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Effect of meteorological conditions on antioxidant flavonoids in Portuguese cultivars of white and red onions

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ABSTRACT

Although onion (*Allium cepa* L.) bulbs are good sources of phenolic compounds, the levels of these secondary metabolites are highly variable, depending on the cultivar, production, metereological conditions and post-harvest practices. The aim of this study was to characterize the interannual variation of flavonoid content in two Portuguese landrace varieties of onion ('Branca da Póvoa', white, and 'Vermelha da Póvoa', red), grown in the Spring–Summer of 2004, 2005, 2006, 2007 and 2008. HPLC-DAD was used to determine flavonoid concentration.

Quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside were the main flavonols in both varieties. Additionally, six cyanidin derivatives were identified in the red variety. Total and individual flavonoids levels varied significantly among seasons, with higher levels in 2005, a very dry and hot season. The red onion variety did not accumulate detectable amounts of anthocyanins in 2007, the year with the lowest air and soil temperature and highest soil water content.

1. Introduction

Onions (*Allium cepa* L.) are one of the world's oldest cultivated vegetables and are the second most produced vegetable crop after tomatoes (Griffiths, Trueman, Crowther, Thomas, & Smith, 2002). Over the past 15 years, the total surface area dedicated to onion crops in the world has doubled, now reaching 3.07 million ha with a production of 53.6 Mt (FAOSTAT, 2004). In Portugal, onion is the third vegetable in consumption (13 kg per capita and year), after potato and cabbage, and the landrace cultivars 'Branca da Póvoa' and 'Vermelha da Póvoa' are predominant in the northwestern region of the country. Onions contain high levels of no nutrient antioxidant compounds (phenolics) which have protective effects against different degenerative pathologies such as cardiovascular and neurological diseases, cancer and other dysfunctions based on oxidative stress (Griffiths et al., 2002).

Flavonoids are the largest group of polyphenolic plant, secondary metabolites (Schijlen, Ric de Vos, van Tunen, & Bovy, 2004). Epidemiologic studies show that dietary intake of flavonoids is inversely associated with risk of cardiovascular disease and cancer (Arts & Hollman, 2005). The beneficial health effects of flavonoids are attributed to their antioxidant, anti-estrogenic, anti-inflammatory, chelating properties and prevention of platelet aggregation (Heim, Tagliaferro, & Bobilya, 2002). Considering the health benefits of flavonoids, there is currently a growing interest in the development of agronomically important food crops with optimized levels and composition of flavonoids (Schijlen et al., 2004). Additionally, the results of the epidemiological studies can only be accurately interpreted if an extensive database of the flavonoid content of foods is available for a given population, since cultural dietary habits often dictate which food are consumed and, in turn, the subclasses and the amount of flavonoids ingested (Beecher, 2003).

Flavonoids (C_6–C_3–C_6) are major phenolics in onions and can be classified into different subclasses (flavones, flavanones, flavonols, isoflavones, flavanonols, flavanols, chalcones and anthocyanins) according to the degree of unsaturation and degree of oxidation of the three-carbon skeleton. Subclasses of flavonoids can be further differentiated on the basis of the number and nature of substituent groups attached to the rings (Robards & Antolovich, 1997). Flavonols and anthocyanins are the main subclasses of flavonoids present in onions, the latter being found only in red onions. Many of these compounds are glycosylated, and some of these glycosyl derivatives are esterified with aromatic or aliphatic acids whose combinations yield a large variety of compounds (Slimestad, Fossen, & Vagen, 2007). Aromatic acylation increases the stability of anthocyanins by intramolecular stacking of anthocyanins with polyphenols. Malonylation is one of the most common forms of aliphatic acylation of anthocyanins and is important for enhancing

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pigment solubility in water, protecting glycosyl moiety from enzymatic degradation, and stabilizing anthocyanin structures, which contributes to keeping the colour hue. Within a vegetable family, the quality and quantity of the phenolic pool may change with the cultivar, growth stage, and environmental conditions. Patil, Pike, and Hamilton (1995) have shown that metereological factors (including temperature and rainfall patterns) have a stronger influence on quercetin concentration in onion cultivars than soil factors or plant maturity. Thus, the most critical factors for the accumulation of flavonoids like quercetin (e.g. temperature and rainfall) are also the ones that are more difficult to control in open air cultivation.

