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Morphometric Characteristics, Polyphenols and Ascorbic Acid Variation in *Brassica oleracea* L. Novel Foods: Sprouts, Microgreens and Baby Leaves

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Abstract: In the present study, we investigated the content and profile of polyphenols (PPH), ascorbic acid (AA), the Folin–Ciocalteu index (FCI), and antioxidant activity (1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxyl radical (ROO)) variation during three different plant growth stages (sprouts, microgreens and baby leaves) of two broccoli types, the traditional Sicilian sprouting broccoli landrace ('Broccolo Nero') and the broccoli standard ('Cavolo broccolo Ramoso Calabrese'), and the standard commercial cultivar of kale ('Cavolo Lacinato Nero di Toscana'). All biomasses collected were freeze-dried for PPH, AA, FCI, DPPH and ROO analysis. The highest polyphenol content was observed for 'Broccolo Nero' (BN) and 'Cavolo Broccolo Ramoso Calabrese' (CR), and generally sprouts showed significantly higher values compared to the microgreens and the baby leaves. The AA, FCI, DDPH and ROO significantly vary with regards to the cultivar and the plant growth stage, showing interaction between the two experimental factors analyzed. The interaction detected showed higher values for the antioxidant traits of the proposed novel food, especially for the two broccoli cultivars in the sprout growth stage in comparison to the microgreens and baby leaves. Our results suggest that the antioxidant activity is partially dependent on kaempferol and apigenin. The PPH compounds showed the highest values of kaempferol and apigenin for 'Broccolo nero', whereas for the other two cultivars studied, only kaempferol was the main compound represented. The data acquired are of interest for increasing the healthy traits of the novel food proposed showing the contribution offered by the neglected LRs until now underutilized and at risk of extinction. The germplasm conserved in several world genebanks could support and diversify the organic vegetable items, providing us with added-value products for organic food supply chains.

Keywords: antioxidants; functional foods; plant growth stage; broccoli; kale; landraces

1. Introduction

Brassica oleracea vegetables are a good source of many phytochemicals with health-related activity, and their dietary consumption is associated with the reduction in the incidence of several human chronic inflammatory diseases [1–4]. Both the profile and the amount of these phytochemicals are strongly affected by the genotype (different species/cultivar/landraces), by the environmental conditions

during plant growth and by the different plant growth stages. Some authors have detected great variation of several antioxidant compounds, as such as glucosinolates, carotenoids, polyphenols and ascorbic acid, and of the related bioactivity of several landraces (LRs) and crop wild relatives (CWRs) of *B. oleracea*, evidencing elite breeding materials useful for improving the nutraceutical traits of the products [5,6].

Among the detected phytochemicals, polyphenols are a large class of organic compounds including several aromatic rings associated with different phenolic groups. Phenolics range from simple and single aromatic-ringed compounds, with a low molecular-weight, to large and complex tannins and derived polyphenols [7,8]. They can be classified based on the number and arrangement of their carbon atoms in flavonoids (flavanols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) and they are commonly found in plants as conjugated to sugars and organic acids [7].

Polyphenols are often produced in response to biotic or abiotic stress [9] and act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers. The multifunctionality of polyphenols is due to their distribution in different tissues and organs of plants at different concentrations. The most widespread and different groups of polyphenols in *Brassica* species are the flavonoids (mainly flavonols but also anthocyanins) and the hydroxycinnamic acids [10,11]. Phenolic compounds play a role in different stages of cancer development, exerting their activity in regulating different signaling pathways involved in cell survival, growth and differentiation of the reproductive organs.

Vitamins are indispensable micronutrients for humans with antioxidant activity. Studies in vitro demonstrated vitamin (E and C) consumption decreases the risks of chronic diseases by acting as direct antioxidants and electron donors [12,13].

Today, new natural products containing high levels of bioactive compounds consumed through food are being researched and studied. Sprouts, microgreens and baby leaves represent a growing market segment within vegetable products, mainly consumed raw for their high nutritional value and sensory traits [14]. Candidate genotypes are expanding based on sensory and health criteria; however, currently, most of the species belong to Brassicaceae, Asteraceae, Chenopodiaceae, Lamiaceae, Apiaceae, Amarillydaceae, Amaranthceae, and Cucurbitaceae families [15].

In the last few years, the request of seeds and sprouts of broccoli and of other Brassicaceae species has become increasingly popular among consumers interested in improving and maintaining their health status by changing dietary habits [16].

The production and commercialization of sprouts are legally defined, and generally they are grown in dark conditions, without growing medium, fertilizers and agrochemicals [14]. Studies carried out on broccoli sprouts exposed to low intensity and high intensity of ultraviolet A (UVA) or ultraviolet B (UVB) demonstrated an enhancement of bioactive compounds, suggesting their exploitation as a functional food with potential industrial applications [17].

A new class of specialty salad crops exploited for their color- and flavor-enhancing phytonutrient content are the microgreens [18,19]. They differ from sprouts for the fully developed cotyledons and the appearance of the first true leaf; they are defined as young and tender vegetables, with a species-dependent production cycle of 1–3 weeks from seed germination, and they are harvested at soil level [20,21]. Compared to their mature-leaf counterparts, microgreens contain higher amounts of important phytonutrients (ascorbic acid, β-carotene, phylloquinone, etc.), minerals and lower nitrate levels [20,22]. Microgreens have been proposed as 'super foods' for their favorable content in micronutrients and bioactive compounds, whose yield can be increased by modulating the blue light proportion in red and blue light-emitting diode lighting [23–25]. Due to their phytonutrient compounds, a recent study supported the idea that microgreens could be considered as a resilient phytochemical factory for the dietary and psychological needs of crew members in orbital flights and platforms [26]. They are thought to be a new food for solving malnutrition problems affecting over two-thirds of the world's people living in countries of every economic status.

Among the ready-to-eat products, the baby leaf vegetable market has been growing and offering to consumers convenient, healthy and appealing items, which may contain useful bioactive compounds. The consumer demand for more convenient fresh food products led to a rapid growth in the fresh-cut industry, which became a multi-billion-dollar sector worldwide in the last decade [27,28]. Fresh-cut vegetables can meet the consumer demands for their relationship among food, healthy lifestyle and convenience [29].

