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Low soil water content during growth contributes to preservation of green colour and bioactive compounds of cold-stored broccoli (*Brassica oleraceae* L.) florets

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ABSTRACT

Broccoli, cultivated under low (0.40 MPa) and normal (0.04 MPa, equivalent to field capacity) soil water content, and stored under low (1 °C) and room (23 °C) temperature, was assessed for changes in colour, bioactive compounds, and antioxidant activity. Results demonstrated a significant interaction between cultivation and storage conditions. Low soil water content during plant growth and postharvest cold storage were the conditions that, combined, gave the best preservation of colour, antioxidant activity, and L-ascorbic acid and 5-methyl-tetrahydrofolate contents. Carotenoid preservation was dependent on postharvest storage conditions while the contents of phenolic compounds were reduced over time, independent of cultivation and postharvest storage conditions.

1. Introduction

Broccoli is an important source of L-ascorbic acid, folates, glucosinolates, flavonoids, vitamins and dietary fibres (Vallejo et al., 2003). However, significant postharvest loss of nutritional and functional properties, including that of bioactive compounds such as ascorbic acid, glucosinolates, and folic acid, have been shown to occur (Jones et al., 2006; Moreno et al., 2006). Attempts to minimize the problem include the use of cold storage (Able et al., 2005), heat treatment (Funamoto et al., 2003), improvement of cytokinin synthesis through transgenic plants (Chen et al., 2001), application of cytokinin (Costa et al., 2005), ethanol vapours (Asoda et al., 2009), 1-MCP treatment (Able et al., 2002), storage under modified (Rai et al., 2008) or controlled atmosphere (Watada et al., 1996; Eason et al., 2007), and UV-C treatment (Costa et al., 2006). Among these methods, cold storage with or without controlled or modified atmosphere is the most widely employed strategy (Toivonen, 1997).

Recently we demonstrated that low soil water content (0.40 MPa of soil water tension) during broccoli growth led to leaf size reduction, without affecting weight or yield, and contributed to the maintenance of green colour, possibly due to induced cytokinin synthesis (Zaicovski et al., 2008). Cytokinins, which are considered senescence inhibitors acting as ethylene antagonists and protectors of membranes, mitochondria, and plastid metabolism are also known to be induced in response to stresses (Xu and Huang, 2009). However, Wurr et al. (2002) observed that severe stress (−0.6 MPa of soil water pressure) led to negative effects on broccoli yield and morphology.

Cultivation under biotic and abiotic stress conditions can stimulate synthesis of bioactive compounds (Oh et al., 2009), mostly associated with antioxidant systems linked to plant defense mechanisms (Mittler, 2002). Fortier et al. (2010) observed that broccoli grown under nitrogen and water stress had an increased content of phenolic compounds. In addition, contents of bioactive compounds in fruit and vegetables can also be influenced by genotype, maturity at harvest, weather, cultivation, and storage conditions (Podsedek, 2007).

In this context, we have investigated the influence of soil water content during plant growth and postharvest storage conditions on colour, antioxidant activity, and contents of bioactive compounds of broccoli florets, including carotenoids, phenolic compounds, L-ascorbic acid, and 5-methyl-tetrahydrofolate.

2. Materials and methods

2.1. Plant material

'Green Star' broccoli (*Brassica oleraceae* L. var. italica) was cultivated in a greenhouse, with air temperatures of 15.5–24.9 °C and natural light of 6.7–15.7 mol m^{-2} day^{-1}, in Pelotas, RS, Brazil, from April through September of 2009. The soil was an Acrisol (dark red podzolic by Brazilian soil taxonomy and palendult by US soil taxonomy), with 530 g kg^{-1} of sand, 210 g kg^{-1} of silt and 230 g kg^{-1} of

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clay, pH 6.2, organic matter 3.8%, phosphorus 29.7 mg dm^{-3}, potassium 180 mg dm^{-3}, sodium 8 mg dm^{-3}, aluminium 1.3 mmol dm^{-3}, magnesium 3 mmol dm^{-3}, and boron 0.06 mg dm^{-3}. Soil fertility was adjusted using 60 kg ha^{-1} of nitrogen (ammonium sulphate), 300 kg ha^{-1} of P_2O_5 (simple superphosphate), 240 kg ha^{-1} of K_2O (potassium chloride) and 6 kg ha^{-1} of boron (borax). In addition, three nitrogen applications (20 kg ha^{-1} of nitrogen as urea) were performed at 15, 30, and 45 d after transplanting. Soil temperature measured at 20 cm varied from 19.8 to 20.9 °C.

