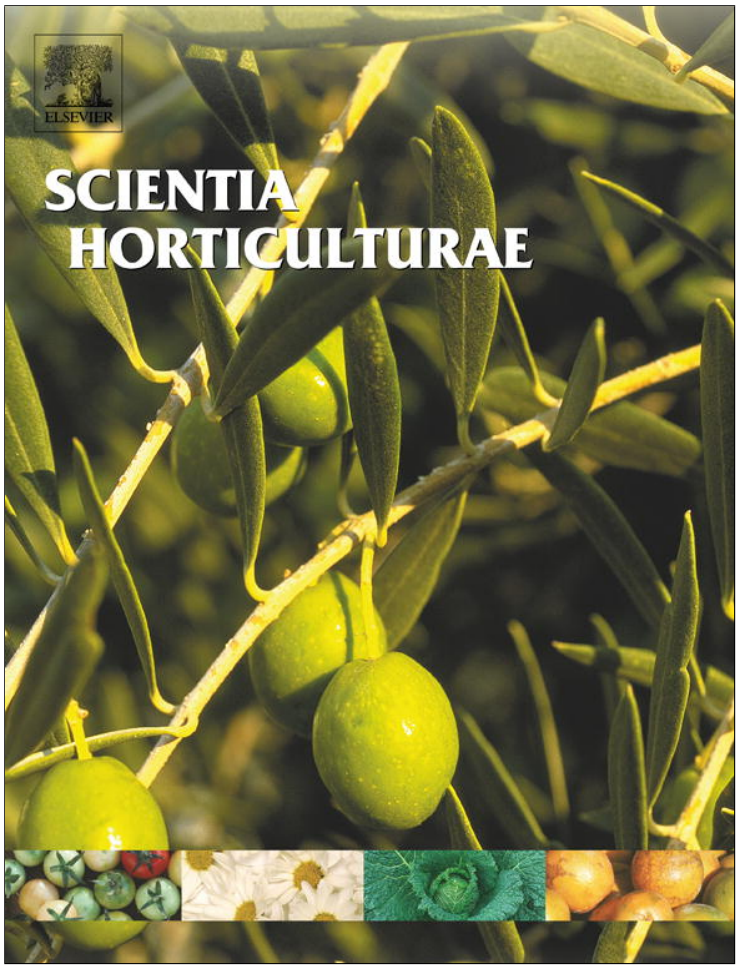

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Effect of 1-MCP on the expression of chlorophyll degrading genes during senescence of broccoli (*Brassica oleracea* L.)

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ABSTRACT

Degreening caused by chlorophyll degradation is the most evident visual manifestation of broccoli senescence and deterioration. In this work, the effect of 1-MCP on color change, chlorophyll degradation and expression of genes associated to chlorophyll catabolism was evaluated during postharvest storage. 1-MCP treatment markedly delayed degreening and reduction of chlorophyll content. The expression of genes encoding chlorophyllases showed different behaviors. In controls, the expression of *BoCLH1* decreased during postharvest storage while that of *BoCLH2* increased after 72 h and then decreased. The treatment with 1-MCP did not cause any effect on *BoCLH1* but induced a higher expression of *BoCLH2*. A high *BoPPH* expression level was found in senescent broccoli florets, but the up-regulation of this gene was delayed by 1-MCP treatment. The expression of *BoPaO*, a gene encoding a pheophorbide a oxygenase, increased after three days and then decreased. In contrast, this expression was reduced in treated broccoli. Finally, the expression of *BoRCCR* decreased during senescence and the treatment with 1-MCP caused a higher decrement. These results show that postharvest 1-MCP inhibit selectively some of the genes encoding enzymes related chlorophyll catabolism.

1. Introduction

Broccoli is a product that has recently acquired a growing demand and increased consumption due to its high content of nutrients and nutraceuticals such as ascorbic acid, phenolics and glucosinolates. However, this vegetable is highly perishable and its visual and organoleptic qualities greatly decrease during postharvest storage. Broccoli inflorescences are harvested while they are in development. Since inflorescences require high levels of nutrients, water and hormones, harvesting causes a severe stress and determines the appearance of accelerated senescence symptoms and the consequent degreening. The green color is the main important commercial quality index of broccoli and its lost is the result of the breakdown of chlorophylls (Amir-Shapira et al., 1987). The chlorophyll degradation pathway can be divided into early steps, which are localized in the chloroplast, followed by species-specific modification of chlorophyll breakdown products which are stored in the vacuole. Until recently it was believed that chlorophyll is first dephytylated to chlorophyllide (Chlide) by chlorophyllase (CLH) and then, a metal chelating substance (MCS) removes Mg^{+2}. The resulting product, pheophorbide (Pheide) a, is converted to blue-fluorescing intermediate (pFCC) in a two-step reaction by Pheide a oxygenase (PaO) and red Chl catabolite reductase (RCCR). pFCC undergoes several modifications and products (non-fluorescent chlorophyll catabolites or NCCs) generated are finally stored inside the vacuole (Hörtensteiner, 2006).

