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Antioxidant characterization of different italian broccoli landraces

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ABSTRACT

The *Brassicaceae* plants are among the most consumed vegetables in the world. They feature a large biodiversity, in which landraces and primitive cultivars still play a major role on the cultivation systems of many countries. Brassicas and especially broccoli are closely linked to antioxidant compounds that play a key role for human health. This experiment aimed to characterize some Italian unknown broccoli landraces from the antioxidant point of view. Five landraces named BF, BF4, BFT, BB and BS were evaluated. The total antioxidant properties of broccoli were significantly different among ecotypes and the higher values were recorded in BS and BF4. Total phenols showed the higher values in BF4 and concerning the ascorbic acid content found in the different ecotypes results can be summarized in three groups: the first one is represented by BB with higher values, the second one by BF4 and BS and then BF and BFT. BB represented the lower amount of hydroxycinnamic acids, whereas BF4 showed quite higher sulforaphane content (+95%) than other cultivars. The high content of phenolic acids, vitamin C and antioxidant compounds make these broccoli landraces, still little known, an important natural source of dietary antioxidant.

Keywords: *Brassica oleracea* var. *italica*, antioxidant activity, total phenols, vitamin C, hydroxycinnamic acids, sulforaphane.

RESUMO

Caracterização antioxidante de diferentes variedades crioulas de brócolis italiano

As Brassicaceaes estão entre as hortaliças mais consumidas no mundo. Elas possuem uma grande biodiversidade, em que variedades locais e cultivares primitivas continuam a desempenhar papel importante nos sistemas de cultivo de muitos países. Brassicas e especialmente brócolis estão intimamente ligados a compostos antioxidantes que desempenham papel fundamental para a saúde humana. Este experimento teve como objetivo caracterizar algumas variedades desconhecidas crioulas de brócolis italianos do ponto de vista antioxidante. Cinco variedades crioulas nomeados BF, BF4, BFT, BB e BS foram avaliados. As propriedades antioxidantes totais de brócolis foram significativamente diferentes entre ecotipos e os valores mais elevados foram registrados em BS e BF4. BF4 apresentou os valores mais elevados de fenóis totais e, no que concerne ao teor de ácido ascórbico encontrado nos diferentes ecotipos, pode ser resumido em três grupos: o primeiro é representado pelo BB com valores mais elevados, o segundo por BF4 e BS e, em seguida, BF e BFT. BB representa a menor quantidade de ácidos hidroxicinâmicos, enquanto BF4 mostrou teor de sulforafano (+95%) bastante mais elevado do que outras cultivares. Os altos teores de ácidos fenólicos, vitamina C e compostos antioxidantes fazem essas variedades crioulas de brócolis, ainda pouco conhecidas, uma importante fonte natural de antioxidantes na dieta.

Palavras-chave: *Brassica oleracea* var. *italica*, atividade antioxidante, fenóis totais, vitamina C, ácidos hydroxycinnamicos, sulforaphano.

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Epidemiological studies indicated that the consumption of cruciferous vegetables is expected to be beneficial for human health (van Poppel *et al*., 1999; Voorrips *et al*., 2000) and this finding is related to the significant increase in consumption in many markets. Cruciferous vegetables, indeed, contain lots of antioxidant compounds, especially glucosinolates, which are not present in other vegetables.

The demand for broccoli is increasing worldwide, especially in developed countries, resulting from changes in eating habits. Globally, the latest FAO statistics highlight the increasing cultivation of broccoli and cauliflower with a total area of over $20x10^6$ ha and about $1.2x10^6$ tonnes produced. As part of these crops Italy appears to be the seventh Country for acreage preceded by China, India, Ecuador, Spain, Mexico, France, and 5th for production with more than 420,000 tonnes a year preceded by China, India, Spain and Mexico.