This study addresses the flavonoid composition in two Portuguese landrace varieties of onion, one of the most important vegetables consumed in Portugal. The effect of metereological conditions on antioxidant flavonoid concentration was studied in five consecutives years. As far as we know, nobody has studied the effect of these conditions in the regional varieties of Portuguese onion bulbs we focused. This work is part of an ongoing project where the effects of different processes on onion flavonoids are being monitored as is the case of post-harvest practices (Rodrigues, Pérez-Gregorio, García-Falcón, Simal-Gándara, & Almeida, 2010) or cooking treatments (Rodrigues, Pérez-Gregorio, García-Falcón, & Simal-Gándara, 2009).

2. Material and methods

2.1. Plant material and growing conditions

Two Portuguese landrace varieties of onion, 'Branca da Póvoa' (white) and 'Vermelha da Póvoa' (red) were grown in Póvoa do Varzim (Northwest of Portugal) in 2004, 2005, 2006, 2007 and 2008, using standard cultural practices. The onion cultivars were grown under the same conditions in a farm located in a traditional onion-growing region in northwestern Portugal (Póvoa do Varzim, at 41°22′57″N and 8°45′46″W). The crop was established by transplanting in March and harvested in July, when 60% of foliage had fallen over. Bulbs were then cured in field for 10 days before analysis. Metereological data during all experimental years were obtained from the meteorological station of Pedras Rubras (Institute of Meteorology of Portugal).

2.2. Extraction and determination of flavonoids

A group of 30 onion bulbs from each cultivar was selected. From this initial group, 10 onion bulbs with a representative average weight (67 and 150 g, respectively, for red and white onions) were selected and individually analyzed in triplicate. Each bulb was skinned, cut longitudinally into quarters and a representative sample of each quarter (15 g) was immediately homogenized at 1200 rpm for 3 min with 25 ml of methanol:formic acid:water (MFW; 50:5:45; v:v:v) and stabilized with 2 g l^{-1} of tertbutylhydroquinone. The homogenate was agitated for 15 min in an alternative shaker and centrifuged. Each homogenate was extracted three times and the combined fractions diluted to a final volume of 60 ml with the MFW solution, and a 50 μl aliquot used for analysis.

HPLC measurements were made using a Thermo Separation Products (TSP) P2000 binary pump, equipped with a TSP AS1000 autosampler, a TSP SCM1000 vacuum membrane degasser and a UV6000LP diode array detector (DAD). The chromatographic data were collected and processed using the Chrom-Quest software version 2.51. The optimized instrumental and conditions parameters for the chromatographic analysis of flavonoids were as follows: guard column Pelliguard LC-18 (50 × 4.6 mm id, 40 μm) (Supelco) and analytical column Water Symmetry C18 (150 × 4.6 mm id, 5 μm), both at 40 °C; Mobile phase A (5% formic acid in water):B (methanol), using a gradient elution of: 0–5 min: 100:0, 15–20 min: 85:15, 25–40 min: 45–55, 41–45 min: 0:100 (washing step), 46–51 min: 100:0 (conditioning step), eluted at flow rate of 1 ml min^{-1}; scanning 200–600 nm, with a detection wavelength of 360 nm for flavonols and 520 nm for anthocyanins; scan rate of 1 Hz, step of 2 nm, bandwidth of 3 nm.