Chlorophyllases catalyze the hydrolysis of chlorophyll to chlorophyllide and phytol and has been considered the first enzyme in the chlorophyll catabolic process (Matile et al., 1999; Jacob-Wilk et al., 1999). Recently, it was questioned the true involvement of CHLs, since some of the isolated genes have not a chloroplast transit peptide, suggesting alternative pathways occurring outside of chloroplast or involvement of enzymes other than CHL (Hörtensteiner, 2006). Schelbert et al. (2009) revealed the existence of a new enzyme, termed pheophytinase (PPH), which would act as a pheophytin hydrolase. In this new model, Mg^{+2} released seems to precede phytol cleavage, producing pheophytin, which is dephytilated by PPH to give pheophorbide (Schelbert et al., 2009). In broccoli, it was shown that PPH expression seems to be strictly

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Table 1
Hue values and chlorophyll *a* and *b* contents (mg chl. g^{-1} Fw tissue) in controls and 1-MCP treated broccoli florets during postharvest senescence at 22 °C.

	Hue		Chlorophyll *a*		Chlorophyll *b*	
	Control	1-MCP	Control	1-MCP	Control	1-MCP
0 h	125.7 ± 2.9	125.7 ± 2.9	0.075 ± 0.008	0.075 ± 0.008	0.052 ± 0.002	0.052 ± 0.002
72 h	122.4 ± 3.6	126.1 ± 3.4*	0.058 ± 0.005	0.073 ± 0.006*	0.040 ± 0.004	0.051 ± 0.003*
120 h	101.6 ± 6.0	118.7 ± 2.4*	0.022 ± 0.007	0.056 ± 0.008*	0.018 ± 0.002	0.031 ± 0.004*

Asterisks show statistical differences between treated samples and controls at the same time ($P < 0.005$).

related to chlorophyll breakdown during senescence (Büchert et al., 2011a, 2011b).

The effect of different postharvest treatments like hot air (Costa et al., 2005, 2006a), UV-C (Costa et al., 2006b), modified atmosphere (Eason et al., 2007) or visible light (Büchert et al., 2011a, 2011b) on broccoli shelf life, visual quality and degreening have been widely investigated.

The commercialization of 1-methylpropene (1-MCP, an effective inhibitor of ethylene action) has provided a new tool to improve maintenance of horticultural products, delaying ripening or senescence, and extending the shelf-life of fruit, vegetables, and ornamental crops (Watkins, 2008). 1-MCP acts by binding irreversibly to ethylene receptor, even at very low concentrations; and it has been considered non-toxic for both humans and the environment (Luo et al., 2007; Yuan et al., 2010). In broccoli, application of 1-MCP can delay yellowing, decreases respiration and extends the shelf-life (Gong and Mattheis, 2003; Ku and Wills, 1999; Yuan et al., 2010).

The objective of the present study was to investigate the effect of a treatment with 1-MCP on superficial color, chlorophyll contents and expression of chlorophyll degrading related genes during postharvest senescence of broccoli florets.

2. Materials and methods

2.1. Plant material

Broccoli (*Brassica oleracea* var. *Italica* cv. Cicco) heads were harvested at a local farm in La Plata, Argentina, and immediately refrigerated at 0 °C with ice, transported to the laboratory within 2 h and processed. Heads of uniform size and shape and free of damage were selected.

2.2. 1-MCP treatment

Broccoli heads were placed in plastic bowls containing a small amount of distilled water and treated with 1-MCP (1 $\mu L\,L^{-1}$) in a hermetic container for 16 h at 22 °C. Controls were kept under the same conditions without 1-MCP. Heads were subjected to color measurement at 0, 72 and 120 h. After that, the florets of five heads were separated from stems, randomly grouped and frozen in liquid nitrogen and stored at −20 °C until analysis.