From a qualitative point of view, broccoli is a food often consumed for its potential health-promoting properties and there are many different varieties all over the world. The health benefits of broccoli are partly associated with secondary plant compounds that have bioactivity; glucosinolates and phenolic acids are two of the most abundant and important in broccoli (Zhang, 2004). Among glucosinolates, glucoraphanin (4-methylsulfinybutyl glucosinolate), is a glucosinolate found in broccoli that produces mostly sulforaphane (4-methylsulfinybutyl isothiocyanate) when it is hydrolyzed by myrosinase

(Liang *et al*., 2006; Vermeulen *et al*., 2008). The sulforaphane has attracted much recent interest since it was found to be the most potent naturally occurring inducer of phase-II detoxification enzyme (Kushad *et al*., 1999; Sarikamis *et al*., 2006; Zhang *et al*., 2006).

Broccoli can vary widely in terms of both its content and the composition of its glucosinolates and other chemical properties, depending on the variety and the growing environment (Vallejo *et al*., 2003). In Italy there exist a large number of broccoli varieties that are quite unknown from a qualitative point of view, especially concerning antioxidant properties. These less known cultivars are quite interesting to be studied in order to characterize them and find higher qualitative properties than conventional cultivars. This could be useful also for the consumer that could find new healthy vegetables products on the market. Therefore the purpose of the present work was to evaluate five Italian broccoli landraces describing their antioxidant and other qualitative properties.

MATERIAL AND METHODS

Plant material

Qualitative analyses of Italian broccoli landraces were carried out in 2012 at the Department of Agronomy, Food, Natural Resources, Environment and Animals at University of Padova. Broccoli materials came from the Institute of Genetics and Agricultural Research "N. Strampelli" located in Lonigo in the Province of Vicenza (45°23'0"N, 11°23'0"E). Here five broccoli (*Brassica oleracea* var. *italica*) landraces were grown: Broccolo Fiolaro (BF), Broccolo Fiolaro 4 (BF4), Broccolo Fiolaro tardivo (BFT), Broccolo di Bassano (BB) and Broccolo Spigariello (BS). The first three ecotypes (BF, BF4 and BFT), belonging to the same type, but different in terms of precocity, are characterized by the presence of inserted buds along the stem leaves that are harvested from November to February, following the first frost. In relation to BB, it is a plant of small size (0.40-0.60 m), whose edible part is a medium size inflorescence (100-150 mm large). Finally, BS is a genotype for which the young shoots with the inflorescence at the early stages of development is consumed.

A randomized block experimental design with three replications was used and each plot was 25 m^2 wide (5x5 m). Transplant was realized on 7th August adopting a plant spacing of 65 cm between rows and 50 cm in the row. During crop cycle two pesticide treatments were carried out: the first on August 27th with Actara (thiamethoxam 25%) at a dose of 20 g/hL and the second on September 23rd, with Trebon (etofenprox 20%) at a dose of 100 mL/hL mixed with 2 kg of Ossiclor 35 WG (copper oxychloride 35%) and 250 mL of Irol Plus (wetting adhesive). The harvest was conducted on December 17th and, within each thesis, nine plants were considered for qualitative analysis. To further characterize the different genotypes the average plant weight was also measured. BB showed values of 294 g followed by BF, BF4, BFT and BS with respectively 318, 370, 399 and 912 g.

For each landrace were collected nine plants at commercial maturity. Once collected they were immediately weighed, vacuum packed and subjected to sampling. For each repetition (nine plants) the edible part obtained was mixed to have a homogeneous sample. For each sample a portion was used to obtain the dry matter in a ventilated oven at 105°C and the left part was immediately frozen in a freezer at -80°C to be freeze-dried later and used for qualitative analysis.

Chemical analysis

The pH, electrical conductivity (EC), refraction index (Brix°) and titratable acidity (ISO750 method) were measured on sample juice obtained after filtering unfrozen sample fractions. The nitrogen content was measured by the Kjeldhal method (ISO1656).

About phenols extraction broccoli tissues (5 g) were homogenized in methanol (20 mL) with an Ultra Turrax T25 until uniform consistency at 13500 rpm. Samples were filtered (filter paper, 589 Schleicher) and appropriate aliquots of extracts were assayed by a FC assay for total phenols (TPs) content. For HPLC analysis, extracts were further filtered through cellulose acetate syringe filters (0.45 μm) after dilution.