Quantification of single flavonols and anthocyanins was achieved by calibrations curves obtained using pure quercetin (from Sigma–Aldrich, Madrid, Spain; CAS No. 6151-5-3) and cyanidin 3-glucoside (from Extrasynthese, Genay Cedex, France; CAS No. 7084-4-4) as standards, respectively. To evaluate the recovery and repeatability of the analytical method, the levels of flavonoids that remain in the onion matrix after three successive extractions were determined. Recovery percentages were higher than 92% with relative standard deviations lower than 4% (Table 1). Phenolic compounds were quantified using external standards and subsequent regression of the peak areas for the different analytes against their concentrations. Flavonol and anthocyanin concentrations were expressed in mg g^{-1} quercetin and cyanidin 3-glucoside, respectively. Table 1 shows the linearity parameters for these compounds. Detection and quantitation limits (American Chemical Society, 1980) were lower than 0.5 mg kg^{-1} quercetin for flavonols, and lower than 0.2 mg g^{-1} cyanidin 3-glucoside for anthocyanins.

2.3. Acid and alkaline hydrolyses for flavonoid identification

Onion flavonoids occur mainly as glycosyl derivatives, and in the case of anthocyanins, some of these glycosyl derivatives are esterified with aromatic or aliphatic acids. Acid and alkaline hydrolysis, enzymatic autolysis and spectral analysis were performed for identification purposes (Pérez-Gregorio, García-Falcón, Simal-Gándara, Rodrigues, & Almeida, in press). Standards used for identification purposes were: cyanidin 3-glucoside (CAS 7084-24-4, from Extrasynthese, 95% pure) and quercetin (CAS 6151-25-3, from Sigma–Aldrich, 98%), kaempferol (CAS 520-18-3, from Sigma–Aldrich, 96%) and isorharmnetin (CAS 480-19-3, from Sigma–Aldrich, 98%).

The transformation of glycosides into aglycones was done by acid hydrolysis of the glycosidic compounds. ten ml of onion extract was concentrated on a rotary evaporator (during 10 min) to eliminate the methanol fraction and the extract obtained was reconstituted with water. To eliminate the sugars and prevent caramelization during the hydrolysis process, the solution was purified by loading in a Sep Pak Plus C_{18} cartridge (Waters), previously activated. The loaded cartridge was washed with 10 ml water, dried with N_2 (15 min) and the flavonoids were eluted with 3 ml of 1 N HCl in methanol and then dissolved in 2 ml of 2 N HCl in methanol and heating at 90 °C for 2 h under N_2 atmosphere. The hydrolyzed solution was analyzed in HPLC as described.

To confirm the identity of anthocyanins, alkaline hydrolysis was performed by adding 10 ml of 10% KOH to 5 ml of onion extract, in sealed vessels under N_2, and maintained in dark for 8 min at room temperature. The solution was then purified in a Sep Pak Plus C_{18} cartridge, previously activated. The loaded cartridge was washed with 5 ml of 0.001% HCl in distilled water, and the elution of flavonoids was made with 5 ml of 0.001% HCl in methanol. The methanol fraction was concentrated to 1 ml, in a rotary evaporator and the extract obtained was reconstituted with 5% formic acid in water to a final volume of 5 ml and separated by HPLC, to identify the deacylated anthocyanins.

2.4. Statistical analysis

Data were subjected to one-way analysis of variance at the 95% level to assess the differences in major individual flavonols concen-

Table 1
Quality parameters of the optimized HPLC/DAD for flavonol and anthocyanin quantification ($n = 6$).

Flavonols	% recovery	% RSD	Quercetin calibration range (mg/l)	r^2
(1) Quercetin 7,4-diglucoside	97	3	0.25–8	0.995
(2) Quercetin 3,4-diglucoside	97	2		
(3) Isorhamnetin 3,4-diglucoside	98	2		
(4) Quercetin 3-glucoside	98	2		
(5) Quercetin 4-glucoside	92	4		
(6) Isorhamnetin 4-glucoside	96	2		
Anthocyanins	% recovery	% RSD	Cyanidin 3-glucoside calibration range (mg/l)	r^2
(1) Cyanidin 3-glucoside	99	1	0.04–10	0.999
(2) Cyanidin 3-laminaribioside	99	2		
(3) Cyanidin 3-(6″-malonylglucoside)	99	1		
(4) Cyanidin 3-malonyl-laminaribioside	99	1		
(5) Cyanidin 3-dimalonylaminaribioside	93	1		

tration among cultivars. When significant effects were observed, means were separated by the least significant difference multiple range test with a, b and c. All statistical analyses were performed with the Statgraphics Plus statistical software (v. 5.1, Statistical Graphics Corp., Herndon, VA 20171, USA).