Broccoli was cultivated under low (L) and normal (N) soil water contents (SWC), corresponding to soil water tensions of 0.40 ± 0.02 MPa and 0.04 ± 0.02 MPa (equivalent to field capacity), respectively. SWC were established 7 d before transplanting (plants with four or five leaves) and maintained until harvest, using an automated irrigation and monitoring system (Irrigás PRO® and MDI-10S®), as described by Zaicovski et al. (2008). One dripper for every two plants, and SWC sensors installed at 20 cm bellow soil surface, were used. The cultivation system described above is commonly used for commercial production of high quality broccoli in Brazil.

65 d after transplanting, 140 broccoli heads from each SWC were selected for uniformity, harvested at sunset (between 6 and 7 pm), weighed (450–500 g), and placed in ventilated plastic boxes (14 broccoli heads per box). Out of the 140 broccoli heads from each SWC (L and N), half was stored at room temperature (RT), at 23 ± 2 °C and 75 ± 5% relative humidity (RH), (LRT and NRT treatments), and the other half was kept under cold storage (CS), at 1 ± 1 °C and 85 ± 5% RH, (LCS and NCS treatments). Daily, for 7 d, 9 broccoli heads were sampled randomly from each treatment (LCS, LRT, NCS and NRT), pooled in triads, weighed, measured for colour, cut up into 2 cm long florets, mixed well, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Analyses

2.2.1. Colour

Colour of intact broccoli heads was measured with a chromameter (Minolta™ CR-300) and reported as hue angle [$h = \tan^{-1}(b/a)$ when $a > 0$ and $b > 0$ or $h = 180 + \tan^{-1}(b/a)$ when $a < 0$ and $b > 0$)] as previously described by Zaicovski et al. (2008).

2.2.2. Carotenoids

Broccoli (5 g fresh fw) was finely ground in liquid nitrogen, suspended in 10 mL of 80% acetone (v/v), stirred for 15 min and filtered (the extraction was repeated three times). The filtrate was centrifuged at 10,000 × *g* for 10 min and the supernatant brought to 40 mL with the acetone solution. Absorbance was measured at 647, 663 and 470 nm in an UV/Vis spectrophotometer. Carotenoids content was determined using the equations described by Lichtenthaler (1987) and expressed in μg g^{-1} fw.

2.2.3. L-Ascorbic acid

Broccoli (5 g fw) was finely ground in liquid nitrogen, suspended in 30 mL of a cold (4 °C) metaphosphoric acid solution (4.5%, w/v in water), and stored at 4 °C for 1 h in the dark and then brought to 50 mL with deionized water. The sample was filtered and the filtrate centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 μm Durapore membrane and 25 μL was injected in a HPLC Shimadzu system, using a reverse phase Shim-Pak CLC-ODS column (4.6 mm × 150 mm × 5 μm). An elution gradient started at 100% acetic acid (0.1%, v/v, A), then linearly reduced at 5 min to 98% of A and 2% of methanol (B), then held for 2 min and returned to initial conditions at 10 min. Flow rate was 0.8 mL min^{-1} and the UV detector was set at 254 nm. Identification was based on retention time comparison to an L-ascorbic acid standard. Quantification was based on external standard calibration curve and results were expressed as L-ascorbic acid in mg 100 g^{-1} fw.