The production of sprouts, microgreens and baby leaves from local varieties and wild edible species may provide novel and nutraceutical vegetables, which can satisfy the demand of modern consumers. Moreover, they represent further expressions of biodiversity in vegetable production, contributing to preserve and to increase the value of many vegetable LRs at risk of genetic erosion or extinction. The phytochemical composition of Brassicaceae varies considerably as a consequence of the plant growth stage and of the species considered [30]. For these reasons, we investigated the content of bioactive compounds at three stages of plant growth (sprouts, microgreens and baby leaves) of a traditional Sicilian broccoli LR and of two commercial standard cultivars, one of broccoli and one of kale.

2. Materials and Methods

2.1. Plants Materials, Seed Morphological Characteristics and Germination Test

Seeds of the standard commercial cultivars of broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Cavolo Broccolo Ramoso Calabrese' (CR) and of kale (*Brassica oleracea* L. var. *acephala*) 'Cavolo Lacinato Nero di Toscana' (CL) were purchased by the S.A.I.S. S.p.A. seed company (Cesena, Italy). The Sicilian landrace of sprouting broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Broccolo Nero' (BN) belongs to the Di3A active genebank collection (BR 354, UNICT 4939).

The weight of 1000 seeds, number of seeds per gram, perimeter, longitudinal and transversal lengths were registered. The germination test was carried out at the laboratory of the Department of Agriculture, Food and Environment (Di3A) of Catania University (Italy). Four replicates of 50 seeds for each cultivar (cv) were placed in 90 mm Petri dishes, between two layers of filter paper (Whatman® no. 2), imbibed with 10 mL of distilled water. Petri dishes were placed in dark condition in five incubators (FTC 90E, Refrigerated Incubator, VELP Scientifica, Italy) set at constant temperatures of 5, 10, 15, 20 and 25 °C. At the end of the experiment, the following indices were calculated: germinability (%) = ((N/NT) × 100), where N is the number of germinated seeds and NT is the number of the total seeds utilized; Mean Germination Time (MGT) = $\sum$(ni × di)/n, where n indicates the number of germinated seeds at day i, d is the incubation period in days, and n is the number of the total seeds germinated.

2.2. Sprouts, Microgreens and Baby Leaves Production and Characterization

For the sprouts, microgreens and baby leaf production, seeds were sown in cellular trays placed in cold greenhouse under natural light (4.6 to 9.2 $MJ \cdot m^{-2} \cdot d^{-1}$) and temperature (15.4 ± 5.8 °C) conditions, from November to December 2017 at Catania (South Italy, 37°31'10" N 15°04'18" E; 105 m above sea level (m a.s.l.) using organic growing practices. We utilized the organic substrate Brill®semina bio (Geotec, Italy) to fill the cellular trays and we treated the baby leaves once by BTK ® 32 WG (Xeda, Italy) based on *Bacillus thuringiensis* sub. *kurstaki* for controlling *Pieris brassicae*. The plantlets were irrigated on the basis of the ordinary techniques. Plants were collected at the three plant growth stages analyzed: sprouts (germinated seeds without coats and roots), microgreens (plantlets with the first true leaf) and baby leaves (plantlets with almost three true leaves), weighted and stored immediately at −80 °C, and then freeze-dried for analyzing them.

Plants were characterized for the main morphological descriptors. Longitudinal and transversal lengths of the cotyledons, number of true leaves, if present, and the hypocotyl length were measured. The morpho-biometric parameters of sprouts, microgreens and baby leaves were acquired using the software IM50 v. 117 (Leica, Germany).

2.3. Polyphenol Analysis

The polyphenol compounds were analyzed in accordance with Soengas et al.'s method [31] with some modifications. For the extraction, 60 mg of lyophilized powder was dissolved in 1.5 mL of a 1:1 v/v mixture of methanol and 0.06 N HCl. The mixture was vortexed and put in an ultrasonic bath for 10 min. After the thermal hydrolysis which was performed by putting samples in hot bath (80 °C for 1 h), the mixture was centrifuged (13,000 rpm, 10 min, 4 °C). The supernatant was stored at −20 °C and analyzed by HPLC Agilent 1200 series system equipped with a diode array detector (DAD). We utilized the analytical column Lichrospher 100RP-18 (240 × 4 mm i.d., particle size = 5 µm). The mobile phase contained water/acetic acid (90:10, v/v, A) and acetonitrile/acetic acid (90:10, v/v, B). Chromatography was performed with 0.6 mL/min flow rate and the following gradient program: 0–7 min 1% B, 7–20 min 30% B, 20–28 min 50% B, 28–33 min 50% B, 33–38 min 1% B, 38–48 min 1% B. The injection volume of sample was 30 µL. Polyphenols were detected by DAD monitoring the absorbance at 280, 310, 325, 350, 520 nm. Hence, the characterization of each phenol compound was based on its characteristic absorption spectra. Each sample was analyzed in triplicate. Quantification was based on the calibration curves of external standards, by comparing each compound through the absorption spectra for apigenin, caffeic acid, chlorogenic acid, cyanidin chloride, gallic acid, coumaric acid, kaempferol and sinapic acid; polyphenols concentrations were expressed in mg g^{-1} d.w.

2.4. Ascorbic Acid Analysis

For the ascorbic acid (AA) measurements, the titration method reported by Thillmans et al. [32] was used. Freeze-dried samples were treated by 3% metaphosphoric acid by shaking. Extracts after filtration on filter paper (size: 110 mm, medium filtration rate, particle retention: 5–13 µm) were titrated by 1.5 mM 2,6-dichlorophenol-indophenol (DCIP) water solution at room temperature. The ascorbic acid contents were quantified by comparing them with the standard curve obtained for the known ascorbic acid concentrations. Each sample was analyzed in triplicate, and the results were expressed as mg g^{-1} d.w.

2.5. Folin–Ciocalteu Index

The Folin–Ciocalteu index (FCI) was calculated on methanolic extracts as described by Meda et al. [33], with slight modifications. Twenty milligrams of freeze-dried material were dissolved in 0.5 mL of 80% ethanol (EtOH). The mixture was vortexed and put in ultrasonic bath for 10 min and then centrifuged (13,000 rpm, 10 min, 4 °C). The supernatant was collected and the remaining pellet was re-extracted as described above. The collected supernatant was pooled. Twenty microliters of the extract were diluted with 1580 µL of distilled water and 100 µL of Folin-Ciocalteu reagent and then we left it at room temperature for 5 min. The solution was then treated with sodium carbonate (20%, 300 µL) and incubated in the dark for 2 h at room temperature. The absorbance of the blue-colored solution was measured at 750 nm. Each sample was analyzed in triplicate. The total polyphenol content was expressed as gallic acid equivalents (GAE mg g^{-1} d.w.) after using a standard calibration curve obtained by the known concentrations of the gallic acid standard.