3. Results and discussion

3.1. Identification of flavonols and anthocyanins

Six flavonols were identified in each of the onion varieties analyzed (Fig. 1). In the red onion variety six anthocyanins were also isolated (Fig. 2). The acid hydrolyzed extracts separated by HPLC revealed two peaks at 360 nm, identified as quercetin and isorhamnetin, and two peaks at 520 nm, identified as cyanidin and peonidin, by comparison with their commercial standards. Upon alkaline hydrolysis the area of peaks 1 and 2 increased, due to the hydrolysis of acylated forms of cyanidin (peaks 3, 4 and 5), and a new peak (peonidin 3-glucoside) resulted from the hydrolysis of the acylated form of peonidin (peak 5).

The identity of the compounds (Figs. 1 and 2) was ascertained based on chromatographic retention times, acid and alkaline hydrolyses, and spectral analysis, taking into account published data (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2005; Donner, Gao, & Mazza, 1997). The six flavonols were quercetin 7,4′-diglucoside, quercetin 3,4′-diglucoside, isorhamnetin 3,4′-diglucoside, quercetin 3-glucoside, quercetin 4′-glucoside and isorhamnetin 4′-glucoside and the six anthocyanins were cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-(6″-malonylglucoside), cyanidin 3-(6″-malonyl-laminaribioside), cyanidin 3-dimalonylaminaribioside and peonidin 3-malonylglucoside. The absence, in the edible portion of onion, of quercetin aglycone and other structures derived from myricetin and kaempferol is in agreement with the observations of Lombard, Geoffriau, and Peffley (2002), Nuutila, Kammiovirta, and Oksman-Caldentey (2002) and Sellappan et al. (2002). Only cyanidin- and peonidin-derivatives were found in red 'Vermelha da Póvoa' onions, but very low amounts of delphinidin, and petunidin derivatives have been reported in onions elsewhere (Gennaro et al., 2002).

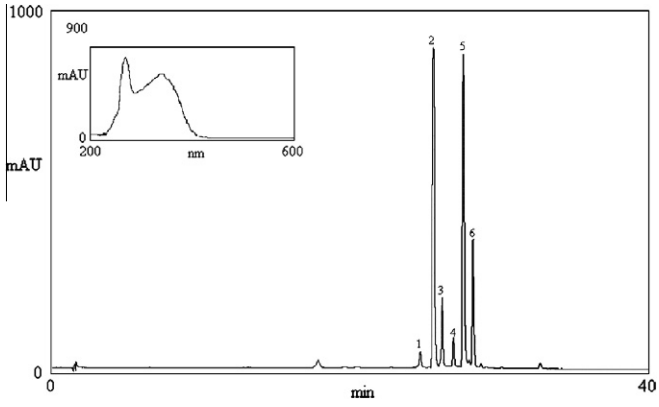

Fig. 1. Chromatogram ($\lambda = 360$ nm) of the red onion extract with the typical flavonol absorption spectrum. Peak: (1) quercetin 7,4′-diglucoside, (2) quercetin 3,4′-diglucoside, (3) isorhamnetin 3,4′-diglucoside, (4) quercetin 3-glucoside, (5) quercetin 4′-glucoside and (6) isorhamnetin 4′-glucoside.

