per gram of fresh tissue) in broccoli heads stored during five days at 20 °C. Data represent a mean ± standard deviation. Different letters indicate significant differences at the same storage time ($P < 0.05$).

	Hue		**Total Chlorophylls**	
	Control	**Light**	**Control**	**Light**
Day 0	125 ± 7 **a**	125 ± 7 **a**	0.06 ± 0.01 **a**	0.06 ± 0.01 **a**
Day 3	103 ± 7 **a**	118 ± 9 **b**	0.03 ± 0.01 **a**	0.05 ± 0.01 **b**
Day 5	88 ± 6 **a**	101 ± 9 **b**	0.014 ± 0.006 **a**	0.039 ± 0.008 **b**

Data represent a mean ± standard deviation. Different letters indicate significant differences at the same storage time ($P < 0.05$). (n = 25 for Hue determinations and n = 5 for chlorophyll determinations)

Table 2. Changes in the content of glucosinolates (µmol / g dry tissue) of broccoli heads stored during five days at 20 °C.

	Day 0	Day 3		Day 5	
		Control	Light	Control	Light
Glucoraphanin (aliphatic)	1.9 ± 0.4	1.0 ± 0.2	0,9 ± 0.1	0.71 ± 0.02	1.10 ± 0.06 *
Glucobrassicin (indolic)	4.9 ± 0.8	1.6 ± 0.5	1.6 ± 0.2	0.48 ± 0.06	1.31 ± 0.09 *
Neoglucobrassicin (indolic)	2.9 ± 0.4	0.6 ± 0.1	0.59 ± 0.06	0.16 ± 0.01	0.52 ± 0.04 *
4-hydroxyglucobrassicin (indolic)	0.06 ± 0.01	0.07 ± 0.01	0.17 ± 0.02 *	0.05 ± 0.01	0.03 ± 0.01
4-methoxyglucobrassicin (indolic)	0.36 ± 0.05	0.15 ± 0.04	0.12 ± 0.01	0.02 ± 0.01	0 .06 ± 0.01 *

Data represent a mean ± standard deviation (n = 5). Asterisks indicate significant differences at the same storage time ($P < 0.05$).