The content of TPs was determined using the FC assay with gallic acid as calibration standard, by a Shimadzu UV-1800 spectrophotometer (Columbia, MD, USA). The FC assay was carried out by pipetting 200 μL of broccoli extract into a 10 mL PP tube. This was followed by addition of 1 mL of Folin-Ciocalteau's reagent. The mixture was vortexed for 20-30 s and 800 μL of filtered 20% sodium carbonate solution was added after 1 min and before 8 min of addition of the FC reagent. This was recorded as time zero; the mixture was then vortexed for 20-30 s after addition of sodium carbonate. After 2 h at room temperature, the absorbance of the colored reaction product was measured at 765 nm. The TPs content in the extracts was calculated from a standard calibration curve obtained with different concentrations of gallic acid, ranging from 0 to 600 μg/mL (correlation coefficient: R^2= 0.9996). Results were expressed on the basis of mg of Gallic Acid Equivalent per kg (mg GAE/kg) of fresh broccoli (Singleton *et al*., 1974).

The Ferric Reducing Antioxidant Power (FRAP) reagent was prepared fresh and it contained 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate at pH 3.6. A 100 μL aliquot of the methanol extract prepared as above was added to 1900 μL of FRAP reagent, and accurately mixed. After leaving the mixture at 20°C for 4 min, the absorbance at 593 nm was determined. Calibration was against a standard curve (0-1200 μg/mL ferrous ion) (correlation coefficient: R^2= 0.9987) obtained by the addition of freshly prepared ammonium ferrous sulfate. The total antioxidant activity (AOA) values were calculated as μg/mL ferrous ion (ferric reducing power) from three determinations and are presented as mg/kg of Fe^{2+} equivalent (Benzie & Strain, 1996).

For the extraction of ascorbic acid 5 g samples were homogenized until uniform consistency in a meta-phosphoric acid and acetic acid solution. Ascorbic acid was determined following the ISO 6557 method.

Free phenolic acids

Fresh sample (5 g) was treated in the same way as reported for phenols extraction. The chlorogenic, *p*-coumaric, caffeic, ferulic and sinapic acid were separated and quantified using a HPLC-DAD constituted of a Jasco X-LC system, consisting of a model PU-2080 pump, a multiwavelength detector (mod. MD-2015), an autosampler (mod. AS-2055) and a column oven (mod. CO-2060). ChromNAV Chromatography Data System software was used for result analyses. The separation of phenolic acids was achieved on a Tracer Extrasil OSD2 column (5 µm, 250×4.6 mm), operating at 35°C, at a flow rate of 1 mL/min. The mobile phase consisted of 0.1% acid formic (A) and methanol (B). Gradient elution was as follows: 0-100% B over 50 min and held at 100% B for an additional 10 min to clean the column. HPLC analysis at 325 nm was used to detect sample. Phenolic acids were quantified following a calibration method. Five standards ranging from 0.1 mg/L to 50 mg/L of chlorogenic, *p*-coumaric, caffeic, ferulic and sinapic acid were used.

Sulforaphane determination

Fresh sample (5 g) was treated in the same way as reported for TPs extraction. The sulforaphane was separated (Liang *et al*., 2006) and quantified using a Jasco X-LC system, consisting of a model PU-2080 pump, a multiwavelength detector (mod. MD-2015), an autosampler (mod. AS-2055) and a column oven (mod. CO-2060). ChromNAV Chromatography Data System software was used for result analyses. The separation of sulforaphane was achieved by a Tracer Extrasil OSD2 column (5 µm, 250×4.6 mm), operating at 30°C, at a flow rate of 1 mL/min. The mobile phase consisted of water (A) and methanol (B). Gradient elution was as follows: 20-60% B over 15 min and held 60-100% B for 2 min. HPLC analysis at 254 nm was used to detect sample. Sulforaphane was quantified following a calibration method ranging from 5 mg/L to 500 mg/L.