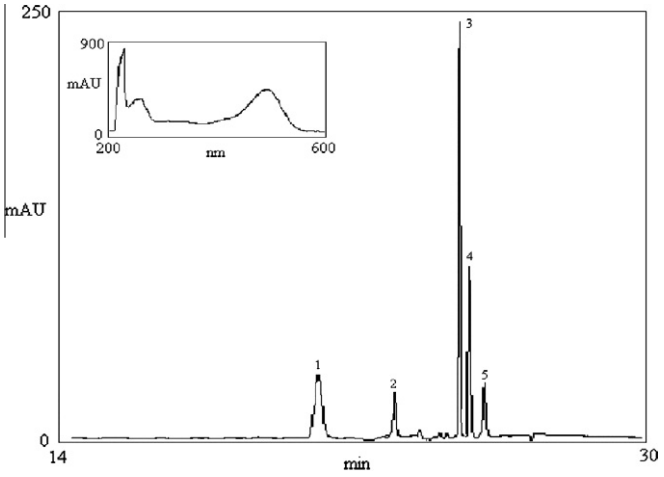

Fig. 2. Chromatogram ($\lambda = 520$ nm) of the red onion extract with the typical anthocyanin absorption spectrum. (1) Cyanidin 3-glucoside, (2) cyanidin 3-laminaribioside, (3) cyanidin 3-(6″-malonylglucoside), (4) cyanidin 3-(6″-malonyl-laminaribioside), (5) cyanidin 3-dimalonylaminaribioside + peonidin 3-malonylglucoside.

3.2. Differences in flavonoids between white and red varieties

Significant differences in total flavonols concentration were observed between varieties. Total flavonol content was lower in the white variety 'Branca da Póvoa' than in the red variety 'Vermelha da Póvoa', ranging from 78.9 to 186.8 mg kg^{-1} FW and from 226.7 to 402.5 mg kg^{-1} FW, respectively (Table 2). Most studies (Arabbi, Genovese, & Lajolo, 2004; Lin & Tang, 2007; Marotti, Piccaglia, & Venturi, 2002) report higher levels of flavonols in red onions than in other varieties, but Crozier, Lean, Mc Donald, and Black (1997) obtained the opposite result. In general, our levels of total (Table 2) and individual flavonols (Fig. 3) were similar to those reported by Mattila, Astola, and Kumpulainen (2000) (192–307 mg kg^{-1} FW), Nuutila et al. (2002) (306 mg kg^{-1} FW) but considerably lower than those reported by Gennaro et al. (2002)

Table 2
Total flavonols in onion varieties and metereological conditions in each of the three years. Total Rainfall–R, Mean Air Temperature–T, Mean Soil Temperature (10 cm)-ST_{10}, Mean Air Relative Humidity–RH, Total Global Radiation–GR and Mean Soil Water Content–SWC for the growth period from March through July 2004–2006.

Total flavonols (mg kg^{-1} FW)			Total anthocyanins (mg kg^{-1} FW)	Meteorological conditions					
Year	White	Red	Red	R (mm)	T (°C)	ST10 (°C)	RH (%)	GR (kj m^{-2})	SWC (%)
2004	93.5	239.5	5.6	227.7	15.8	17.8	72.8	3197163.4	49.5
2005	186.8	402.5	5.9	222.9	16.3	18.2	69.4	3244902.8	36.9
2006	109.2	260.5	2.6	352.5	16.8	19.4	73.2	2399870.3	50.5
2007	78.9	226.7	<0.5	231.8	15.4	17.5	73.4	2997781.6	51.2
2008	185.0	338.1	3.7	224.1	16.5	18.6	69.6	3301023.1	37.3