2.2.4. 5-Methyl-tetrahydrofolate (5-mthf)

Broccoli (5 g fw) was finely ground in liquid nitrogen, then 15 mL of ammonium acetate solution (0.05 M) was added, and the extract placed in a sonic bath for 10 min. The mixture was transferred to a volumetric flask, to which 500 μL of trichloroacetic acid was added and final volume brought to 10 mL with the ammonium acetate solution. After homogenization, the sample was centrifuged for 20 min at 12,000 × g and filtered through a 0.45 μm Durapore membrane. An aliquot of 10 μL was injected in the previously described HPLC system. Analytical conditions were as follows: flow rate of 0.8 mL min^{-1}, initially 100% of acetic acid (2%, v/v, pH 2.8, A), at 20 min 70% A and 30% acetonitrile (B), at 25 min 100% A, held until 40 min. The fluorescence detector excitation wavelength was set at 290 nm and emission at 360 nm. 5-Methyl-tetrathydrofolate (5-mthf) identification was based on retention time comparison with a standard. Quantification was obtained from an external standard calibration curve and results were expressed as μg 100 g^{-1} fw.

2.2.5. Phenolic compounds

Broccoli (5 g fw) finely ground in liquid nitrogen was suspended in 20 mL of cold methanol (4 °C) and stirred for 24 h at 4 °C in the dark; then centrifuged at 4 °C for 15 min at 12,000 × *g*. Phenolic compounds were measured at 760 nm and quantification was expressed as gallic acid equivalent in mg g^{-1} fw, following the Folin–Ciocalteu method.

2.2.6. Antioxidant activity

Antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described by Brand-Williams et al. (1995). Briefly, broccoli (5 g fw) was finely ground in liquid nitrogen, suspended in 20 mL of cold methanol (4 °C), stirred for 24 h at 4 °C in the dark; and centrifuged at 4 °C for 15 min at 12,000 × *g*. The reaction was carried out using 100 μL of the supernatant and 3.9 mL of DPPH (100 μM) in methanol. After 60 min (exponential phase of the reaction curve) absorbance (Abs) was measured at 517 nm and the radical sequestering capacity was expressed as percent inhibition, calculated as follows: %Inhibition = [(Abs blank − Abs sample)/Abs blank] × 100.

2.3. Experimental design and statistical analysis

The experiment was set up in a completely randomized design with three replicates per treatment. Three experimental factors were considered: (1) soil water content (SWC) during plant growth, with two levels (L, 0.40 MPa and N, 0.04 MPa); (2) postharvest storage condition (PSC), with two levels (CS, 1 °C at 85% RH and RT, 23 °C at 75% RH); and (3) postharvest storage period (PSP), with eight levels (0, 1, 2, 3, 4, 5, 6 and 7 d). Antioxidant activity expressed as percentage was normalized according to the equation $f(x) = \text{arcsine}\, X^{1/2}$. Statistical analysis of the data was performed using SAS version 9.1 for Windows (SAS Institute, Cary, NC, USA) and Metrixus (Élin Duxus, São Paulo, SP, Brazil), an *add-in* program for Microsoft Office Excel. Using SAS, data were subjected to an analysis of variance, performed using the F test. Using Metrixus, data were subjected to Pearson's correlation test and principal component analysis (PCA).

3. Results

From 0 d through 3 d, as expected, LCS and NCS broccoli were greener than LRT and NRT broccoli (Fig. 1a), reinforcing that cold storage contributes to a better preservation of the green colour.