2.6. Antioxidant Activity

The antioxidant capacity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quenching and peroxyl radical (ROO) scavenging methods. For the sample preparations, 100 mg of lyophilized powder was extracted by 1.5 mL of a 1:1 v/v mixture of ethanol (EtOH) and 0.06 N HCl, homogenized and centrifuged at 25,000 g for 5 min at 4 °C. The DPPH was measured using electronic paramagnetic resonance (EPR) with a MiniScope MS200 Magnettech (Berlin, Germany) following the protocol detailed in Picchi et al. [34]. The antioxidant activity towards ROO was determined spectrophotometrically by measuring the enzymatic peroxidation of linoleic acid after the addition of lipoxygenase, as described in Lo Scalzo et al. [35].

2.7. Statistical Analysis

The experiments were performed using a completely randomized design. The significance of differences between the main factors of morphometric characteristics was evaluated by one-way analysis of variance (ANOVA). Data were reported as mean ± standard error (S.E.). To evaluate the effect of the genotype and of the stage of growth, the results of the Folin–Ciocalteu index, ascorbic acid and antioxidant activity were analyzed through a multifactorial ANOVA. The mean values associated with the main factors as well as their interactions were evaluated using Tukey's test ($p < 0.05$). All statistical analyses were performed using CoStat release 6.311 (CoHort Software, Monterey, USA).

3. Results

3.1. Germination Process, Seed and Plant Characteristics

The seed characteristics of BN, CR and CL are shown in Table 1. The weight of 1000 seeds was higher for BN (4.5 g) and lower for CR (3.9 g); the number of seeds per gram showed the statistically lower value for BN (223.6) in comparison to CL (242.1) and CR (254.4). No significant differences for the other morphometric parameters were registered (Table 1).

Table 1. Seed morpho-biometric characteristics of 'Broccolo Nero' (BN), 'Cavolo Laciniato Nero di Toscana' (CL) and 'Cavolo Broccolo Ramoso Calabrese' (CR).

Characteristics	BN	CL	CR	*p* Value
Weight of 1000 seeds (g)	4.5 ± 0.1a	4.1 ± 0.0b	3.9 ± 0.0c	***
Seeds per gram (n)	223.6 ± 2.5c	242.1 ± 2.7b	254.4 ± 2.4a	***
Perimeter (mm)	6.0 ± 0.1	6.1 ± 0.1	5.8 ± 0.1	ns
Longitudinal length (mm)	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.1	ns
Transversal length (mm)	2.0 ± 0.0	2.1 ± 0.1	2.0 ± 0.1	ns

Data are reported as mean ± S.E. Means were compared using Tukey test (*** $p < 0.001$; ns: $p > 0.05$). Values in the same row followed by the same letter are not significantly different at $p < 0.05$.

For all genotypes no germination occurred at 5 °C. In this sense, the genotypes seem to be more sensitive to low temperatures than high ones, during germination; in fact, all genotypes showed the highest germinability (100%) at highest temperature (25 °C) (Figure 1).

MGT was significantly affected by temperature for all the genotypes studied (Table 2); the number of days for germination decreased by increasing the temperature (from 4.5 days at 10 °C to 2.1 days at 25 °C). Among the genotypes studied, CL was the fastest to germinate at 25 °C (2.1 days) while the slowest was CR (2.6 days), showing a significant interaction between the two experimental factors analyzed.

With regards to the morphometric characteristics of the sprouts, significant differences were observed among the cultivars (cvs) studied (Table 3). The sprouts were collected seven days after the sowing when all of them showed the cotyledons well disclosed without any seed coat. The weight of ten sprouts varied from 1.0 (BN) to 1.4 g (CR), showing a very sensible increment from the weight of the seed. The weight of the sprouts did not show any significant difference among the cvs, despite the size of the cotyledons and of the hypocotyl varying among the genotypes (Table 3).

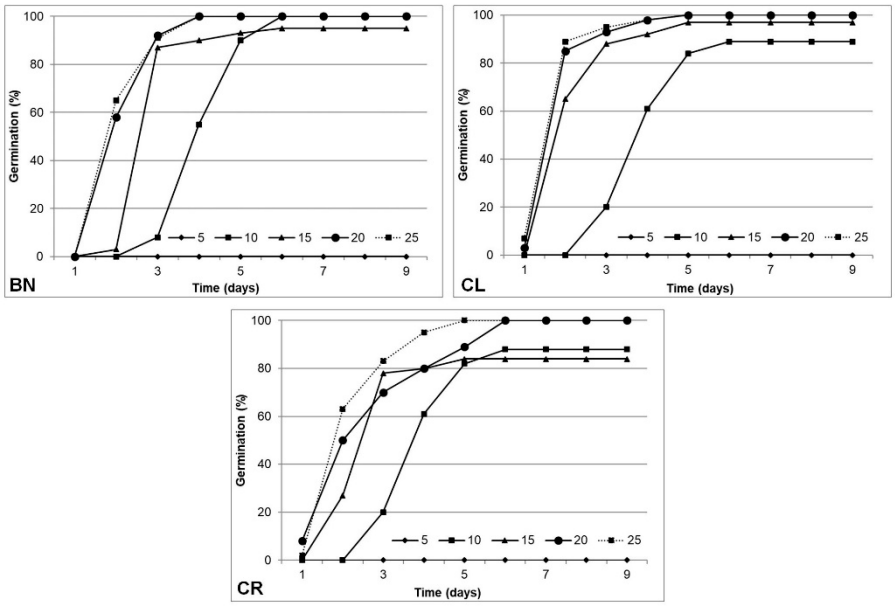

Figure 1. Progress of germination percentage (%) of BN, CL and CR seeds in relation to temperature (5, 10, 15, 20 and 25 °C). Each point represents the mean of four replications of 50 seeds.

Table 2. Effect of growing temperature on the mean germination time (MGT) of studied genotypes.