2.3. Superficial color measurement

Superficial color was evaluated by measuring the parameters *a* and *b* in five positions of each broccoli head with a chromameter (Model CR-300, Minolta, Osaka, Japan). Hue angle (h°) was calculated as $h^\circ = \tan^{-1}(b/a)$ when $a > 0$ and $b > 0$ or as $h^\circ = 180^\circ - \tan^{-1}(b/a)$ when $a < 0$ and $b > 0$.

2.4. Determination of chlorophyll contents

Frozen broccoli florets were ground in liquid nitrogen and 0.5 g of the resulting powder was mixed with 5 ml of 80%(v/v) acetone and centrifuged at 10,000 × *g* for 10 min at 4 °C. Chlorophyll content was measured in supernatant by spectrophotometry (Inskeep and Bloom, 1985) and results were expressed as mg of total chlorophyll per gram of fresh tissue. All measurements were performed by quintuplicates.

2.5. RNA extraction and real-time PCR

Broccoli florets were ground in liquid nitrogen, and total RNA was obtained by hot borate method (Wan and Wilkins, 1994). Then, approximately 6 μg of total RNA were treated with RQ1 DNAse (Promega), purified with chlorophorm:1-octanol (24:1) and precipitated with 3 M sodium acetate. Purified RNA was quantified again and 4 μg were used for cDNA synthesis using MML-V reverse transcriptase (Promega) and random primers hexamers. Resulting cDNA was employed as template for two-step qPCR reactions using an Mx3005P real-time PCR system (Stratagene) and FastStart Universal SYBR Green Master (Roche). Sequences of primers utilized were as follows: Actin (AF044573) (used as normaliser), forward: 5′-CCAGAGGTCTTGTTCCAGCCATC-3′and reverse:5′-GTTCCACCACTGAGCACAATGTTAC-3′; Chlorophyllase 1 (*BoCLH1*) forward: 5′-AGACCCATCCATCAAGTTTTCAGC-3′ and reverse: 5′-AGATTTCGGGATCGGTTCTTATGC-3′; Chlorophyllase 2 (*BoCLH2*) forward: 5′-AGATGCCTGTTCTAGTTATTGG -3′ and reverse: 5′-CACGCTGGACCTTGACATTC-3′; pheophytinase (*BoPPH*) forward: 5′-AGAGGTTATCGGTGAGCCA-3′ and reverse: 5′-GACGAGATGAGGATGGG-3′; phephorbide a oxygenase (*BoPaO*) forward: 5′-GCGAAATTCCCGTCCAGAGTCTC-3′ and reverse: 5′-TTATCTCCGCCGTGCTCTTCTTC-3′, and red catabolite chlorophyll reductase (*BoRCCR*) forward: 5′-CCTCCCTCATCGCAAAGACCTAG-3′and reverse: 5′-AGCAGAGCGGACAAAGAGAGAC-3′. Each measurement was performed by quintuplicate.

2.6. Statistical analysis

Experiments were performed according to a factorial design. Data were analyzed using ANOVA, and the means were compared by the LSD test at a significance level of 0.05.

3. Results and discussion

Several studies have shown that 1-MCP delays the development of broccoli senescence during postharvest (Ku and Wills, 1999; Able et al., 2002; Gong and Mattheis, 2003). 1-MCP not only blocks the action of ethylene as an accelerator of senescence but also inhibits the activities of ethylene biosynthesis enzymes and reduces the expression of genes encoding these enzymes as well as those of ethylene receptors (Ma et al., 2009). One of the central aspects of the use of 1-MCP is the delay in the degreening, through a reduced degradation of chlorophylls. To analyze the effect of 1-MCP on the expression of genes associated with chlorophyll degradation, we performed a treatment with a concentration of 1 $\mu L\,L^{-1}$ for 16 h. The progress of senescence was followed by Hue values and chlorophyll content. The treatment caused a delay in degreening evidenced by a lower decrease in the Hue value and in the rate of degradation of both chlorophylls *a* and *b* (Table 1). This effect was similar to those