Statistical analysis

Statistical analysis was performed comparing five broccoli landraces with three field replications (blocks). Data were analyzed using ANOVA. In the case of a significant F-value, the means were compared by Tukey's HSD test at the significance level of $p \leq 0.01$.

RESULTS AND DISCUSSION

In relation to the qualitative aspects, interesting differences emerged among the different genotypes of broccoli examined. Concerning the dry matter content (Table 1), BFT and BF4 showed the highest amount followed by BF with 13.4%. No differences between BB and BS were observed with values close to 12.5%. About the ash content values were quite heterogeneous, BS provided the highest results with more than 30% ash followed by BB and, finally, the type fiolaro, where all three landraces showed values significantly lower. This result is mainly due to the different tissues used as marketable production: BB and BS are represented by an inflorescence, whereas all fiolaro types are young sprouts. Looking at the pH values, the responses were quite stable, with slight differences that were significant only between BF4 and Broccolo Spigariello. The soluble solids content (TSS) was different among ecotypes presenting the highest concentrations in BFT and BF4 followed by BF and BB. BS, however, showed lower values with less than 7.5°Brix. No difference was observed in connection with titratable acidity where all values were close to 0.4% of citric acid. Finally, as regards the qualitative aspect linked to the nitrogen content, the concentration of crude protein was significantly different among the ecotypes and the highest values were found in BS. BFT and BF showed intermediate levels, whereas BF4 and BB, with 27.5% and 26.4%, respectively, expressed lower concentrations.

About the antioxidant properties of broccoli, the total antioxidant activity (Figure 1A) was significantly different among ecotypes. The higher AOA was recorded in BS, BF4 and BB with values ranging between 50000 and 70000 mg Fe^{2+} E/kg dry weight (dw). Intermediate concentration was observed for BF, while BFT showed the lower values less than 40000 mg Fe^{2+} E/kg dw.

These results statistically significant are first of all linked with the different organs of the plant that are consumed for each landrace. The antioxidant content varies considerably in relation to different parts of the plant (Nilsson *et al*., 2006). The total antioxidant capacity detected for different landraces appears to be high and in line with other tests performed on other *Brassica* species (Aires *et al*., 2011). Ou *et al*. (2002) found that the total antioxidant activity of broccoli turns out to be among the

Table 1. Dry matter, ash, pH, total soluble solids, titratable acidity and nitrogen contents in different Italian broccoli landraces (teores de matéria seca, cinzas, pH, sólidos solúveis totais, acidez titulável e nitrogênio em diferentes variedades crioulas de brócolis italianos). Padova, UNIPD, 2012.

Genotypes	Dry matter (%)	Ash (% dw)	pH	TSS (°Brix)	Titratable acidity (% citric acid)	Kjeldhal N	Crude protein
						(%)	
Bassano (BB)	12.7 b	28.8 b	5.73 ab	8.2 ab	0.42 a	4.22 b	26.4 b
Fiolaro (BF)	13.4 b	13.0 c	5.67 ab	8.6 ab	0.40 a	4.48 ab	28.0 ab
Fiolaro 4 (BF4)	14.7 a	11.1 d	5.83 a	9.0 a	0.41 a	4.40 b	27.5 b
Fiolaro Tardivo (BFT)	15.7 a	11.6 d	5.73 ab	9.3 a	0.43 a	4.44 ab	27.8 ab
Spigariello (BS)	12.5 b	30.8 a	5.47 b	6.9 b	0.40 a	4.81 a	30.1 a

Column values with no letter in common significantly differ at $p \leq 0.01$ (Tukey HSD test) {valores da coluna com letras iguais não diferem significativamente, $p \leq 0.01$ (teste de Tukey HSD)}.

Table 2. Sum of main hydroxycinnamic acids and their amount (%) in different Italian broccoli landraces (soma dos principais ácidos hidroxicinâmicos e seu valor (%) em diferentes variedades crioulas de brócolis italianos). Padova, UNIPD, 2012.