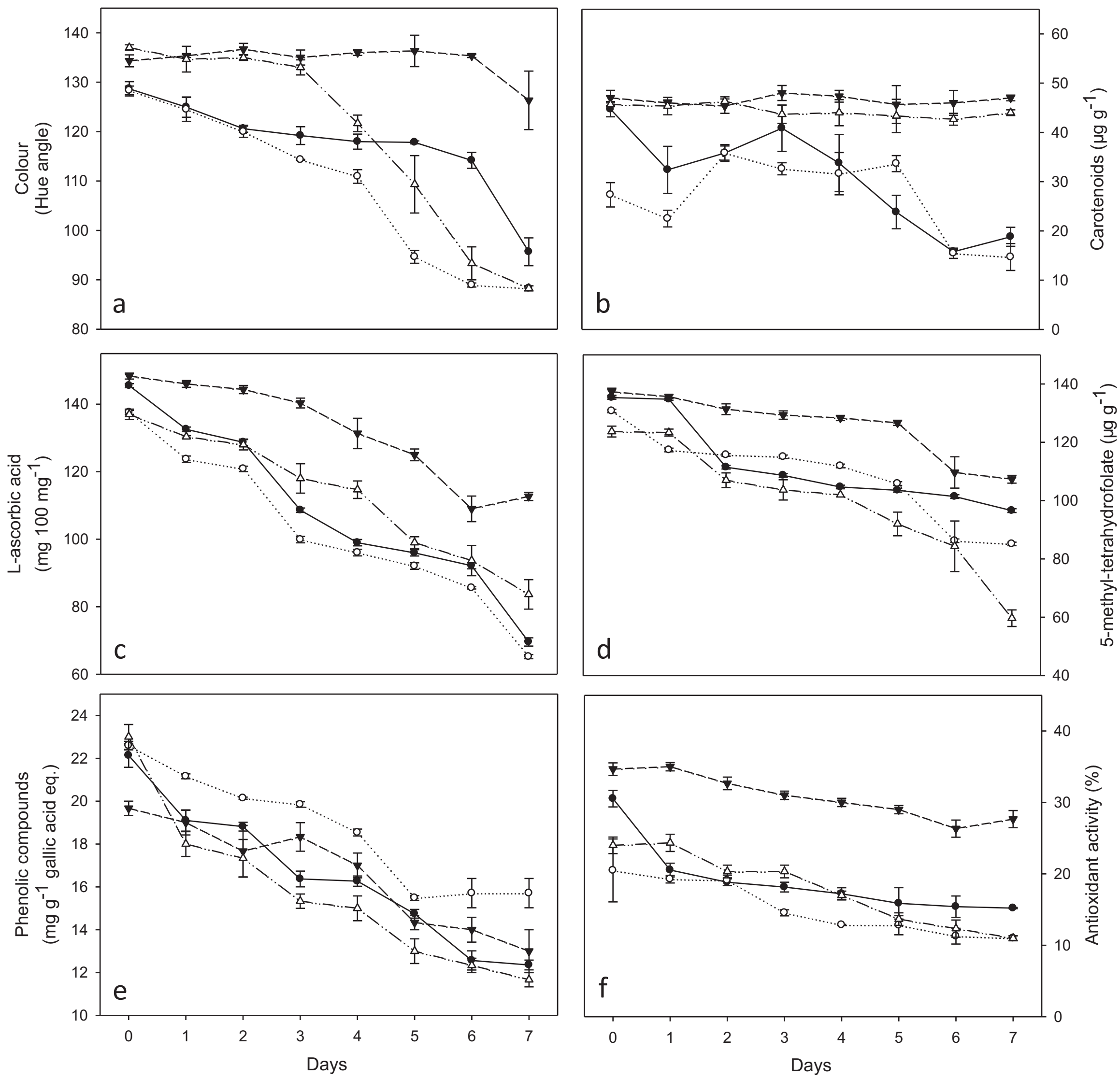

Fig. 1. Colour (a), carotenoids (b), L-ascorbic acid (c), 5-methyl-tetrahydrofolate (d), phenolic compounds (e), and antioxidant activity (f) of 'Green Star' broccoli cultivated under low (L) and normal (N) soil water content, 0.40 ± 0.02 MPa and 0.04 ± 0.02 MPa (equivalent to field capacity), respectively, and postharvest stored under room temperature (RT), at 23 ± 2 °C and 75 ± 5% relative humidity (● LRT and ○ NRT), and under cold storage (CS), at 1 ± 1 °C and 85 ± 5% relative humidity (▼ LCS and △ NCS), for 7 d. Results (charts b–f) are expressed on fresh weight basis. Error bars represent standard error of the mean (n = 3).

However, from 4 d through 7 d the response was dependent on the SWC. As mentioned above, no yellowing was observed in LCS broccoli (hue angle > 120) for at least 6 d, while in broccoli stored at room temperature (LRT and NRT) yellowing was observed 3 d after harvest. These results indicate that cold storage potentiates the effect of low SWC during plant growth in the preservation of the green colour. Moreover, a significant interaction between SWC during plant growth and PSC was observed (Table 1). While yield was not affected by SWC, postharvest weight loss was affected by PSC only. On average, cold-stored broccoli lost 0.76% of its weight per day, for a total of 5.31% weight loss, while broccoli stored at room temperature lost 4.75% of its weight per day, for a total of 33.24% weight loss (data not shown).