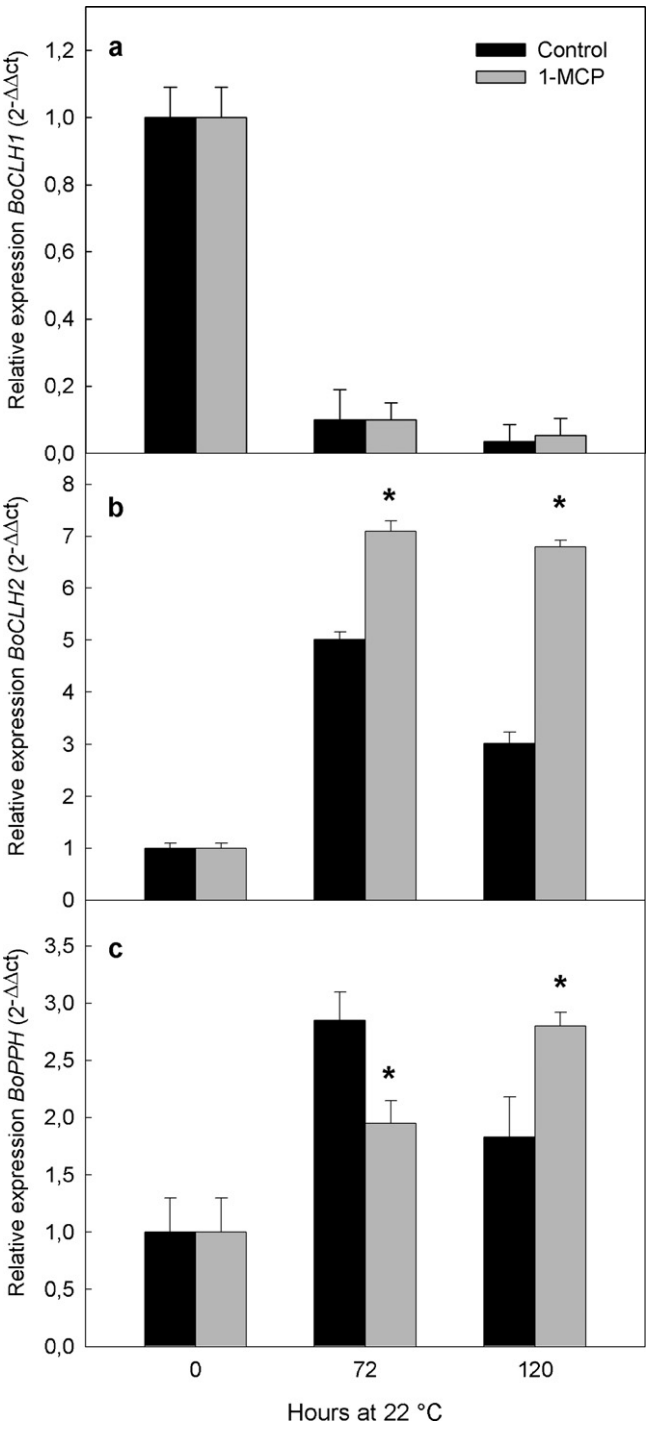

Fig. 1. Relative expression as measured by RT-PCR of (a) *BoCLH1*, (b) *BoCLH2* and (c) *BoPPH* in broccoli florets subjected to 1-MCP treatment during 5 days of induced senescence. *Value significantly different from the corresponding control ($P < 0.05$).