MGT (days)	10 °C	15 °C	20 °C	25 °C
BN	4.5 ± 0.04a	3.1 ± 0.04a	2.5 ± 0.04b	2.4 ± 0.05b
CL	4.0 ± 0.06b	2.5 ± 0.04c	2.2 ± 0.11c	2.1 ± 0.08c
CR	4.1 ± 0.04b	2.8 ± 0.05b	3.0 ± 0.24a	2.6 ± 0.03a

Data are reported as mean ± S.E. Means were compared using Tukey test. Values in the same column followed by the same letter are not significantly different at $p < 0.05$.

The microgreens were harvested 19 days after the sowing when the first true leaf of all the individuals reached the minimum size of 20 mm. The weight of ten microgreens varied from 2.7 (CL) to 3.4 g (BN), increasing by about three-fold compared to the sprouts (Table 3). The weight of the microgreens did not show any significant difference among the cvs (Table 3), despite the differences in hypocotyl and cotyledon length (Table 3). The first true leaf showed a similar size among the cvs (Table 3).

The baby leaves were harvested 39 days after the sowing when the plants showed about 3–4 true leaves. The weight of ten baby leaves varied from 7.0 to 8.4 g, respectively, for CL and BN, and the length of the stem ranged from 107.8 to 125.15 mm for BN and CL, respectively (Table 3).

The weight of baby leaves increased more than two-fold in comparison to the microgreens (Table 3). The number of the true leaves varied from 3.0 to 3.6 and their length and width ranged significantly from 113.5 to 124.3 mm and from 32.6 and 42.5 mm, respectively, for CL and BN (Table 3).

The stem, the cotyledons and the leaf midribs and veins were well distinguishable from both CL and CR ones for their reddish color dues to the high content of anthocyanins (Figure S1).

Four grams of seeds, on the average of the three genotypes studied, produced 120 g of sprouts, 300 g of microgreens and 760 g of baby leaves after 7, 19 and 39 days from sowing, respectively. The great increment of the weight of these products in so short a time offers an idea of the high added value of these novel foods that could be of great interest for seed companies.

Table 3. Morpho-biometric characteristics of the sprouts, microgreens and baby leaves of the three cultivars studied. Data are reported as mean ± S.E. (n = 10).

Characteristics	Sprouts				Microgreens				Baby leaves			
	BN	**CL**	**CR**	**Means**	**BN**	**CL**	**CR**	**Means**	**BN**	**CL**	**CR**	**Means**
Weight of 10 individuals (g)	1.0 ± 0.1a	1.2 ± 0.2a	1.4 ± 0.3a	**1.2 ± 0.0**	3.4 ± 0.5a	2.7 ± 0.4a	2.8 ± 0.3a	**3.0 ± 0.1**	8.4 ± 0.7a	7.0 ± 0.8a	7.4 ± 0.5a	**7.6 ± 0.5**
Hypocotyl length (mm)	42.1 ± 0.8a	29.2 ± 3.6b	36.6 ± 2.3ab	**36.0 ± 1.7**	43.3 ± 0.6a	29.5 ± 2.6b	28.9 ± 3.2b	**33.9 ± 1.5**	-	-	-	**-**
Cotyledon length (mm)	10.6 ± 0.2ab	10.3 ± 0.8b	14.2 ± 1.2a	**11.7 ± 0.8**	32.4 ± 0.4a	23.5 ± 0.2b	25.7 ±1.6b	**27.2 ± 0.6**	-	-	-	**-**
Cotyledon width (mm)	14.5 ± 0.2b	12.2 ± 0.8b	16.4 ± 1.1a	**14.4 ± 0.8**	23.1 ±0.4a	17.6 ±1.0a	17.8 ±2.2a	**19.5± 1.2**	-	-	-	**-**
Stem length (mm)	-	-	-	**-**	-	-	-	**-**	107.8 ± 3.9a	125.5 ± 6.9a	122.4 ± 5.3a	**118.6 ± 3.9**
Number of true leaf (n)	-	-	-	**-**	1.0 ± 0.0a	1.0 ± 0.0a	1.0 ± 0.0a	**1.0 ± 0**	3.5 ± 0.2a	3.6 ± 0.3a	3.0 ± 0.0a	**3.4 ± 0.1**
Leaf length (mm)	-	-	-	**-**	25.6 ± 2.4a	23.7 ± 1.7a	27.2 ± 2.7a	**25.2 ± 2.0**	124.3 ± 2.1a	113.5 ± 1.8b	120.7 ± 1.5ab	**119.5 ± 0.6**
Leaf width (mm)	-	-	-	**-**	16.4 ± 1.5a	17.6 ± 1.0a	19.0 ± 2.0a	**17.7 ± 0.9**	42.5 ± 1.4a	32.6 ± 0.9b	35.3 ± 0.9b	**36.8 ± 0.7**
Weight of 10 individuals (g)	42.1 ± 0.8a	29.2 ± 3.6b	36.6 ± 2.3ab	**36.0 ± 1.7**	43.3 ± 0.6a	29.5 ± 2.6b	28.9 ± 3.2b	**33.9 ± 1.5**	-	-	-	**-**
Hypocotyl length (mm)	10.6 ± 0.2ab	10.3 ± 0.8b	14.2 ± 1.2a	**11.7 ± 0.8**	32.4 ±0.4a	23.5 ± 0.2b	25.7 ±1.6b	**27.2 ± 0.6**	-	-	-	**-**

Means between genotypes (BN, CL and CR) for each growth stages were compared using Tukey test ($p < 0.05$). The statistical analysis was performed via one-way ANOVA. Values in the same row followed by the same letter are not significantly different at $p < 0.05$.

The data show the variation of the size of the plant during the first growing stages, which depends on the average of the genotypes utilized, mainly for the cotyledon length and width in the sprouts, on the enlarged cotyledons and on the first true leaf size for the microgreens, and on the number and size of the true leaves for the baby leaves (Table 3).

3.2. Multifactorial ANOVA

The multifactorial ANOVA showed that the Folin–Ciocalteu index (FCI), ascorbic acid (AA) and antioxidant activity (DPPH and ROO) were significantly affected by genotype and the stage of plant growth, as well as by their interaction (Table 4). The highest FCI values were measured for BN and CR, and generally sprouts showed significantly higher values compared to microgreens and baby leaves. Similarly, AA content was higher for BN and CR compared to CL, and it tended to increase with the growth stage, since its level was higher for microgreens and baby leaves compared to the sprouts.