Table 1
Interactions between soil water content (SWC), postharvest storage condition (PSC), and postharvest storage period (PSP) for all the measured variables, including colour, carotenoids, phenolic compounds, L-ascorbic acid, 5-methyl-tetrahydrofolate (5-mthf), and antioxidant activity, determined by analysis of variance (ANOVA).

Source	F values					
	Colour	Carotenoids	Phenolics compounds	L-Ascorbic acid	5-Mthf	Antioxidant activity
SWC*PSC	17.85***	1.11NS	83.21***	80.41***	195.94***	79.67***
SWC*PSP	36.92***	2.66*	1.56NS	1.79NS	9.72***	2.36*
PSC* PSP	2.59*	10.33***	2.67*	24.13***	4.39**	1.92NS
SWC*PSC*PSP	6.47***	2.82*	4.39**	3.44*	11.84***	2.33*

NS: non-significance.
* Indicates significance at the 0.05 level.
** Indicates significance at the 0.001 level.
*** Indicates significance at the 0.0001 level.

PSC had greater impact than SWC on carotenoid preservation (Fig. 1b), without a significant interaction between these experimental factors (Table 1). Broccoli kept under CS (LCS and NCS) maintained higher contents of carotenoids than those stored at RT (LRT and NRT), independent of SWC. During 7 d of storage, the contents of carotenoids were not significantly affected in broccoli under CS (LCS and NCS) while in broccoli under RT (LRT and NRT) an average loss of 84% occurred (Fig. 1b).

CS alone was not enough to avoid significant losses of L-ascorbic acid. Although L-ascorbic acid content dropped during storage, in LCS broccoli the losses were limited (Fig. 1c). This indicates that preservation of this compound depends on the association of SWC and PSC (Table 1). After 7 d of storage, LCS broccoli had lost about 24% of L-ascorbic acid, while NCS broccoli had lost about 40% and LRT and NRT broccoli had lost 52% each. Following a similar trend, 5-mthf preservation was a consequence of the interaction between SWC and PSC (Fig. 1d and Table 1). At the end of 7 d of storage, LCS broccoli (best treatment) and NCS broccoli (worst treatment) had lost about 21% and 52% of 5-mthf, respectively.

Carotenoids, L-ascorbic acid, and 5-mthf contents and antioxidant activity in LCS broccoli were higher than in other treatments (Fig. 1). In contrast to the other measured variables, the content of phenolic compounds at harvest was higher in broccoli grown under normal SWC than in broccoli grown under low SWC (Fig. 1e). During storage, the content of phenolic compounds was reduced in all treatments over time. SWC (L and N) or PSC (RT and CS) induced no clear effect on phenolic content.

Principal component analysis (PCA) of the data, led to a reduction of the variables and establishment of two principal components (PC1 and PC2) that represented the majority of the observed variation (87.12%). In this manner it was possible to identify a general trend of the treatments considering the contribution of all measured variables (Fig. 2). PC1 represented 71.06% of the total variation, with major contribution from the variables colour, carotenoids, and antioxidant activity; while PC2 represented 16.06% of total the variation, with main contribution from phenolic compounds and 5-mthf. Broccoli stored under CS and RT formed groupings (CS solid line and RT dotted line, Fig. 2) separated along the *Y*-axis represented by PC2. LRT broccoli did not separate from NRT broccoli, indicating a limited effect of the SWC on broccoli stored at room temperature. On the other hand, NCS broccoli separated from LCS broccoli, illustrating the influence of the SWC on cold stored broccoli. Within groupings (circled in Fig. 2), a continuous progression from 0 d to 7 d indicated a postharvest storage period influence, which was correlated with the general trend observed for all variables in which values decreased over time (Fig. 1).

The highest correlations found for the measured variables were between L-ascorbic acid and colour (0.87), L-ascorbic acid and 5-mthf (0.85), and L-ascorbic acid and antioxidant activity (0.83); meanwhile the lowest correlations were between phenolic compounds and carotenoids (0.08), followed by carotenoids and 5-mthf (0.34), and phenolic compounds and antioxidant activity (0.37) (Table 2).