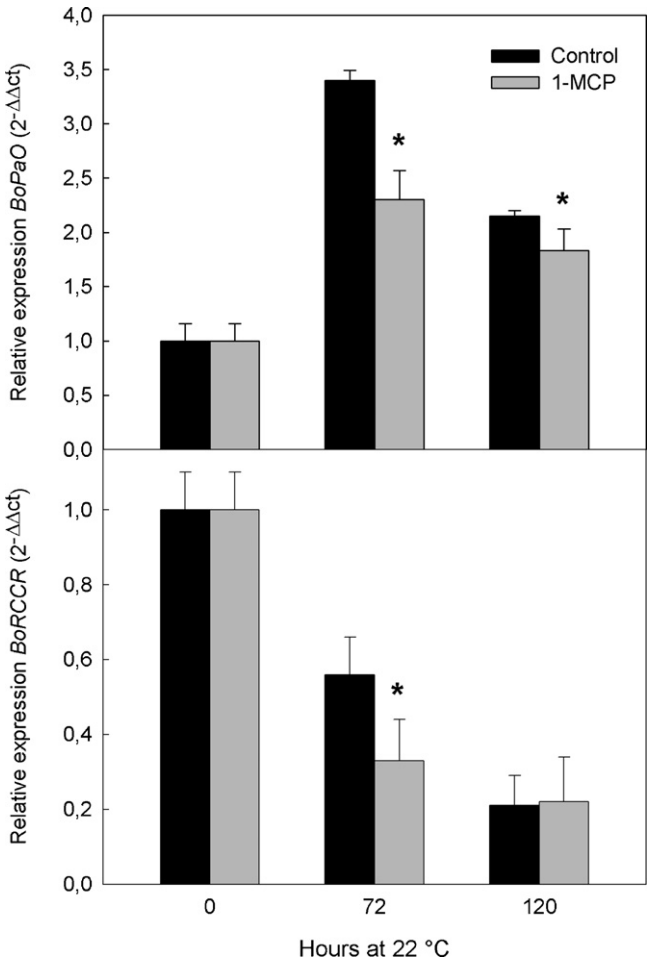

Fig. 2. Relative expression as measured by RT-PCR of (a) *BoPaO* and (b) *BoRCCR* in broccoli florets subjected to 1-MCP treatment during 5 days of induced senescence. *Value significantly different from the corresponding control ($P < 0.05$).

reported in several previous works (Fan and Mattheis, 2000; Able et al., 2002; Gong and Mattheis, 2003).

3.1. Effect of 1-MCP treatment on BoCLH1, BoCLH2 and BoPPH expression

In previous works, genes encoding chlorophyllases and pheophytinase from broccoli were cloned and their expression characterized under different conditions and postharvest treatments (Büchert et al., 2011a, 2011b). *BoCLH1* does not have a chloroplast transit peptide and does not respond to hormonal regulation. On the contrary, *BoCLH2* have a typical transit peptide to chloroplast and it is believed to be involved in chlorophyll homeostasis. Büchert et al. (2011a, 2011b) proposed that *BoCHL1* expression is negatively regulated during senescence, whereas *BoCLH2* expression is enhanced during senescence.

In relation to PPH, Hörtensteiner (2006) proposed that the absence of this enzyme causes indefinite retention of greenness in Arabidopsis. This group provided *in vitro* and *in vivo* evidence that PPH specifically cleaves the phytol ester of pheophytin, but not chlorophyll and proposed that this enzyme is the major dephytilating activity in chlorophyll breakdown during senescence (Hörtensteiner, 2006).

To compare levels of gene expression, control broccoli florets at harvest (day 0) were used as calibrators. The expression of *BoCLH1* greatly decreased after 72 h and maintained lower until the end of incubation. Treatment with 1-MCP did not cause any effect on the expression of this gene (Fig. 1a. The *BoCLH2* expression increased four times after 72 h and then decreased but the values remained higher than the initials. In contrast, the expression in 1-MCP treated samples had higher values than those of controls at 72 h and maintained this level of expression until 120 h (Fig. 1b). Finally, the expression of *BoPPH* in controls increased after 72 h and then decreased at the end of incubation. In contrast, expression in treated samples increased slowly and their values after 120 h reached levels similar to those of controls at 72 h (Fig. 1c).

As noted previously, the expression of *BoCLH1* decreases during the incubation period in a pattern not characteristic of senescence-associated genes (Büchert et al., 2011a, 2011b) In the case of *BoCLH2*, although the expression of this gene was increased during senescence, it was not affected by inhibitors of the process, both 1-MCP (this work) and cytokinins (Büchert et al., 2011a), suggesting the enhanced expression of *BoCLH2* with no regulation by senescence-related hormones could be related to other facts caused by harvest. On the contrary, expression of *BoPPH* followed a characteristic pattern of a gene that enhance their expression during senescence and respond to related hormones. The 1-MCP treatment avoided the increase of expression detected at 72 h and delayed it until 120 h.

3.2. Effect of 1-MCP treatment on BoPaO and BoRCCR expression

Pheophorbide a oxygenase is considered to play an important regulatory role in chlorophyll catabolism since PaO activity correlates positively with chlorophyll degradation (Pruzinská et al., 2003; Chung et al., 2006). In this work, the expression of *BoPaO* increased at 72 h and then decreased at 120 h in controls, while heads treated with 1-MCP showed lower *BoPaO* expression both at 72 and 120 h (Fig. 2a). This pattern coincides with recent researches that have shown that *BoPaO* has an enhanced expression during postharvest senescence and that such expression can be modulated by hormones and postharvest treatments (Aiamla-or et al., 2012; Gómez-Lobato et al., 2011; Zhang et al., 2011).