Table 4. Results of the multifactorial ANOVA for Folin–Ciocalteu index (FCI), ascorbic acid (AA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxyl radical (ROO) scavenging activity according to the factors "genotype" and "stage of growth" and their interaction.

			FCI	AA	DPPH	ROO
Genotype (G)						
		BN	39.06a *	6.67a	3.29b	82.55a
		CL	34.48b	5.48b	2.91c	80.19a
		CR	40.02a	6.34a	3.81a	70.53b
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Stage of growth (S)						
		Sprouts	41.9a	5.7c	3.73a	86.89a
		Microgreens	36.9b	6.1b	3.09b	72.28b
		Baby leaves	34.8c	6.3a	3.20b	74.11b
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
G x S						
		Sprouts	42.9b	6.4b	3.22c	93.28ab
	BN	Microgreens	37.2cd	7.5a	3.23c	89.66b
		Baby leaves	37.1d	6.1bc	3.43bc	64.71d
		Sprouts	35.9de	4.8d	3.54b	99.40a
	CL	Microgreens	34.2ef	5.9c	2.61d	63.23d
		Baby leaves	33.3f	5.8c	2.59d	77.95c
		Sprouts	46.9a	6.1bc	4.41a	67.99d
	CR	Microgreens	39.2c	6.5b	3.43bc	63.94d
		Baby leaves	34.0ef	6.5b	3.59b	79.66c
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

* The mean values associated with the two factors and their interaction were evaluated according to Tukey's test. Means significantly different are indicated by different letters.

With regard to the antioxidant activity, the DPPH quenching capacity provided similar results of the FCI analysis, since it was higher for CR and BN and it decreased during the growth stage. The ROO quenching capacity showed the highest values for BN and CL and it decreased during growth, similarly to what was observed for DPPH analysis.

3.3. Total Polyphenol Content

The FCI showed the highest value for the CR sprouts, whereas the lowest ones were ascertained for the microgreens of CL and the baby leaves of CL and CR (Figure 2). The FCI of microgreens did not significantly differ for BN and CR, while with regard to the baby leaves, BN showed significant higher value compared to CL and CR. CL showed the lowest FCI value in all growth stages (33.3, 34.2, 35.9 mg GAE g^{-1} d.w., for the baby leaves, microgreens and sprouts, respectively) (Figure 2).

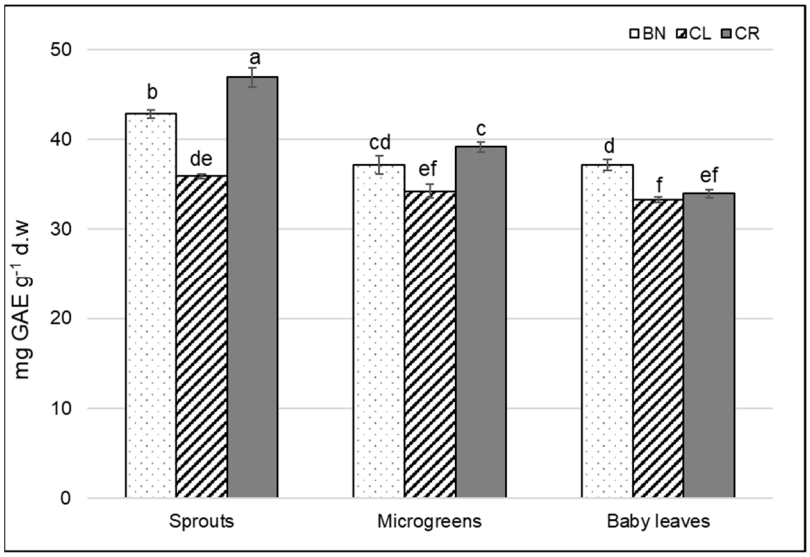

Figure 2. Folin–Ciocalteu index in sprouts, microgreens and baby leaves of BN, CL and CR. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

3.4. Polyphenol Analysis

The seeds of the three cultivars showed significant difference of the polyphenols content. HPLC analysis showed the main PPH compounds of the cultivars studied are represented by sinapyl and gallic acid derivatives (Table 5). The highest content of gallic acid derivate was found in CL (0.525 mg g^{-1} d.w.) while the lowest one was found in BN (0.206 mg g^{-1} d.w.). The highest content of sinapyl acid derivates was observed for CR (3.572 mg g^{-1} d.w.) and the lowest for BN (2.826 mg g^{-1} d.w.).

Table 5. Polyphenol content in seeds of BN, CR and CL.

Genotypes	Gallic Acid Derivate (mg g^{-1} d.w.)	Sinapyl Acid Derivate (mg g^{-1} d.w.)	Total Polyphenol Content (mg g^{-1} d.w.)
BN	0.206 ± 0.021b	2.826 ± 0.051b	3.032 ± 0.252b
CL	0.525 ± 0.062a	3.413 ± 0.251a	3.937 ± 0.313a
CR	0.261 ± 0.047b	3.572 ± 0.412a	3.832 ± 0.456a

Data are reported as mean for each genotype ± S.E. The statistical analysis was performed via one-way ANOVA. Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (Tukey's test).

The content of the main polyphenols compounds of the three cultivars are reported in Figures 3–5. The PPH compounds showed a significant interaction between the cultivars and the plant growth stage.

The phenolics identified by HPLC were caffeoyl, chlorogenic acid, cyanidin, gallic acid, kaempferol, p-coumaric acid, sinapyl, and apigenin derivates. They were identified by their UV-VIS DAD spectral properties, which were compared with the used external standards (Figures 3–5).

For BN, for all the plant growth stages studied, kaempferol (15.1, 20.8 and 50.6 mg g^{-1} d.w. In sprouts, microgreens and baby leaves, respectively), and apigenin derivate (17.9, 30.6 and 43.8 mg g^{-1} d.w. sprouts, microgreens and baby leaves, respectively) predominated. The chlorogenic and p-coumaric acid was not detected in BN baby leaves, and it was observed in higher amount for sprouts than for the other two stages (Figure 3).