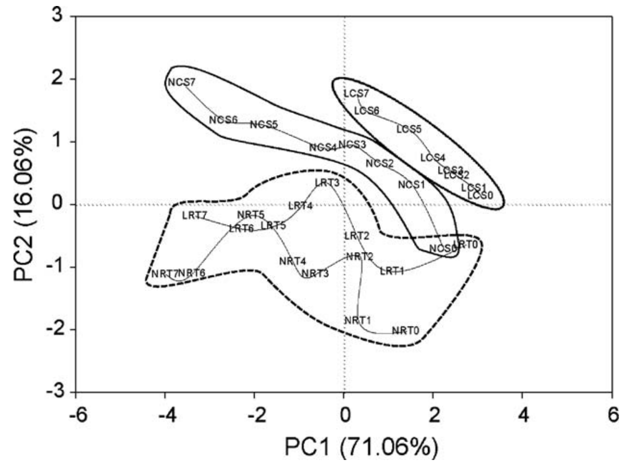

Fig. 2. Principal component analysis (PCA) of 'Green Star' broccoli cultivated under low (L) and normal (N) soil water content, 0.40 ± 0.02 MPa and 0.04 ± 0.02 MPa (equivalent to field capacity), respectively, and postharvest stored under room temperature (RT), at 23 ± 2 °C and 75 ± 5% relative humidity (LRT and NRT, 0–7 d), and under cold storage (CS), at 1 ± 1 °C and 85 ± 5% relative humidity (LCS and NCS, 0–7 d).

4. Discussion

One of the most easily perceived alterations in broccoli leading to loss of commercial value is yellowing which occurs 3–4 d after harvest if maintained at room temperature (Zaicovski et al., 2008). Widely documented in the literature (Zaicovski et al., 2008; Asoda et al., 2009), this problem has various suggested methods of prevention, including CS (Starzynska et al., 2003; Vallejo et al., 2003; Able et al., 2005), with or without controlled or modified atmosphere (Watada et al., 1996; Rakotonirainy et al., 2001; Serrano et al., 2006; Eason et al., 2007; Rai et al., 2008), which contributes to prevent the degradation of chlorophylls caused by enzymes such as chlorophyllase (Hortensteiner, 2006), peroxidase (Funamoto et al., 2003), pheophytinase (Schelbert et al., 2009), and pheophorbide oxygenase (Pruzinska et al., 2005).

In the present study, CS prevented yellowing of 'Green Star' broccoli cultivated under low SWC only; demonstrating an interaction between SWC during plant growth and PSC (Table 1). Zaicovski et al. (2008) observed that low SWC during broccoli growth induced formation of new roots and accumulation of cytokinins in the florets, resulting in better preservation of green colour, possibly a consequence of the protective action of this hormone. In the study of Wurr et al. (2002), water stressed broccoli were also greener and more turgid; however, the more severe stress conditions (−0.6 MPa) negatively affected morphology and yield. Cytokinin application (Costa et al., 2005) or cytokinin synthesis induction by an *ipt* transgene (Chen et al., 2001) protects the inflorescences from yellowing. In addition, Synkova et al. (2006) has shown that tobacco over-expressing an *ipt* gene had increased defense responses, possibly leading to a better preservation of cell homeostasis. 'Green Star' broccoli cultivated under low SWC showed a ten-fold increase in

Table 2
Pearson's correlations between the measured variables: colour, L-ascorbic acid, antioxidant activity, 5-methyl-tetrahydrofolate (5-mthf), carotenoids and phenolic compounds.

Correlations	Colour	L-Ascorbic acid	Antioxidant activity	5-Mthf	Carotenoids	Phenolic compounds
Colour	1.000					
L-Ascorbic acid	0.870	1.000				
Antioxidant activity	0.826	0.835	1.000			
5-Mthf	0.803	0.851	0.786	1.000		
Carotenoids	0.603	0.616	0.633	0.337	1.000	
Phenolic compounds	0.487	0.673	0.375	0.718	0.079	1.000

trans-zeatin riboside when compared to broccoli cultivated under normal SWC (Zaicovski et al., 2008). Xu and Huang (2009) also observed that creeping bentgrass treated with trans-zeatin riboside became more tolerant to abiotic stress.