PaO activity is associated, in a two-step reaction, with the following enzyme, red chlorophyll catabolite reductase, which is localized in chloroplast and is not regulated during senescence (Pruzinská et al., 2005; Wüthrich et al., 2000). Fukusawa et al. (2010) cloned a fragment that codified for a gene encoding RCCR (named *BoRCCR*) characterized its expression during senescence. We utilized the same primers to analyze *BoRCCR* expression under 1-MCP treatment. The *BoRCCR* relative expression decreased during storage, although this decrement was higher in 1-MCP treated samples at 72 h. Fukusawa et al. (2010) found that the expression of *BoRCCR* increases at 48 h and then decreases during postharvest senescence. In our case, since the measurements were performed at 72 h, the peak of expression could have not been detected. Anyway, a lower *BoRCCR* expression was found in samples treated with 1-MCP, in the same manner as observed in broccolis with delayed senescence by treatment with ethanol (Fukusawa et al., 2010).

In conclusion, a 1-MCP treatment that delay senescence and chlorophyll degradation did not have a clear effect on the expression of chlorophyllase related genes (*BoCLH1* and *BoCLH2*). In contrast, the treatment effectively inhibited *BoPPH* and *BoPaO* expression and had a minor effect on *BoRCCR* expression. This and previous results (Pruzinská et al., 2003; Schelbert et al., 2009) suggest that *BoPPH* and *BoPaO* rather than chlorophyllases plays a central role in chlorophyll catabolism during postharvest senescence of broccoli.

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References

Able, A.J., Wong, L.S., Prasad, A., O'Hare, T.J., 2002. 1-MCP is more effective on a floral brassica (*Brassica oleracea* var. *Italica* L.) than a leafy brassica (*Brassica rapa* var. *Chinensis*). Postharvest Biol. Technol. 26, 147–155.

Amir-Shapira, D., Goldschmidt, E.E., Altman, A., 1987. Chlorophyll catabolism in senescing plant tissues: in vivo breakdown intermediates suggest different degradative pathways for citrus fruit and parsley leaves. Proc. Natl. Acad. Sci. U.S.A. 84, 1901.

Aiamla-or, S., Nakajima, T., Shigyo, M., Yamauchi, N., 2012. Pheophytinase activity and gene expression of chlorophyll-degrading enzymes relating to UV-B treatment in postharvest broccoli (*Brassica oleracea* L. *Italica* Group) florets. Postharvest Biol. Technol. 63, 60–66.

Büchert, A.M., Civello, P.M., Martínez, G.A., 2011a. Chlorophyllase versus pheophytinase as candidates for chlorophyll dephytilation during senescence of broccoli. J. Plant Physiol. 168, 337–343.

Büchert, A.M., Civello, P.M., Martínez, G.A., 2011b. Effect of hot air, UV-C, white light and modified atmosphere treatments on expression of chlorophyll degrading genes in postharvest broccoli (*Brassica oleracea* L.) florets. Sci. Horti. 127, 214–219.

Chung, D.W., Pruzinska, A., Hortensteiner, S., Ort, D.R., 2006. The role of pheophorbide *a* oxygenase expression and activity in the canola green seed problem. Plant Physiol. 142, 88–97.

Costa, M.L., Civello, P.M., Chaves, A.R., Martinez, G.A., 2005. Effect of hot air treatments on senescence and quality parameters of harvested broccoli Brassica oleracea L var Italica heads. J. Sci. Food Agric. 85, 1154–1160.

Costa, M.L., Civello, P.M., Chaves, A.R., Martínez, G.A., 2006a. Hot air treatment decreases chlorophyll catabolism during postharvest senescence of broccoli (Brassica oleracea L. var. italica) heads. J. Sci. Food Agric. 86, 1125–1131.

Costa, M.L., Vicente, A.R., Civello, P.M., Chaves, A.R., Martínez, G.A., 2006b. UV-C treatment delays postharvest senescence in broccoli florets. Postharvest Biol. Technol. 39, 204–210.

Eason, J.R., Patel, D., Ryan, D., Page, B., Hedderley, D., Watson, L., West, P., 2007. Controlled atmosphere treatment of broccoli after harvest delays senescence and induces the expression of novel BoCAR genes. Plant Physiol Bioch. 45, 445–456.