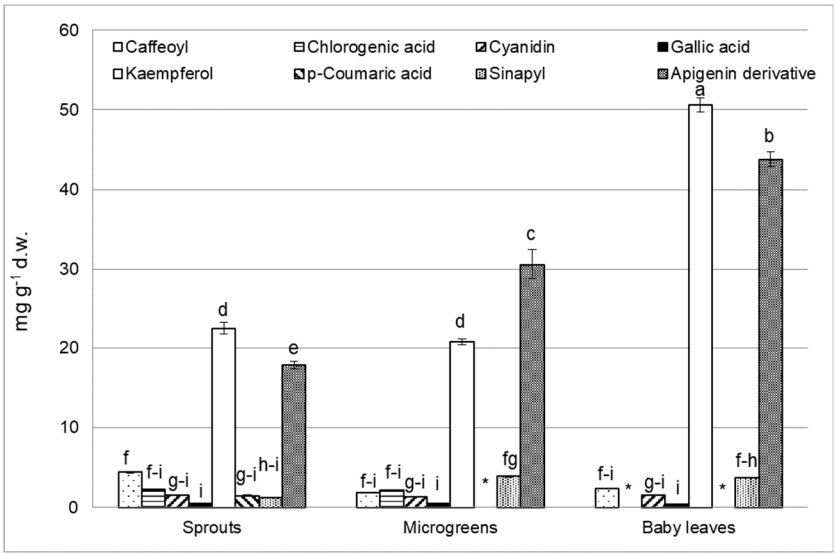

Figure 3. Polyphenol compounds for BN at different growth stages. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$). Symbol * = not detected compound.

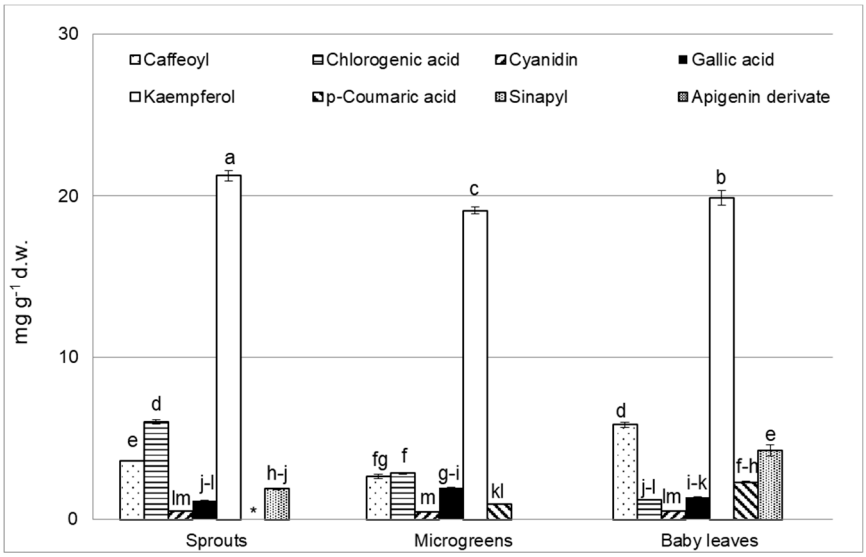

Figure 4. Polyphenol compounds for CL at different growth stages. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$). Symbol * = not detected compound.

With regard to CL, kaempferol (~20.0 mg g^{-1} d.w.) was the main component in all stages of growth. The p-coumaric acid was not detected in sprouts, and sinapyl derivates were present only in sprouts and baby leaves (Figure 4).

CR was characterized by the predominance of caffeoyl, kaempferol, gallic acid, and sinapyl derivatives, and a very small amount of other compounds. Chlorogenic acid was not found in all the plant growth stages studied (Figure 5).

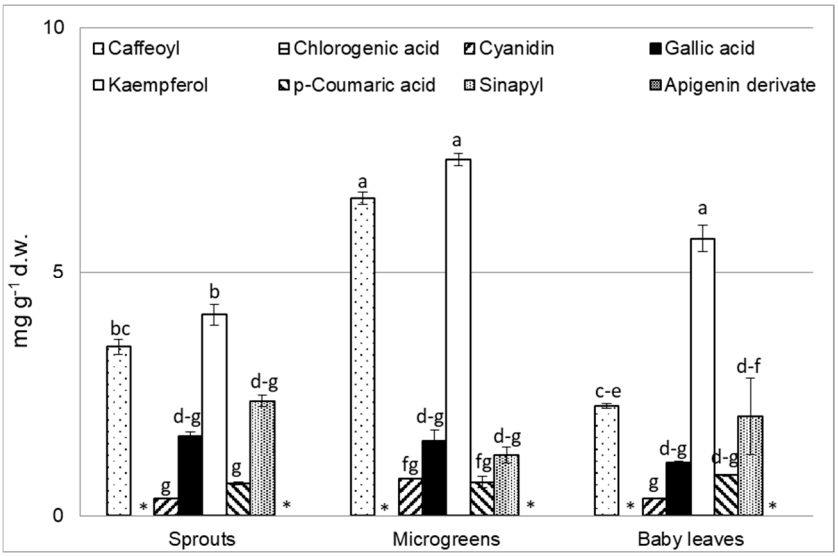

Figure 5. Polyphenol compounds for CR at different growth stages. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$). Symbol * = not detected compound.

3.5. Ascorbic Acid Content

Ascorbic acid (vitamin C) is an essential nutrient for the human body, acting as an antioxidant. The vitamin C content, measured by acid ascorbic (AA), is shown in Figure 6. Significant differences in the content of AA at individual stages of plant growth were noticeable. The highest amount of AA was ascertained for the microgreens of BN (7.5 mg g^{-1} d.w.) while the lowest for the sprouts of CL (4.8 mg g^{-1} d.w.) (Figure 6). A significant increment of the AA amount was observed from the sprouts to the microgreens of BN and CL whereas for CR the value was stable for all the three plant growth stages (Figure 6).