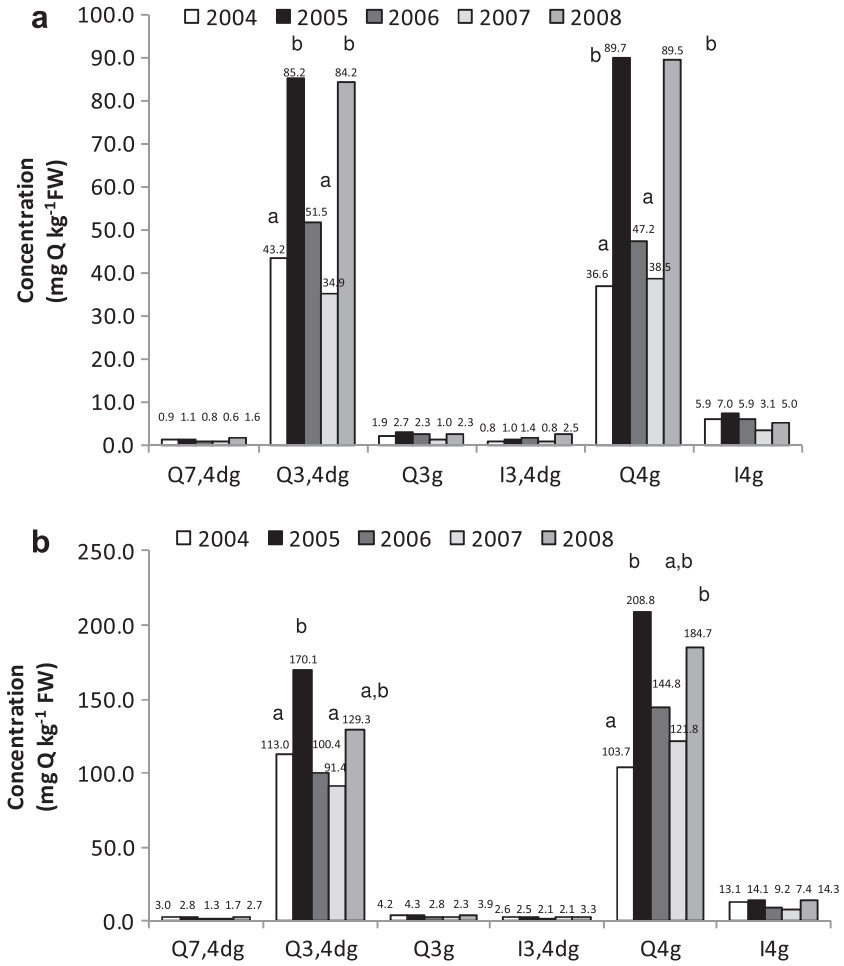

Fig. 3. Individual flavonols content in (a) white onion and (b) red onion. (1) quercetin 7,4′-diglucoside, (2) quercetin 3,4′-diglucoside, (3) quercetin 3-glucoside, (4) isorhamnetin 3,4′-diglucoside, (5) quercetin 4′-glucoside and (6) isorhamnetin 4′-glucoside.

(598 mg kg^{-1} FW), Arabbi et al. (2004) (482–936 mg kg^{-1} FW) and Bonaccorsi et al. (2005) (554–612 mg kg^{-1} FW).

Two flavonols, quercetin 3,4′-diglucoside and quercetin 4′-glucoside (Fig. 3), account for 90–94% of the total flavonol content in each variety, a value slightly higher than reported elsewhere (Bonaccorsi et al., 2005; Lombard et al., 2002).

HPLC chromatograms of the extracts from the red variety (Fig. 1) revealed one large anthocyanin peak [cyanidin 3-(6″-malonylglucoside)] representing 49–58% of total anthocyanins. Similar proportions of this anthocyanin were reported by, Fossen, Andersen, Ovstedal, Pedersen, and Raknes (1996) and Donner et al. (1997) in red onion cultivars. However, the total anthocyanin content in the red onion variety was 5.6 and 5.9 mg kg^{-1} FW in 2004 and 2005, respectively, much lower than reported in other studies. The anthocyanin contents in onions harvested in 2006, 2007 and 2008 were still much lower. In 2007, the red onions did not accumulate detectable concentrations of anthocyanins.