Low SWC contributed synergistically with CS in the maintenance of colour (Fig. 1a) and some bioactive molecules, such as L-ascorbic acid (Fig. 1c), 5-mthf (Fig. 1d) and antioxidant activity (Fig. 1f), but not in the maintenance of carotenoids (Fig. 1b) and phenolic compounds (Fig. 1e). CS was a determinant in the conservation of carotenoids (Fig. 1b), but neither CS nor low SWC was effective in preventing losses of phenolic compounds (Fig. 1e).

Carotenoid contents were kept relatively constant during the course of 7 d under CS (Fig. 1b), which is particularly important from a nutritional point of view since carotenoids are an important source of vitamin A precursors (Zhang and Hamauzu, 2004; Jones et al., 2006). From a physiological perspective, carotenoids protect chloroplasts against photooxidation, preventing chlorophyll degradation and avoiding the formation of free radicals in plastids (Aluru et al., 2009). In this study a large variation in carotenoid contents was observed, although, the exact causes were not identified. Senescence and physical injuries might have accounted for the observed losses.

Contents of phenolic compounds were lower in broccoli cultivated under low SWC, contrasting with the initial hypothesis based on work by Mittler (2002), Oh et al. (2009) and Fortier et al. (2010), who proposed increased accumulation of these compounds upon abiotic stresses. However, not all abiotic stresses have beneficial impacts or always give the same response. For example, under low SWC, accumulation of L-ascorbic acid is increased in some fruit and vegetables but is reduced in strawberry and leafy greens (Cisneros-Zevallos, 2003). Phenolic compounds were dramatically reduced under all treatments, independent of SWC or PSC. Leja et al. (2001) and Zhang and Hamauzu (2004) have reported broccoli with phenolic compounds contents above 34.5 mg 100 g^{-1} fw while in this study, they ranged from 19.7 to 23.0 mg 100 g^{-1} fw at harvest and from 11.7 to 15.7 mg 100 g^{-1} fw after 7 d of storage. Reduction in phenolic compounds during storage could be attributed to the bioconversion of these molecules to antioxidants involved in protection of the cells (Toivonen, 1997; Keles and Oncel, 2002; Vallejo et al., 2003).

Losses of L-ascorbic acid were lower in LCS broccoli (Fig. 1c) indicating that low SWC and CS synergistically contributed to its preservation. L-ascorbic acid reduction involves participation in the protection of the oxidative and respiratory systems, as well as in the recycling of lipid-soluble α-tocopherol synthesized in the mitochondria and transported to the chloroplasts, important organelles in oxidative stress protection (Smirnoff, 2000; Serrano et al., 2006).

Preservation of 5-mthf in LCS broccoli could be associated with the preservation of L-ascorbic acid. Although L-ascorbic acid is mainly synthesized in the mitochondria, higher concentrations are found in the plastids where folates are synthesized. Alhagdow et al. (2007) also mentioned that L-ascorbic acid acting as an electron receptor in enzymatic and chemical oxidation–reduction reactions contributed to the preservation of folates, carotenoids, α-tocopherol and chlorophyll.

Antioxidant activity was highly correlated with L-ascorbic acid (0.84), 5-mthf (0.79) and carotenoids (0.63), and consequently, LCS broccoli with higher contents of these compounds had high antioxidant activity (Table 2). Often antioxidant activity in vegetables is strongly associated with phenolic compounds (Starzynska et al., 2003; Serrano et al., 2006; Koca and Karadeniz, 2008). In this study, this was not the case, possibly due to the fact that contents of phenolic compounds were relatively low when compared to other genotypes (Zhang and Hamauzu, 2004).

In summary, the preservation of colour, L-ascorbic acid, 5-mthf and antioxidant activity was achieved as a consequence of the interaction between low SWC during cultivation and CS after harvest. On the other hand, losses of phenolic compounds were not dependent on SWC or PSC and carotenoid stability was only influenced by PSC.

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