Genotypes	Sum of main hydroxycinnamic acids (mg/kg dw)	Chlorogenic acid	*p*-coumaric acid	Caffeic acid	Ferulic acid	Sinapic acid
		(%)				
Bassano (BB)	393 b	20.0 ab	7.6 b	20.7 a	23.3 a	28.4 a
Fiolaro (BF)	2921 a	28.1 a	27.8 ab	10.1 b	5.1 b	29.0 a
Fiolaro 4 (BF4)	3197 a	9.4 b	42.6 a	9.7 b	27.3 a	11.0 b
Fiolaro Tardivo (BFT)	2741 a	22.2 a	45.5 a	8.5 b	13.5 ab	10.3 b
Spigariello (BS)	3404 a	16.6 ab	49.3 a	8.4 b	17.8 ab	7.9 b

Column values with no letter in common significantly differ at p≤0.01 (Tukey HSD test) {valores da coluna com letras desiguais diferem significativamente, p≤0.01 (teste de Tukey HSD)}.

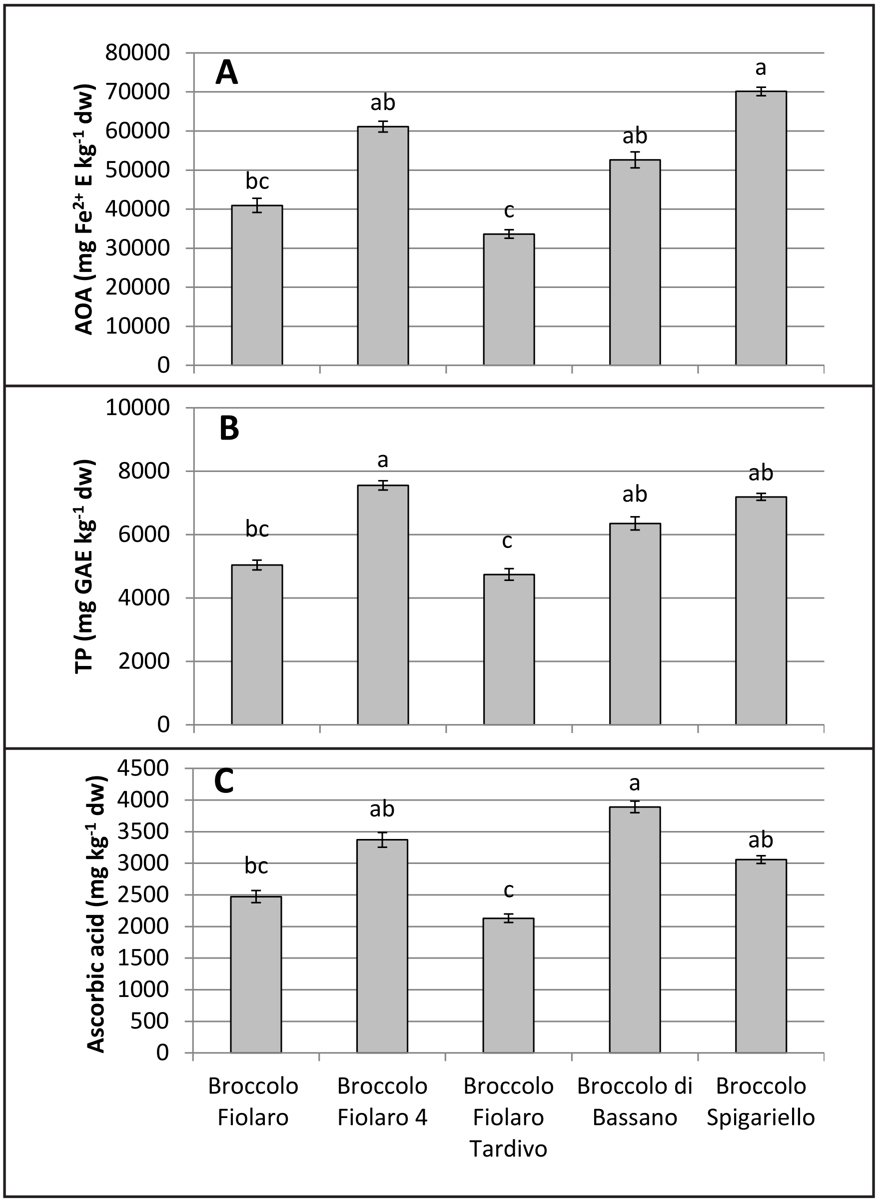

Figure 1. Antioxidant activity (A), total phenols (B) and ascorbic acid (C) contents in different Italian broccoli landraces {atividade antioxidante (A), fenóis totais (B) e ácido ascórbico (C) contidos em diferentes variedades crioulas de brócolis italiano}. Values with no letter in common significantly differ at p≤0.01 (Tukey HSD test) {valores com letras iguais não diferem significativamente, p≤0.01 (teste de Tukey HSD)}. Padova, UNIPD, 2012.

highest compared to other vegetables. This characteristic is of great interest for the consumer, since high antioxidant capacity is linked to the positive control of cancer, cardiovascular and aging-related diseases (Kushad *et al*., 1999; Zhang & Hamauzu, 2004; Chuanphongpanich *et al*., 2006; Kaur *et al*., 2007).

In relation to total phenol content (Figure 1B) results were quite similar to those reported for AOA except for BF4 with the next highest concentration of 7500 mg/kg dw GAE. Comparing the data of this trial with those reported in the literature (Dixon, 2007; Kaur *et al*., 2007), considered landraces have concentrations significantly higher. Anyway, if we refer to other cultivars evaluated in different seasons (Aires *et al*., 2011), TP values are lower than what recorded in this experiment. About BF4, which has provided the higher TP values, differed by more than 60% compared to other cultivars with the greatest concentration of TP (Kaur *et al*., 2007).

Among vegetables, broccoli is an excellent source of vitamin C. Concerning the ascorbic acid content found in the different ecotypes (Figure 1C), results can be summarized in three groups: the first one is represented by BB with almost 4000 mg/kg dw, the second one by BF4 and BS with values between 3000 and 3500 mg/kg dw and then BF and BFT that, with values lower than 2500 mg/kg dw, provided the lower concentrations. These results are just a little lower than what reported in literature for other cultivars (Zhang & Hamauzu, 2004; Kaur *et al*., 2007;).