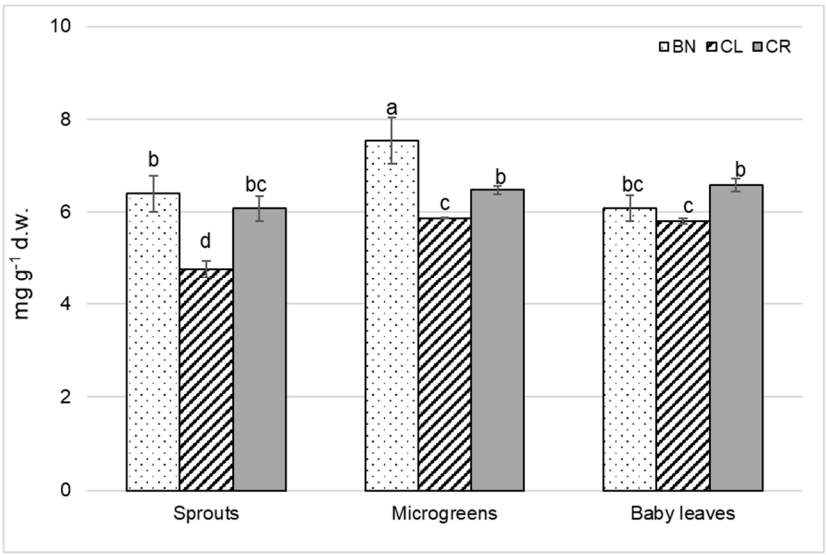

Figure 6. Ascorbic acid (mg g^{-1} d.w.) in BN, CL and CR in sprouts, microgreens and baby leaves. Each value represents mean ± S.E. of three replicates. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

3.6. Antioxidant Activity

The DPPH quenching activity of CR was significantly higher ($p < 0.001$) compared to BN and CL (Figure 7, top). The data of DPPH scavenging ranged from 2.6 to 4.5 mmol AA eq/100 g dw. In particular, CR sprouts were distinguished for the highest DPPH activity (4.41 mmol AsAeq /100 g d.w.) which was 37.0% and 24.4% higher compared to BN and CL sprouts, respectively (Figure 7), with similar values for BN for all three stages. With regard to microgreens and baby leaves, CR and BN showed similar values, while for CL and CR, both microgreens and baby leaves were characterized by lower DPPH quenching activity. In this case, the evolution of the DPPH antioxidant capacity was similar to the Folin–Ciocalteu index (Figure 2; Figure 7) In fact, the two parameters were significantly correlated (r = 0.68, $p < 0.001$), and both had a tendency to decrease from sprouts to baby leaves, particularly for CL and CR.

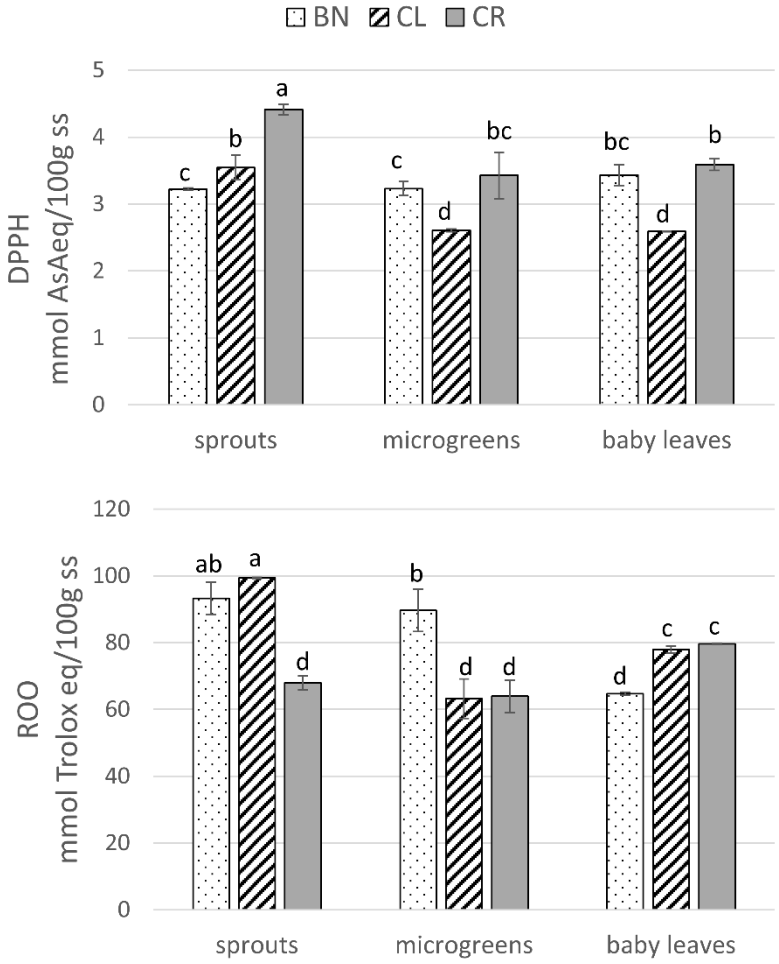

Figure 7. DPPH (mml AsAeq/100 g^{-1} d.w.) and ROO (mmol trolox equ/100 g^{-1} d.w.) in BN, CL and CR in sprouts, microgreens and baby leaves. Each value represents mean ± S.E. of three replicates. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

The data of ROO scavenging ranged from 57 to 100 mmol Trolox eq/100 g dw. As for the ROO scavenging capacity, the highest values were found in sprout samples of the BN and CL genotypes (around 100 mmol Trolox eq/100 g d.w.), while the values tended to decrease during the plant growth, particularly for BN, for which the baby leaves reached values of around 60 mmol Trolox eq/100 g d.w. (Figure 7, bottom). On the contrary, for CL and CR, we ascertained a slight but significant increment of the ROO scavenging activity values from microgreens (around 60 mmol Trolox eq/100 g d.w.) to the

baby leaves (around 80 mmol Trolox eq/100 g d.w.), not detected in DPPH scavenging data, for which we registered similar scavenging values.

4. Discussion

Several authors focused their attention on the optimization of the germination process in order to maximize the content in health-promoting compounds of the sprouts [36,37]. Seeds can germinate at a wide range of temperatures, but the germinability is drastically reduced at extreme temperatures [38]. Previous investigation showed a good germinability at 30 °C but with a higher MGT values in comparison to 25 °C [37]. That has been observed in our study in which no germination occurred at 5 °C for all the genotypes analyzed.

The phenolic profile of vegetables can be influenced by intrinsic factors related to the plant growth stage and to genetic variability, also within the same species. In seeds, the differences between varieties and genotypes suggested that the genotype was the main factor of variation; in fact, our results show that the main content in polyphenols was shown for CL and CR. During the germination process, reactivation of seed metabolism takes place, promoting the hydrolysis of storage proteins and carbohydrates by the synthesis/accumulation of metabolites with health-promoting properties [39].