Fan, X., Mattheis, J.P., 2000. Yellowing of broccoli in storage is reduced by 1-methylcyclopropene. HortScience 35, 885–887.

Fukusawa, A., Suzuki, Y., Terai, H., Yamauchi, N., 2010. Effects of postharvest ethanol vapor treatment on activities and gene expression of chlorophyll catabolic enzymes in broccoli florets. Postharvest Biol. Technol. 55, 97–102.

Gómez-Lobato, M.E., Civello, P.M., Martínez, G.A., 2011. Effects of ethylene, cytokinin and physical treatments on BoPaO gene expression of harvested broccoli. J. Sci. Food Agric. 92, 151–158.

Gong, Y., Mattheis, J.P., 2003. Effect of ethylene and 1-methylcyclopropene on chlorophyll catabolism of broccoli florets. Plant Growth Regul. 40, 33–38.

Hörtensteiner, S., 2006. Chlorophyll degradation during senescence. Annu. Rev. Plant Biol. 57, 55–77.

Inskeep, W.P., Bloom, P.R., 1985. Extinction coefficients of chlorophyll a and b in N,N-dimethylformamide and 80% acetone. Plant Physiol. 77, 483–485.

Jacob-Wilk, D., Holland, D., Goldschmidt, E.E., Riov, J., Eyal, Y., 1999. Chlorophyll breakdown by chlorophyllase: isolation and functional expression of the *Chlase1* gene from ethylene-treated *Citrus* fruit and its regulation during development. Plant J. 20, 653–661.

Ku, V.V.V., Wills, R.B.H., 1999. Effect of 1-methylcyclopropene on the storage life of broccoli. Postharvest Biol. Technol. 17, 127–132.

Luo, Z., Xu, X., Cai, Z., Yan, B., 2007. Effects of ethylene and 1-methylcyclopropene (1-MCP) on lignification of postharvest bamboo shoot. Food Chem. 105, 521–527.

Ma, G., Wang, R., Wang, C.-R., Kato, M., Yamawaki, K., Qin, F., Xu, H.-L., 2009. Effect of 1-methylcyclopropene on expression of genes for ethylene biosynthesis enzymes and ethylene receptors in post-harvest broccoli. Plant Growth Regul. 57, 223–232.

Matile, P., Hörtensteiner, S., Thomas, H., 1999. Chlorophyll degradation. Annu. Rev. Plant Biol. 50, 67–95.

Pruzinská, A., Tanner, G., Anders, I., Roca, M., Hörtensteiner, S., 2003. A chlorophyll breakdown: pheophorbide *a* oxygenase is a Rieske-type iron–sulfur protein, encoded by the accelerated cell death 1 gene. Proc. Natl. Acad. Sci. U.S.A. 100, 15259–15264.

Pruzinská, A., Tanner, G., Aubry, S., Anders, I., Moser, S., Müller, T., Ongania, K.-H., Kräutler, B., Youn, J.-Y., Liljegren, S.J., Hörtensteiner, S., 2005. Chlorophyll breakdown in senescent Arabidopsis leaves: characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction. Plant Physiol. 139, 52–63.

Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., Hörtensteiner, S., 2009. Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in arabidopsis. The Plant Cell 21, 767–785.

Wan, C.H., Wilkins, T.A., 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). Anal. Biochem. 223, 7–12.

Watkins, C.B., 2008. Overview of 1-methylcyclopropene trials and uses for edible horticultural crops. HortScience 43, 86–94.

Wüthrich, K.L., Bovet, L., Hunziker, P.E., Donnison, I.S., Hortensteiner, S., 2000. Molecular cloning, functional expression and characterisation of RCC reductase involved in chlorophyll catabolism. Plant J. 21, 189–198.

Yuan, G., Sun, B., Jing Yuan, J., Wang, Q., 2010. Effect of 1-methylcyclopropene on shelf life, visual quality, antioxidant enzymes and health-promoting compounds in broccoli florets. Food Chem. 118, 774–781.

Zhang, X., Zhang, Z., Li, J., Wu, L., Guo, J., Ouyang, L., Xia, Y., Huang, X., Pang, X., 2011. Correlation of leaf senescence and gene expression/activities of chlorophyll degradation enzymes in harvested Chinese flowering cabbage (*Brassica rapa* var. parachinensis). J. Plant Physiol. 168, 2081–2087.