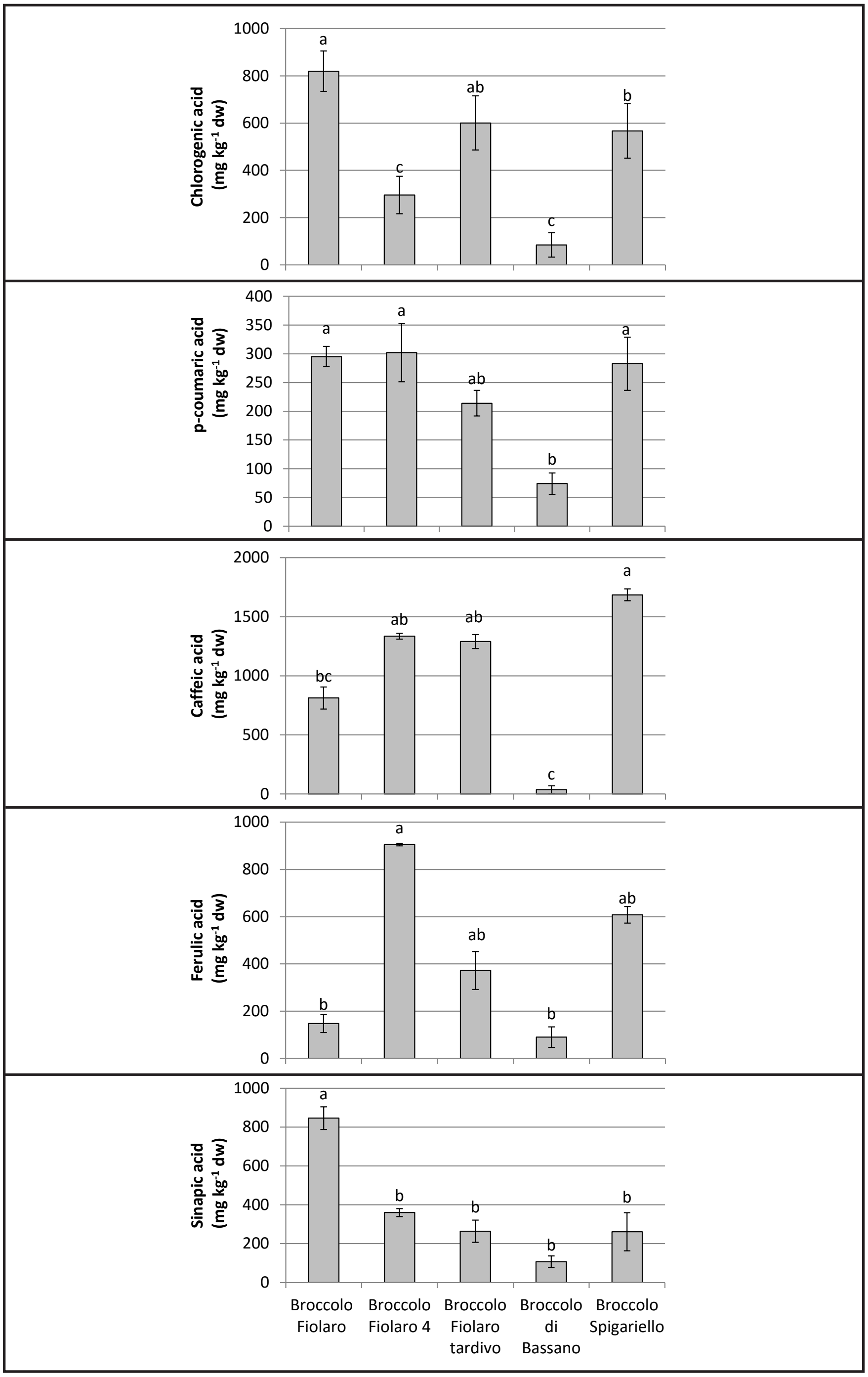

Figure 2. Free phenolic acids contents in different Italian broccoli landraces (teores de ácidos fenólicos livres em diferentes variedades crioulas de brócolis italianos). Values with no letter in common significantly differ at p≤0.01 (Tukey HSD test) {valores com letras iguais não diferem significativamente, p≤0.01 (teste de Tukey HSD)}. Padova, UNIPD, 2012.

This variability, in addition to be linked to genetic aspects, is also connected to different climatic conditions in which the plants were grown. In fact, this factor can greatly affect the physiology of the plant (Kaur *et al*., 2007; Aires *et al*., 2011).

Data obtained in this experiment are only partly confirmed at bibliographic level as the considered landraces were evaluated for the first time. Furthermore the content of hydroxycinnamic acids appears to be rather heterogeneous among different species of *Brassicaceae* (Nilsson *et al*., 2006) and also in relation to the plant part (Dixon, 2007). In order to obtain an overall view of the hydroxycinnamic acids content, Table 2 shows the sum of detected acids. Appears clearly the low content of phenolic compounds in BB contrary to what occurred for the other landraces. Considering the percentages of the various hydroxycinnamic acids for each landrace clearly points out the different percentage amount of each compound. Chlorogenic acid is dominant in BF and BFT with values higher than 20%. The *p*-coumaric acid, however is high in BF4, BFT and BS, whereas is quite low (7.6%) in BB. The latter landrace instead presents the higher percentage of caffeic acid, ferulic and sinapic together with BF4 and BF respectively. *Brassica* crops, such as broccoli, have special interest because of their sulforaphane content which is associated with the reduction of active oxygen in tissues and may provide protection from cancer and coronary diseases (Liang *et al*., 2006; Sarikamis *et al*., 2006; Dixon, 2007). In this respect the concentration of sulforaphane (Figure 3), compound belonging to the isothiocyanates group, was rather stable in BB, BF, BFT and BS with values lower than 200 mg/kg dw; BF4, however, showed values significantly higher (+92%) than the other ecotypes. The sulforaphane content is in agreement for most of the considered landraces with other 18 varieties of broccoli (Liang *et al*., 2006). However, it is interesting to note that BF4 gave values significantly higher (+95% on average) which enhance health properties for this specific genotype.