According to Paśko et al. [16], higher total phenolic content in sprouts compared to seeds suggest the synthesis of phenolic antioxidants during germination may occur. The strong correlation found between total polyphenol content and antioxidant activity also suggests that this content is a good predictor of the in vitro antioxidant activity. It is thought that seeds mainly act as a reservoir for the development of the sprouts [40]. The PPH compounds increased significantly for the three cultivars considered, from the seeds to the baby leaves stage, showing significant differences among them (Figures 3–5).

Some of the health-promoting factors may be present at a ten times higher level in sprouts than in mature vegetables [41,42]. This is the case of the flavonoids content and of the other phenolic compounds that clearly contribute to the antioxidant potential [43].

Germination determines significant changes in the phenolic content, mainly due to activation of endogenous enzymes and to the complex biochemical metabolism of seeds during this process [44]. For this reason, germination may be a suitable process to obtain functional food with high antioxidant capacity. Sprouts are produced by the seed germination process and they represent an interesting novel food and a valuable alternative to increase the consumption of different seeds in human nutrition [45]. Sprout phenolic composition depends on genotype as well as on numerous environmental factors, including temperature, light or dark condition, humidity and sprouting time [46] and also water quality and salt content [42].

With regard to the cvs analyzed, BN showed the highest increment of the kaempferol and apigenin from the seed to the baby leaves growth stage, whereas for CL and CR, we ascertained the increment of the PPH compounds, mainly represented by kaempferol, caffeoyl and chlorogenic, just after germination process with no significant differences among the three plant growth stages considered (Figures 4–6).

According to the literature data, *Brassica* plants are rich in anthocyanins, phenolic acids and flavanols, in particular, kaempferol and quercetin [47]. In different studies conducted by Cartea et al. [11,48] on qualitative flavonoid evaluation in *B. napus*, quercetin and kaempferol glycosides were found as the major compounds in *Brassica* samples, in full qualitative accordance of previous data [11,48] with the here presented ones. Moreover, a quantitative survey by Velasco et al. [48] on kale and leaf rape reported a total phenol amount of 3–5 mg g^{-1} d.w., very close to the here presented data in seeds (Table 5), and lower than the data in vegetative parts reported in the present work, especially for BN samples (Figure 3).

According to Pająk et al. [49] and Velasco et al. [48], a range of phenolic acids have been identified in sprouts (chlorogenic, gallic, sinapyl, ferulic, p-cumaric). Broccoli sprouts are very rich in phenolic compounds, more than commercial broccoli florets; among them, free phenolic acids, caffeic, chlorogenic

and gallic acids occurred in the largest amounts; the main free phenolic acids found in broccoli sprouts were ferulic and sinapic acids [49]. Elevated levels of flavonoids and phenolic acids, which are highly bioavailable, were observed in a study conducted by Gawlik-Dziki et al. [50] on broccoli sprouts; this is in accordance with our results where sprouts of BN showed the highest content of kaempferol and apigenin derivates.

Phenolic compounds and vitamin C are the major antioxidants of *Brassica* vegetables. The content of vitamin C among *Brassica* vegetables varies significantly among and within each species and interspecific entity [1]. The cause of the reported variations in vitamin C content might be related to the different genotypes studied [51,52]. In particular, in the study by Vallejo et al. [52], the content of vitamin C was very irregular among the genotypes analyzed; the content ranged from 43.1 mg per 100 g fw in Lord (commercial cultivar) to 146.3 mg per 100 g fw in SG-4515 (experimental cultivar). In our work, the highest content in vitamin C was found in BN microgreens. Vitamin C is an important water-soluble dietary antioxidant, which significantly decreases the adverse effects of the free radicals can cause oxidative damage to macromolecules such as lipids, DNA and proteins, which are in turn implicated in chronic diseases, like cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis [53].

The average levels of DPPH and ROO scavenging activity were highest in sprouts and tended to decrease with growth (Figure 7). In particular, as regards to the DPPH quenching activity, CR and CL sprouts showed higher values compared to microgreens and baby leaves, and a similar trend was observed for ROO antioxidant activity, even if with different results depending on the cultivars. Our findings partially agree with the results of Ebert et al. [54], who found that the antioxidant activity for amaranth sprouts was much higher than for microgreens, but not for the fully expanded leaves plant growth phase. Indeed, higher levels of the Folin–Ciocalteu index were found in sprouts (Figure 2), while the highest level in AA was found in microgreens (Figure 6) and single polyphenols usually increased in baby leaves, particularly for BN (Figure 3). These results suggest the presence of other antioxidants than kaempferol and apigenin or AA in Brassica sprouts, which may enhance the antioxidant capacity of the sprouts compared to the microgreens and the baby leaves. Interestingly, the Sicilian landrace BN of broccoli was distinguished for the highest ROO scavenging activity both at the stage of sprouts and of microgreens, but not at the stage of baby leaves, for which its value significantly decreased. On the other hand, for CL and CR, the ROO antioxidant capacity of the baby leaves increased in comparison to microgreens, being finally higher by around 20% compared to the BN baby leaves.

5. Conclusions

The activities carried out provide additional data related to the novel foods proposed, such as sprouts, microgreens and baby leaves. *B. oleracea* crops, and in this case broccoli and kale, are shown to represent good sources of antioxidant compounds, such as polyphenols and ascorbic acid, confirmed also by their high antioxidant capacities. The cultivars utilized showed different polyphenol profiles among them and also in relation to the plant growth stages proposed, correspondent to the three novel foods investigated.

The total polyphenols showed the highest values for the sprouts of both the two cvs of broccoli utilized. The Sicilian landrace of 'Broccolo Nero' showed the highest amount of kaempferol and apigenin in late growth stages, whereas the former represents the main polyphenol compound for the other cultivars of broccoli and of kale studied. Among the plant growth stages analyzed, the baby leaves showed high values of kaempferol and apigenin. The ascorbic acid varied significantly both in relation to the cultivar and to the plant growth stage and the highest value was observed for the microgreens of 'Broccolo Nero'. The antioxidant capacity showed in general the highest values for the sprouts for all the three cultivars analyzed.

The experimental factors analyzed offer the opportunity to improve the knowledge related to these novel foods of interest for diversifying the vegetable items by the exploitation of the *B. oleracea*

landraces. Of interest are the data of the antioxidant traits acquired for implementing the nutraceutical and organoleptic traits of the novel foods proposed for improving the horticultural organic food supply chains.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/6/782/s1, Figure S1: Different growth plant stages of the Sicilian landrace 'Broccolo nero' (BN): from the top to bottom, sprouts, microgreens and baby leaves.

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