3.3. Effect of meteorological conditions on flavonoids

Both varieties present higher levels of total flavonols in 2005 and the lowest levels in 2007 (Table 2). Variability in flavonols content among years was higher in the white variety (100%). Mogren, Olsson, and Gertsson (2006) also found significant differences in

quercetin content among years. The higher levels of flavonols observed in 2005 and 2008 are probably related to the higher global radiation and lower rainfall during the growing season. These meteorological conditions can enhance secondary metabolism, favouring the synthesis of flavonols (Fig. 3). In contrast, in the year with the lowest soil and air temperatures, higher relative humidity and higher soil water availability (2007), onions accumulated less flavonols. Something similar to this happens in 2004. Mogren et al. (2006) also found a high correlation between global radiation and levels of quercetin in onion bulbs. Similar results are obtained for apples, where quercetin glycosides were found to be twice as high on light exposed fruits (Ubi, 2004).

According to Table 2 the two onion varieties have a different quantitative behaviour under the same meteorological conditions:

- The total flavonols of the white variety increase by 100% and 17% between 2004–2005 and 2004–2006, respectively. It decreases by 16% between 2004–2007, and again increases by 98% between 2004–2008.
- The total flavonols of the red variety, instead, increase by 68% and 9% between 2004–2005 and 2004–2006, respectively. It decreases by 5% between 2004–2007, and again increases by 41% between 2004 and 2008.

The meteorological factor with the greatest variability was the total global radiation (GR). This factor increased by 1.5% between 2004 and 2005 and decreased by 25% between 2004 and 2006. The factor again decreased by a 6% between 2004–2007, while increases 3% between 2004–2008.

Accumulation of phenolics and higher activity of their biosynthetic enzymes in response to drought stress have also been reported in other plants. Chaves, Escudero, and Gutierrez-Merino (1997) demonstrated that, in Cistus ladanifer, drought and high temperatures are correlated with the increase of the more methylated flavonoids. In water-stressed Hypericum brasiliense there is a general increase in the levels of phenolic compounds, particularly rutin and quercetin (Abreu & Mazzafera, 2005). Wang and Zheng (2001) found a strong correlation between temperature and production of phenolic in strawberry fruits.

Light and temperature are the most important factors affecting anthocyanin accumulation in fruits. Low light intensity results in reduction of photosynthesis, which reduced the soluble sugar content of tissues and led to a repression of genes that encode enzymes of the anthocyanin biosynthetic pathway and to a reduction in substrates for flavonoid biosynthesis (Ubi, 2004). High temperatures also inhibit anthocyanin accumulation in apples and grapes (Mori, Goto-Yamamoto, Kitayama, & Hashizume, 2007; Reay & Lancaster, 2001). The effect of high temperatures is probably related to increased respiratory consumption of sugars, which are essential substrates to anthocyanin biosynthesis (Ubi, 2004).

Our results show that both genetic and meteorological factors have a marked influence on the content of flavonoids in onion bulbs.

4. Conclusions

The levels of total flavonols were 2–3 times higher in 'Vermelha da Póvoa' (226.7–402.5 mg kg^{-1} FW) than in 'Branca da Póvoa' (78.9–186.8 mg kg^{-1} FW). Quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside were the main flavonols in both varieties. Additionally, six anthocyanins (cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-(6″-malonyl-glucoside), cyanidin 3-(6″malonyl-laminaribioside), cyanidin 3-dimalonyl-laminaribioside and peonidin 3-malonyl-glucoside) were identified in the red variety. Total and individual flavonoids levels varied significantly among seasons, with higher levels in 2005 and 2008, very dry and hot seasons.

The two onion varieties have a different quantitative behaviour with regards to total flavonols under the same meteorological conditions, in good agreement with the meteorological factor with the greatest variability, which was the total global radiation.

The red onion variety did accumulate lower amounts of anthocyanins in 2006 and 2007, the years with the lowest global radiation. Light and temperature are the most important factors affecting anthocyanin accumulation in fruits. Low light intensity results in reduction of photosynthesis, which reduced the soluble sugar content of tissues and led to a reduction in substrates for flavonoid biosynthesis. Instead, the effect of high temperatures is probably related to increased respiratory consumption of sugars, which are essential substrates to anthocyanin biosynthesis.

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