As regards the determination of the specific component with antioxidant action, in Figure 2 is reported the concentration of the main free hydroxycinnamic acids present in the different ecotypes of broccoli analyzed. About the chlorogenic acid content, there is a high heterogeneity of the responses recorded for the different genotypes. BF and BFT showed the highest values respectively of 819 and 600 mg/kg dw. BS presented intermediate values and, finally, there are BF4 and BB with values lower than 300 mg/kg dw. The *p*-coumaric acid concentration has instead presented values enough homogeneous for three ecotypes (BF, BF4 and BS), whereas BFT and BB

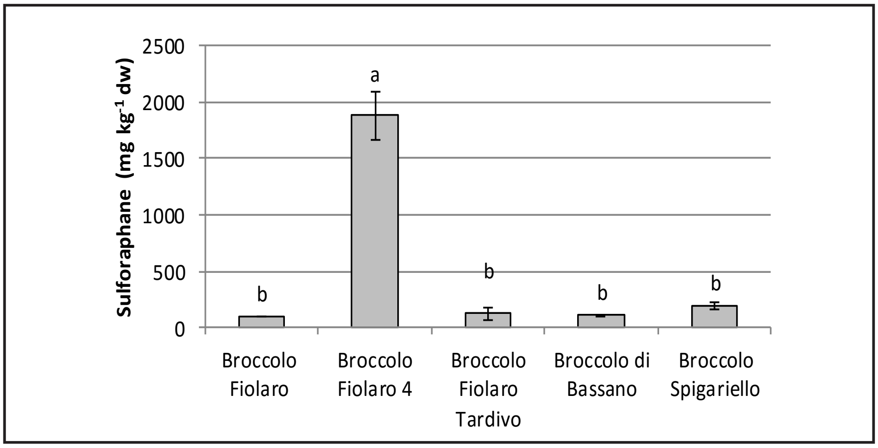

Figure 3. Sulforaphane content in different Italian broccoli landraces (conteúdo de sulforafano em diferentes variedades crioulas de brócolis italianos). Values with no letter in common significantly differ at $p \leq 0.01$ (Tukey HSD test) {valores com letras iguais não diferem significativamente, $p \leq 0.01$ (teste de Tukey HSD)}. Padova, UNIPD, 2012.

showed low concentrations. The higher content of caffeic acid was recorded in BS (1685 mg/kg dw), 97% higher than BB. BF, BF4 and BFT showed intermediate values between 800 and 1400 mg/kg dw. BF4 was characterized by the high content of ferulic acid, higher than 83% and 90% respectively for BF and BB; BFT and BS presented intermediate positions and did not statistically differ between them.

Finally, as regards the sinapic acid content, ecotypes results can be pool into two groups: BF4, BFT, BB and BS with values between 100 and 400 mg/kg dw and BF showing the highest concentration.

Different broccoli landraces considered for the first time in this test have revealed the interesting antioxidant properties that distinguish them and, consequently, represent an important natural resource of antioxidants for the consumer. The high content of phenolic acids, vitamin C and antioxidant compounds also make these ecotypes, still little known, an important resource for the genetic improvement, especially regarding the ecotype BF4 that showed high sulforaphane content. Finally, the set of organoleptic and antioxidants properties found in this experience are a key tool for the promotion of these vegetables at both producers and commercially level especially involving the final consumer obtaining numerous benefits from a qualitative and healthy point of view.

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