Figure 1

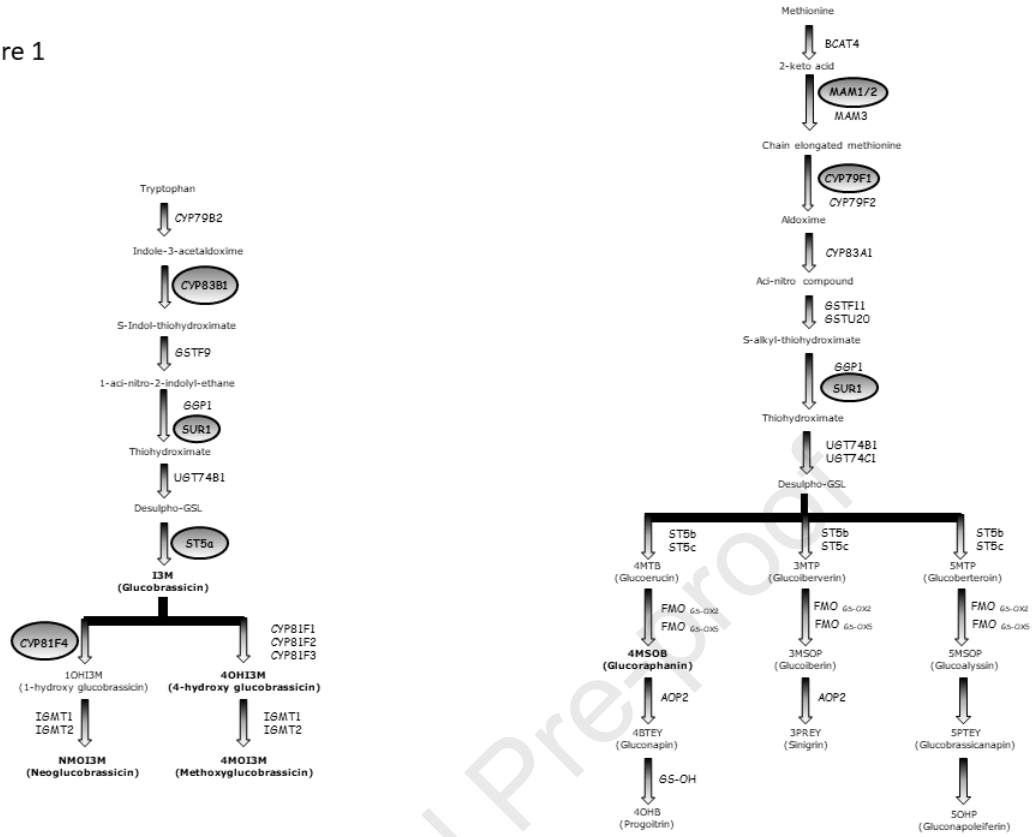


Figure 2

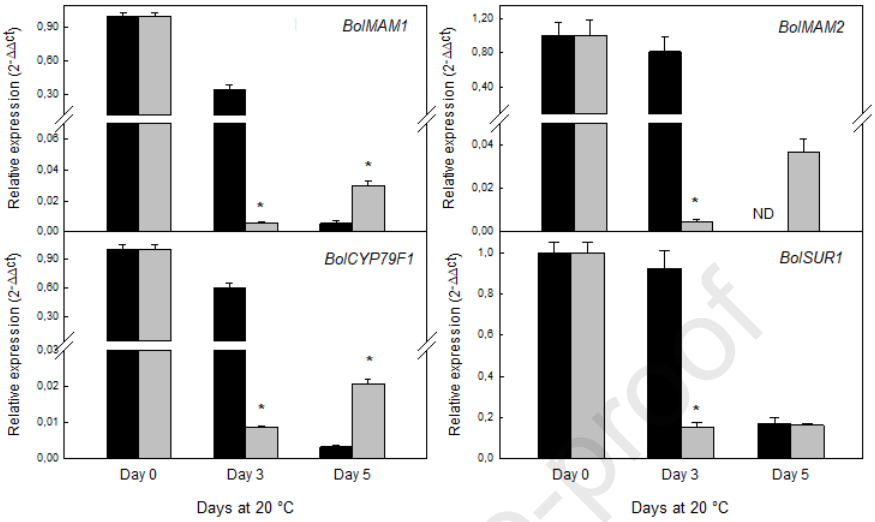


Figure 3

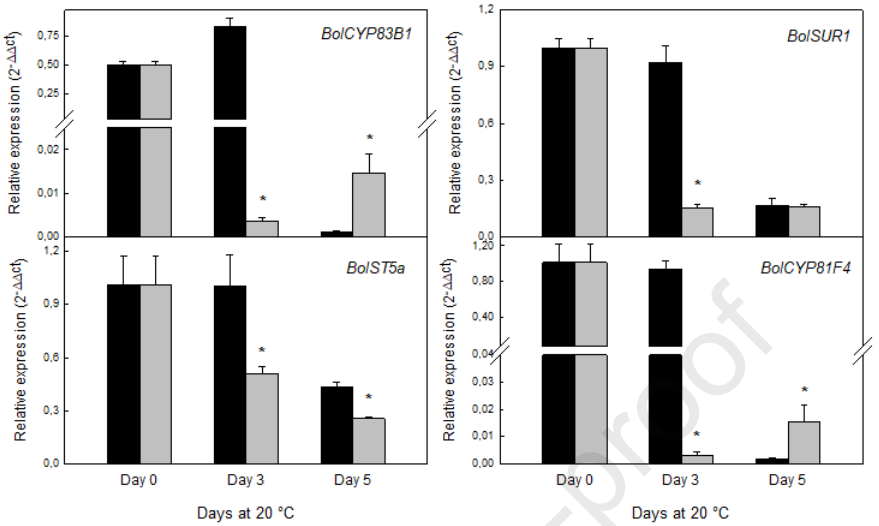
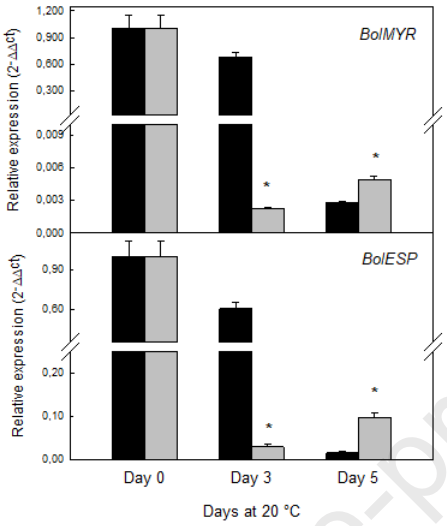


Figure 4



Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: