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Enhancing the Quality of Two Species of Baby Leaves Sprayed with Moringa Leaf Extract as Biostimulant

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Abstract: Natural biostimulants obtained by plants are intensively used nowadays to improve crop yield and quality. The current study aimed to evaluate the effects of leaf extract of moringa (*Moringa oleifera* Lam.) (MLE) in modifying baby leaf characteristics of two genotypes of *Brassica.* The trial was started in October 2020 in a greenhouse; a cultivar of kale 'Cavolo Laciniato Nero di Toscana' (CL) and a Sicilian landrace of sprouting broccoli 'Broccoli Nero' (BN) were used. The plants, after 15, 30 and 40 days from sowing, were treated with MLE, while the control plants (C) with distilled water. Treatment with MLE modified morphological and nutritional value, but with different behavior in the two genotypes. In fact, in BN the treatment reduced the antioxidant activity (2.2-diphenyl-1-picrylhydrazyl (DPPH)) by 54%, while in CL the treatment increased this parameter by 40%. For the phenolic concentration and the sugar content the values recorded were significantly increased by MLE compared to control plants in CL, where in BN a significant reduction was registered. The CL plants treated with MLE showed a significant reduction (−70%) in nitrate content compared to the control plants; a negative effect was, instead, observed in BN, where the plants treated with moringa showed an increase of 60%. Results of this study showed how the foliar application of MLE was effective in improving various nutraceutical parameters, in particular in kale, because it appears to be a species-specific response.

Keywords: brassicaceae; biostimulants; antioxidant activity; total sugars; nitrate content

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1. Introduction

Vegetables are fundamental to human health and nutrition. They represent an important source of essential nutrients such as dietary fiber, vitamins, minerals (macro and microelements), and even phytonutrients such as anthocyanins, carotenoids, and phenolic compounds [1]. Vegetables are considered low calory food and are important to balance or substitute food rich in fats. In recent years, among leafy vegetables, Brassicaceae family species have been the subject of particular attention, due to the health beneficial effects of their products [2,3]. In fact, in recent years, the interest and consumption of *Brassica* vegetables increased thanks to their nutritive value. Among the *Brassica* genera the most studied genotypes are broccoli, cabbage, kale, and some new leaf species like rocket, mizuna, and watercress [4].

The hectic lifestyle reduced the time dedicated to prepare food, so the use of minimally processed leafy vegetables (BLV) sold as ready-to-eat (RTE) salads has increased. To meet the expectations of consumers (convenience, freshness, flavour, quality), in the fresh-cut market, new varieties of raw materials need to be evaluated as potential product innovation [5]. There are many leafy vegetables on the market that are commercialized as baby leaves; among the most popular, there are different varieties of lettuces, lamb's lettuce, purslane, turnip tops or turnip greens, spinach, and Swiss chard. A large number of leafy vegetables from the Brassicaceae family are also used as baby leaf vegetables,

like arugula, garden cress, mizuna, red mustard, tatsoi, watercress, and wild rocket [6]. The production and the market of baby leaves have grown considerably in recent years. They provide consumers attractive products and, at the same time, with a high content of bioactive compounds [7].

The baby leaves with sprouts, microgreens are often considered superfoods and are principally consumed as raw products or minimally processed for commercialization as ready-to-eat vegetables. Because only minimal treatments, cleaning, cutting, packaging, and refrigerated storage are carried out on these products, there is no loss or degradation of the bioactive compounds [8]. Since baby leaves represent a natural source of bioactive compounds, they could become an important source for the development of the fresh-cut market [5]. Ali et al. [9] found different bioactive compounds in Brassicaceae (organosulfuric phytochemicals that have anticarcinogenic activity, and other phytochemicals, with high antioxidant power); the most important compounds are represented by glucosinolates that have several beneficial effects on human health [10]. Green leafy vegetables are excellent source of antioxidants, which act as radical scavengers. The consumption of these baby leaf species can represent, thanks to the high vitamin C concentration, an important source of antioxidants in the diet [4].

The bioactive compounds in the juvenile phase are higher than in mature leaves. Lester et al. [11], in fact, reported that baby spinach had higher levels of bioactive compounds (vitamins C, B9, and K1, and carotenoids) compared to mature leaves. Similarly, the highest total phenolic and antioxidant capacity were found in young lettuce than more mature leaves [12].

In recent years, the use of biostimulant products represents a valid technological innovation with great potential for the sustainable development of plant production. These substances are taking great interest in sustainable agriculture because their applications activate different physiological processes that improve the efficiency of the use of nutrients, stimulate the development of plants and allow the reduction of fertilizer consumption [13,14]. A biostimulant is any substance and/or microorganism, able to improve the nutritional efficiency in plants, the abiotic stress tolerance, and/or quality of the crop [15]. An increase in photosynthetic pigments, net-photosynthesis, and quantum efficiency of photosystem II has been correlated to an increase in sugar biosynthesis in different species treated with biostimulants [14,16–18].

There is an interest in the identification of botanical raw materials rich in bioactive compounds for biostimulant production. The *Moringa oleifera* (Lam.) has been suggested as a potential source of bioactive compounds for biostimulant preparation. Leaf extracts of *M. oleifera* applied as seed soaking and/or foliar spray, which under normal or stressful conditions can positively modify growth and production by altering metabolic processes [19–22]. The *M. oleifera* is a most common species cultivated throughout the tropical regions of the world [23,24]; antioxidant properties are found in moringa leaf extract (MLE) [25]. For its rich source of plant growth hormones, antioxidant capacity, and mineral nutrients in its leaves, this species has received enormous attention from the scientific community among the naturally biostimulants [26,27]. As they have a high content of fibers, proteins, carbohydrates, vitamins, essential amino acids, and minerals, their leaves assure high nutritional and medicinal values [28]. Fuglie [29] showed that the MLE improved the growth of young plants, increased the resistance to diseases and pests, enhanced leaf duration, number of roots, production of fruits, and generally enhanced yield by 20% and 35%. An increase in pigment photosynthetic (chlorophyll a and b) was observed in tomato plants treated with MLE foliar application [26].

Although the leaf extract of moringa has been used in different contexts, such as the increase in yield [30], adaptability to biotic and abiotic stresses [31], extension the duration in post-harvest, such as in the cut flowers [15], so far the use of this biostimulant in baby leaves has not been investigated, despite the possible improvement that could also be obtained for the content of bioactive substances.

Therefore, the objective of this work was to investigate the beneficial effects of the application of MLE foliar spray on growth characteristics, chlorophyll *a* fluorescence, and content of bioactive compounds in two baby leaves of *Brassica* species. The young harvesting stage of these leafy vegetables should get benefit from MLE biostimulants application.

2. Materials and Methods

2.1. Plants Materials

Seeds of the standard commercial cultivars of kale 'Cavolo Laciniato Nero di Toscana' (CL) (S.A.I.S. S.p.A. seed company, Cesena, Italy) and of the Sicilian landrace of sprouting broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Broccolo Nero' (BN) (Di3A active gene bank collection, BR 354, UNICT 4939) were used in the trial. Seeds were grown in cellular trays placed in cold greenhouse under natural light (4.7 to 9.8 $MJm^{-2}d^{-1}$) and temperature (16.4 ± 5.8 °C) conditions, from October to November 2020 at Catania (South Italy, 3731010" N 1504018" E; 105 m above sea level (m a.s.l.). The organic substrate Brill®semina bio (Geotec, Italy) was used to fill the cellular trays and plantlets were irrigated based on the ordinary techniques. The plants were treated once by BTK ® 32 WG (Xeda, Italy) based on *Bacillus thuringiensis* sub. *kurstaki* for controlling *Pieris brassicae*.

The plants were sprayed three times with moringa leaf extract (MLE), at 15, 30 and 1 day before harvest (43 days after planting). The control plants were sprayed with distilled water.

2.2. Preparation and Analysis of Moringa Leaf Extract

The *M. oleifera* leaf extract (MLE) was done by Pervez et al. [32] with slight modifications. The leaves were shade-dried and then finely grounded with a grinder. The MLE extraction was done by 50 g of powdered soaking in 200 mL of distilled water. The mixture was macerated for 48 h at 25 °C and later filtered through filter paper. The extract obtained was indicated as 25% extract which was further diluted to the ratio 1:30 (*v*/*v*). The concentration of MLE was of 200 mg L^{-1} [33]. Tween 20 (0.05%) was added to spray solutions as a wetting agent. Moringa leaf extract was analyzed, and its chemical constituents were reported in the Table 1.

Table 1. Chemical composition of principal component of *Moringa oleifera* leaves.

Component	Value
Phosphorus (P)	4.9 g kg^{-1} DW
Potassium (K)	16.0 g kg^{-1} DW
Calcium (Ca)	16.2 g kg^{-1} DW
Iron (Fe)	0.2 g kg^{-1} DW
Magnesium (Mg)	3.0 g kg^{-1} DW
DPPH [1]	130 mg TE g^{-1} DW
Total polyphenols	22.6 mg GAE g^{-1} DW
Chlorophyll *a*	1.2 µg mg^{-1} FW
Chlorophyll *b*	1.4 µg mg^{-1} FW
Carotenoids	0.10 µg mg^{-1} FW
Nitrate concentration	515.5 mg kg^{-1} FW

[1] Diphenyl-1-Picrylhydrazyl Radical-Scavenging Activity (DPPH)

2.3. Chlorophyll A Fluorescence

The chlorophyll *a* fluorescence was determined by a modulated chlorophyll fluorimeter OS1-FL (Opti-Sciences Corporation, Tyngsboro, MA, USA). Each leaf was dark-adapted using cuvette clips (Opti-Sciences Corporation, Tyngsboro, MA, USA) for 15 min. The maximal quantum yield of PSII photochemistry was expressed as the Fv/Fm ratio, F0 indicated the minimum fluorescence, Fm the maximal fluorescence of the dark-adapted state, and Fv the variable fluorescence.

2.4. Measurement of Growth Parameters

From each replication, nine randomly selected seedlings were chosen and were determined the height, the leaf number, and the fresh and dry biomass. The fresh plants were dried at 70 °C until they reached a constant weight.

2.5. Chlorophyll and Carotenoid Pigments

The photosynthetic pigments (chlorophyll *a* and *b*, and carotenoid) were determined spectrophotometrically from leaf samples. Samples were extracted through methanol (99%, Sigma-Aldrich, Milan, Italy) and incubated in dark room (4 °C for 24 h). The absorbance of samples was read at 665.2 nm, 652.4 nm, and 470 nm. The calculation of chlorophylls was undertaken following the formula reported by Lichtenthaler et al. [34].

2.6. 2.2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical-Scavenging Activity and Total Phenolic Contents

The antioxidant capacity was measured using 2.2-diphenyl-1-picrylhydrazyl (DPPH). We extracted 100 mg of freeze-dried sample by 1.5 mL of methanol solution (80%), sonicated it for 30 min and centrifuged it for 10 min at 5 °C at 5000× *g*. At 0.01 mL of supernatant was added to 1.4 mL of DPPH solution (150 µM) and incubated in the dark for 30. The absorbance was read at 517 nm. The DPPH scavenging activity value was expressed as Trolox equivalent (mg TE g^{-1}) using a standard calibration curve.

The total phenolic content (TPC) was extracted by 0.2 g of freeze-dried using 10 mL of acetone/water (50:50) and incubated for 15 h at 20 °C. At 100 µL of supernatant was added to 0.5 mL of Folin-Ciocalteu reagent, 6 mL of distilled water and 1.5 mL of Na_2CO_3 (20%). The samples were incubated for 2 h at 20 °C. The samples were read at 765 nm. The TPC was expressed as gallic acid equivalent (mg g^{-1}).

2.7. Total Sugars

Total sugars were determined according to Yemm and Willis [35]. For sample preparation, 1 g of fresh sample was extracted in 3 mL of distilled water and centrifuged (3000× *g*, 15 min room temperature (RT)). Then, 1 mL of extract was added to 5 mL of anthrone solution (0.2 g in 100 mL of H_2SO_4), cooled in ice for 5 min and then mixed thoroughly. Samples were incubated at 95 °C for 5 min and then cooled on ice. The samples were read at 620 nm and the concentrations were expressed referring to glucose calibration curve [36].

2.8. Ascorbic Acid Analysis and Nitrate Concentration

The ascorbic acid (Asc) content was determined by the method of Janghel et al. [37]. About 1 g of fresh leaves was mixed in 10 mL of 5% oxalic acid and then centrifuged at 4000 rpm min^{-1} for 5 min. The supernatant was collected and 1 mL, was homogenized with 2 mL of 0.1% methyl viologen and 2 mL NaOH 2 mol L^{-1}. Samples were read at 600 nm against the radical blank. Nitrate concentration was spectrophotometrically determined from leaf samples [38]. About 1 g of fresh sample was homogenized in 3 mL of distilled water and then centrifuged for 15 min at 3000× *g*. The supernatant (20 µL) was collected and used for the colorimetric determination. The supernatant was mixed to 80 µL of 5% (*w*/*v*) salicylic acid dissolved in H_2SO_4 plus 3 mL of 1.5 N NaOH. Samples were cooled at RT and absorbance at 410 nm was measured. Nitrate concentration was calculated referring to a KNO_3 standard calibration curve [39].

2.9. Determination of Mineral Elements

Three biological replicates were used for the determination of the following mineral elements, Na, Mg, K, Ca, Mn, Fe, Ni, Cu, Zn, and P. About 300 mg dry weight (DW) was mineralized at 120 °C in 5 mL 14.4 M HNO_3, clarified with 1.5 mL 33% H_2O_2 and finally dried at 80 °C. The mineralized material was solubilized in 5 mL 1 M HNO_3 and filtered on a 0.45 µM nylon membrane. Mineral content was measured by an inductively-coupled plasma mass spectrometry technique (ICP-MS; Varian 820-MS, Palo Alto, CA, USA ICP Mass Spectrometer).

2.10. Statistical Analysis

Experiments were performed using a completely randomized design. Each treatment was replicated three times. The significance of differences between the main factors (Genotype G, and treatment T) were determined by two-way analysis of variance (ANOVA). The LSD test was applied and the significant differences established as $p < 0.05$ to determine the differences between treatments. The statistical analyses were executed using CoStat release 6.311 (CoHort Software, Monterey, CA, USA). The interactions, when significant, are presented separately. The data presented in Figures are the means ± standard error (SE) (Graphpad 7.0).

3. Results

Light-use efficiency, crop performance, and health conditions were evaluated by non-destructive chlorophyll a fluorescence. Statistical analysis showed that the F0 and the Fm did not significantly differ between the species, the treatments, and their interactions (Table 2).

Table 2. Effect of moringa leaf extract (MLE) application on minimum fluorescence (F0), maximal fluorescence (Fm) and maximal quantum yield of PSII photochemistry (Fv/Fm) on plant of Broccoli Nero (BN) and Cavolo Laciniato Nero di Toscana (CL). MLE extract was sprayed 15, 30 and 43 days after sowing.

Genotype (G)	Treatment (T)	F0	Fm	Fv/Fm
BN	C	433.5 ± 6.4	2379.8 ± 53.2	0.82 ± 0.0 a
	MLE	426.0 ± 20.5	2201.6 ± 28.9	0.82 ± 0.0 a
CL	C	449.5 ± 15.6	2232.8 ± 24.0	0.80 ± 0.0 b
	MLE	449.0 ± 14.1	2364.5 ± 51.9	0.81 ± 0.0 a
BN		429.8 ± 9.2	2290.7 ± 37.8	0.82 ± 0.0
CL		449.3 ± 9.9	2298.5 ± 33.9	0.80 ± 0.0
	C	441.5 ± 8.1	2306.1 ± 36.6	0.80 ± 0.0
	MLE	437.5 ± 11.4	2283.1 ± 34.9	0.81 ± 0.1
Significance				
	G	ns	ns	ns
	T	ns	ns	ns
	$G \times T$	ns	ns	*

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (LSD test). The interactions, when significant, are presented separately in figures. ns = not significant; asterisk(s) = significant at 0.05 (*), 0.01 (**) or 0.001 (***) level of significance.

The Fv/Fm did not show any differences between the genotypes ($p > 0.05$) and between the treatment (T, $p > 0.05$) (Table 2). The effect of interaction was significant ($G \times T$, $p < 0.05$) (Table 3).

Table 3. Effect of MLE application on fresh (g plant^{-1}) and dry biomass (%), height (cm), and leaf number (*n* plant^{-1}) on plant of BN and CL. Extract was sprayed 15, 30, and 43 days after sowing.

Genotype	Treatment	Fresh Biomass (g plant^{-1})	Dry Biomass (%)	Height (cm)	Leaf Number (*n*)
BN	C	1.31 ± 0.06 b	15.83 ± 0.13 a	13.87 ± 0.53 b	3.27 ± 0.12
	MLE	1.44 ± 0.05 b	15.23 ± 0.36 a	15.40 ± 0.24 a	3.47 ± 0.13
CL	C	1.67 ± 0.09 a	12.70 ± 0.80 b	14.47 ± 0.29 ab	3.73 ± 0.15
	MLE	1.52 ± 0.07 ab	14.77 ± 0.22 a	14.20 ± 0.39 ab	3.60 ± 0.13
BN		1.37 ± 0.04 b	15.53 ± 0.22 a	14.63 ± 0.32	3.37 ± 0.09 b
CL		1.59 ± 0.06 a	13.73 ± 0.60 b	14.33 ± 0.10	3.67 ± 0.10 a
	C	1.49 ± 0.06	14.27 ± 0.80	14.20 ± 0.81	3.50 ± 0.10
	MLE	1.48 ± 0.04	15.00 ± 0.21	14.80 ± 0.25	3.53 ± 0.09
Significance					
	G	**	**	ns	*
	T	ns	ns	ns	ns
	$G \times T$	*	*	*	ns

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (LSD test). The interactions, when significant, are presented separately in figures. ns = not significant; asterisk(s) = significant at 0.05 (*), 0.01 (**) or 0.001 (***) level of significance.

The maximum quantum efficiency of PSII (Fv/Fm ratio) showed a significant increment in response to MLE treatment in CL in comparison (0.81 ± 0.0) to control plants (0.80 ± 0.0). No significant differences were observed in BN species (0.82 ± 0.0 for both treatments) (Table 2, Figure 1).

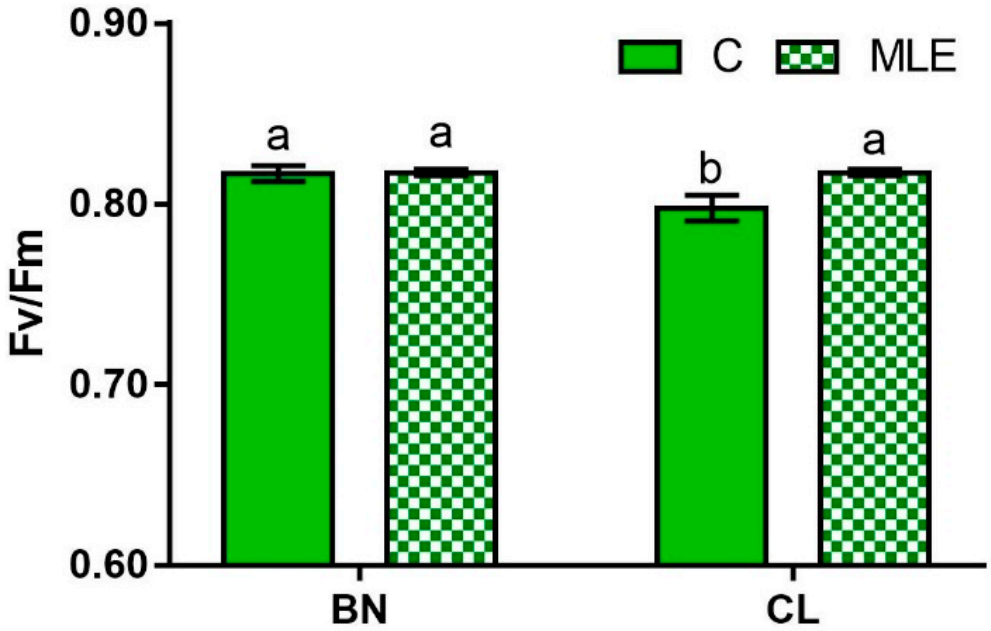
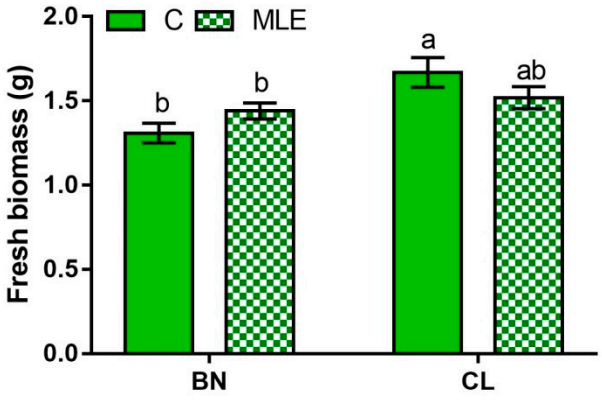
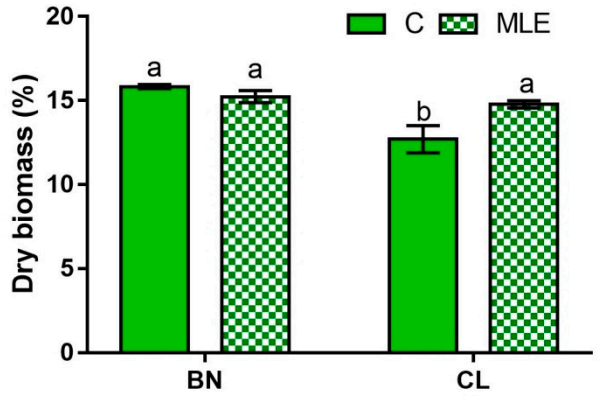


Figure 1. Effect of interaction of maximum quantum efficiency of photosystem II (Fv/Fm) from leaves of BN and CL treated with distilled water (C) and moringa leaf extract (MLE). Values are the means ± standard error (SE, $n = 9$). Different letters: statistically significantly different means for $p < 0.05$ (LSD test).

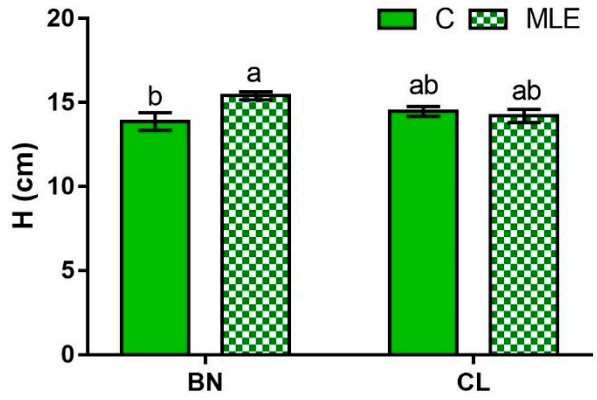
Fresh biomass varied with species ($p < 0.01$) but not with their treatment ($p > 0.05$) (Table 3). Effect of interaction was found ($G \times T$ $p < 0.05$) (Table 3). The fresh biomass showed a higher value in CL control (1.67 ± 0.09) in comparison to BN control (1.31 ± 0.06) (Figure 2a).



(**a**)



(**b**)



(**c**)

Figure 2. Effect of interactions of fresh (g) (**a**) and dry biomass (%) (**b**), height (cm) (**c**) from leaves of BN and CL treated with distilled water (C) and MLE. Values are the means ± SE ($n = 9$). Different letters: statistically significantly different means for $p < 0.05$ (LSD test).

Dry biomass was significantly lowest in CL-C compared to CL-MLE and BN; no significant differences were found for the treatment factor (T, $p > 0.05$) (Table 3). The interaction was statistically significant ($G \times T$, $p < 0.05$) (Table 3). Significant differences for the dry biomass were observed in CL with a reduction by 20% in CL-C compared to the other plants (Figure 2b).

Foliar application of MLE enhanced the height in BN as compared to untreated plants (Table 3 and Figure 2c). No significant differences for CL plants.

The leaf number varied with the genotypes; significantly more leaves were registered on CL than BN genotype ($p < 0.05$); no significant differences were found for the treatment (T, $p > 0.05$) and their interaction ($p > 0.05$) (Table 3).

The application of MLE foliar spray significantly affected the photosynthetic pigment concentration. The genotypes showed a different behavior. The content of Chl *a* and carotenoids presented a different pattern in relation to the treatment (T, $p < 0.01$ and $p < 0.001$ respectively) (Table 4).

Table 4. Effect of MLE application on chlorophyll *a* and *b* (μg mg^{-1} FW), and carotenoids (μg mg^{-1} FW) on plant of BN and CB. Extract was sprayed 15, 30 and 43 days after sowing.

Genotypes	Treatment	Chl *a* (μg mg^{-1} FW)	Chl *b* (μg mg^{-1} FW)	Carotenoids (μg mg^{-1} FW)
BN	C	0.44 ± 0.01 a	1.10 ± 0.13 a	1.29 ± 0.05 a
	MLE	0.48 ± 0.01 a	1.07 ± 0.00 a	1.43 ± 0.04 a
CL	C	0.37 ± 0.02 b	0.77 ± 0.00 b	1.00 ± 0.05 b
	MLE	0.48 ± 0.02 a	1.04 ± 0.08 a	1.37 ± 0.01 a
BN		0.46 ± 0.01	0.91 ± 0.07 b	1.36 ± 0.04 a
CL		0.42 ± 0.03	1.08 ± 0.06 a	1.19 ± 0.08 b
	C	0.41 ± 0.02 b	0.93 ± 0.09	1.15 ± 0.07 b
	ME	0.48 ± 0.01 a	1.06 ± 0.04	1.40 ± 0.02 a
Significance				
	G	ns	*	**
	T	**	ns	***
	$G \times T$	*	*	**

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (LSD test). The interactions, when significant, are presented separately in figures. ns = not significant; asterisk(s) = significant at 0.05 (*), 0.01 (**) or 0.001 (***) level of significance.

Significant was the interactive effect with the treatment for Chl *a*, *b* and carotenoid ($G \times T$, $p < 0.05$, $p < 0.05$ and $p < 0.01$ respectively for chl *a*, *b* and carotenoids) (Table 4).

In particular, no differences were observed in BN for all pigments, while the treatment with MLE enhanced the chlorophyll *a*, *b*, and carotenoids (by ~22%, 26% and 26% respectively) in CL compared to control plants (Figure 3a–c).

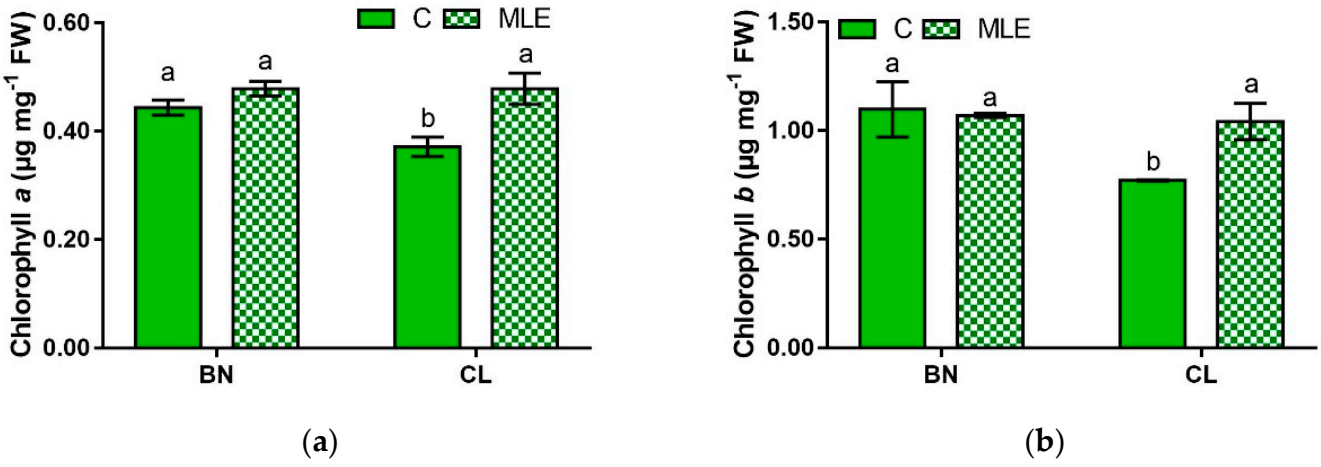
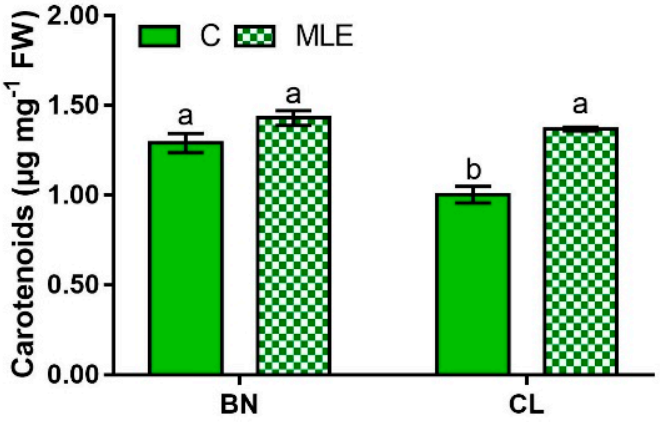


Figure 3. *Cont.*



(**c**)

Figure 3. Effect of interaction ($G \times T$) of chlorophyll a (**a**), chlorophyll b (**b**) and total carotenoid content (**c**), from leaves of BN and CL treated with distilled water (C) and MLE. Values are the means ± S.E (n = 3). Different letters indicate the differences among treatments.

The antioxidant capacity (DPPH) was influenced by genotypes (G, $p < 0.001$). The treatment did not exert any effect on this parameter (T, $p > 0.05$), while significant was the interactive effect with the treatment ($G \times T$, $p < 0.001$) (Table 5).

Table 5. Effect of MLE application on antioxidant activity, DPPH (mg TE g^{-1} DW), total polyphenols, TPC (mg GAE g^{-1} DW), and total sugars (mg g^{-1} FW) on plant of BN and CL. Extract was sprayed 15, 30, and 43 days after sowing.

Genotype	Treatment	DPPH (mg TE g^{-1} DW)	TPC (mg GAE g^{-1} DW)	Total Sugars (mg g^{-1} FW)
BN	C	59.8 ± 3.9 b	10.8 ± 0.2 b	2.10 ± 0.06 a
	MLE	27.7 ± 1.3 c	10.0 ± 0.4 c	1.29 ± 0.03 b
CL	C	63.8 ± 6.9 b	11.4 ± 0.0 b	0.8 ± 0.04 c
	MLE	89.0 ± 3.4 a	12.0 ± 0.2 a	1.3 ± 0.14 b
BN		43.7 ± 17.9 b	10.4 ± 0.2 b	1.08 ± 0.13 b
CL		76.4 ± 31.2 a	11.7 ± 0.2 a	1.70 ± 0.19 a
	C	61.8 ± 3.7	11.1 ± 0.2	1.48 ± 0.29
	LME	58.4 ± 13.8	11.0 ± 0.5	1.31 ± 0.06
Significance				
	G	***	***	***
	T	ns	ns	ns
	$G \times T$	***	*	***

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (LSD test). The interactions, when significant, are presented separately in figures. ns = not significant; asterisk(s) = significant at 0.05 (*), 0.01 (**) or 0.001 (***) level of significance.

Treatment with MLE had different behavior in the two genotypes. In fact, in BN the treatment reduced the antioxidant activity by 54%, while in CL the treatment increased this parameter by 40% (Figure 4a).

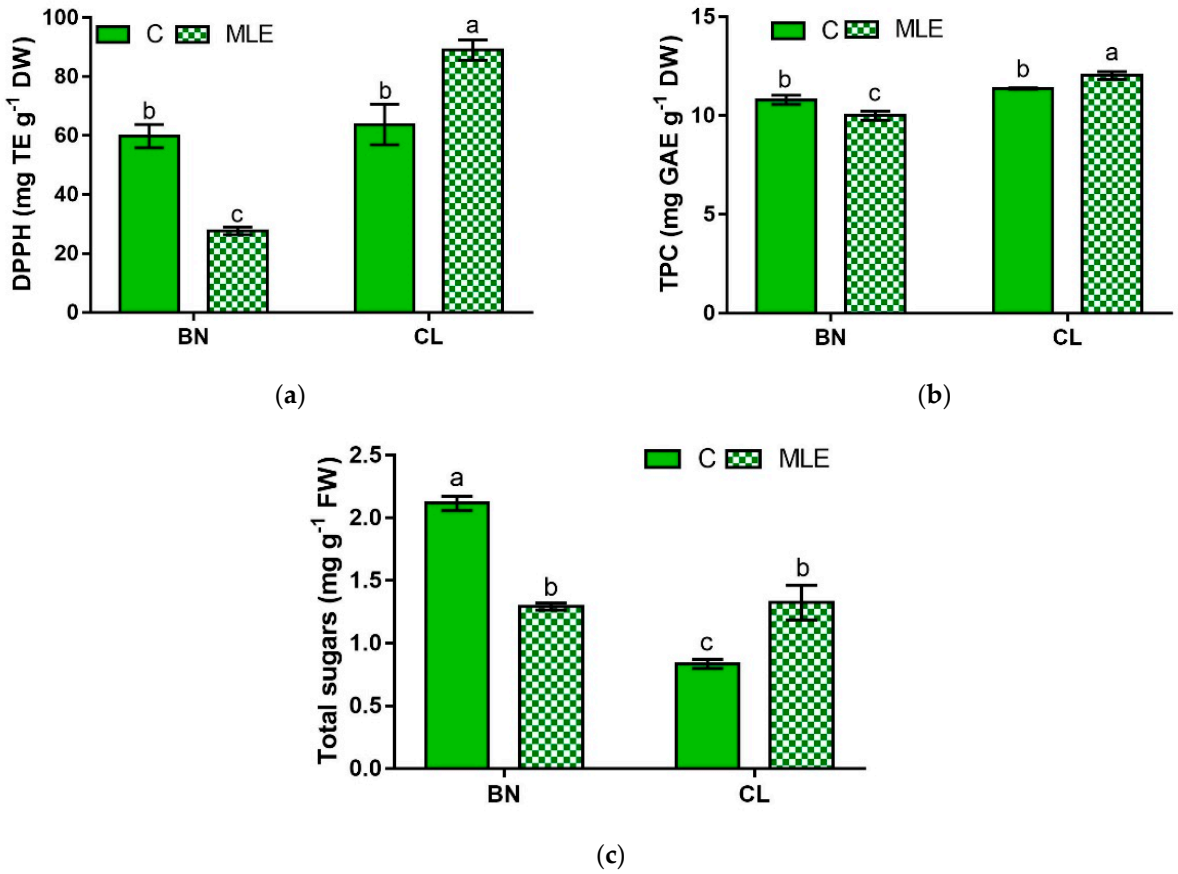


Figure 4. Effect of interaction of antioxidant activity, DPPH (**a**), total phenol content, TPC (**b**), and total sugar content (**c**), from leaves of BN and CL treated with distilled water (C) and MLE. Values are the means ± SE (n = 3). Different letters indicate the differences among treatments.

The phenolic concentration showed similar behavior of the DPPH. The TPC showed significant differences in relation to the genotypes (G, $p < 0.001$) (Table 5). The treatment did not exert any effect on this parameter (T, $p > 0.05$), while the interactive effect with the treatment ($G \times T$, $p < 0.001$) was significant (Table 5). The genotypes showed a different behavior. In particular, the values recorded were significantly increased in CL-MLE (by 6%) compared to control plants whereas in BN, the treatment with MLE affected the TPC with a reduction by 8% (Figure 4b).

A similar trend was recorded for total sugars. Also for this parameter, significant differences were registered for the genotypes (G, $p < 0.001$) (Table 5). Interaction effects were also recorded ($G \times T$, $p < 0.001$). Treatment with MLE had a negative effect on BN, with a decrease by 39%, while CL showed a 40% increase in plants treated with the extract (Figure 4c).

The genotype did not exert any clear effect on this parameter (G, $p > 0.05$), and the interactive effect with the treatment ($G \times T$, $p > 0.05$) was not significant. The highest concentration of ascorbic acid (Table 6) was found in MLE treatment in the mean of the genotypes (3.96 mg g^{-1} FW).

Table 6. Effect of MLE application on ascorbic acid, Asc (mg g^{-1} FW) and Total nitrate (mg kg^{-1} FW) on plant of BN and CL. Extract was sprayed 15, 30 and 43 days after sowing.

Genotype	Treatment	Asc (mg 100 g^{-1} FW)	Nitrate (mg kg^{-1} FW)
BN	C	129.0 ± 16.2	213.1 ± 1.3 c
	MLE	149.2 ± 2.0	475.0 ± 38.3 b
CL	C	132.8 ± 1.6	803.0 ± 0.1 a
	MLE	167.6 ± 0.4	240.1 ± 45.0 c
BN		139.1 ± 8.6	344.1 ± 61.0 b
CL		150.2 ± 7.8	521.6 ± 127.5 a
	C	130.9 ± 7.4 b	508.1 ± 131.9 a
	LME	158.4 ± 4.2 a	357.6 ± 58.8 b
Significance			
	G	ns	***
	T	*	***
	$G \times T$	ns	***

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (LSD test). The interactions, when significant, are presented separately in figures. ns = not significant; asterisk(s) = significant at 0.05 (*), 0.01 (**) or 0.001 (***) level of significance.

Nitrate concentration showed differences between the genotypes (G, $p < 0.001$) and between the treatment (T, $p < 0.001$) (Table 6). A greater quantity was registered in CL plants (>500 mg kg^{-1} FW). Between the treatment, control plants showed a greater quantity (>500 mg kg^{-1} FW). Nitrate concentration was also significantly affected by G and T ($G \times T$, $p < 0.001$) (Table 6).

The CL plants treated with MLE showed a significant reduction (−70%) compared to the control plants. A negative effect was instead observed in BN, where the plants treated with moringa showed an increase in the nitrate content of 60% (Figure 5).

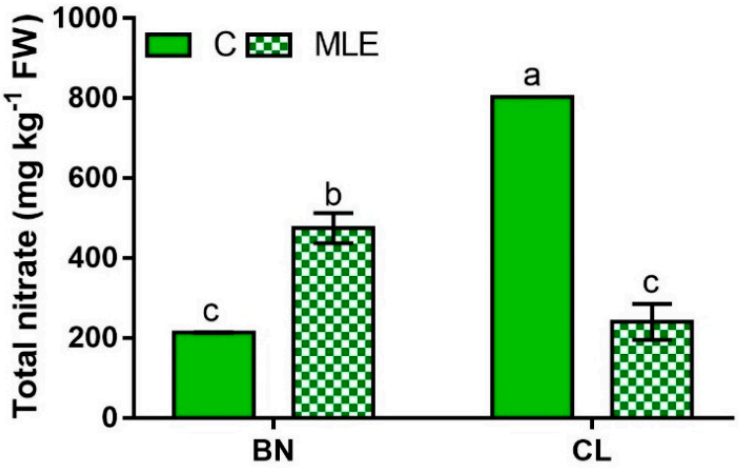


Figure 5. Effect of interactions of total nitrate content from leaves of BN and CL treated with distilled water (C) and moringa leaf extract (MLE). Values are the means ± SE ($n = 3$). Different letters indicate the differences among treatments.

The mineral composition was different in the two species; significant lower values were, in fact, found in the BN species. The MLE extracts reduced the concentrations of Na, Mg, K and Ca (Table 7).

Table 7. Effect of MLE on sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn) and phosphorus (P) concentrations of CL and BN baby leaf.

		Na (g kg^{-1} DW)	**Mg (g kg^{-1} DW)**	**K (g kg^{-1} DW)**	**Ca (g kg^{-1} DW)**	**Mn (g kg^{-1} DW)**	**Fe (mg kg^{-1} DW)**	**Ni (mg kg^{-1} DW)**	**Cu (mg kg^{-1} DW)**	**Zn (mg kg^{-1} DW)**	**P (g kg^{-1} DW)**
BN	C	1.0 ± 0.0 c	2.5 ± 0.0 c	8.4 ± 0.1 d	14.8 ± 0.2 d	51.2 ± 0.7 bc	137.3 ± 8.7	2.2 ± 0.6	8.0 ± 0.8	24.1 ± 1.4	7.8 ± 0.1 b
	MLE	1.1 ± 0.0 d	2.6 ± 0.1 c	9.1 ± 0.1 c	16.1 ± 0.4 c	58.0 ± 0.7 a	139.8 ± 10.8	1.6 ± 0.1	7.7 ± 1.1	27.9 ± 2.0	8.1 ± 0.2 ab
CL	C	1.8 ± 0.0 a	3.4 ± 0.0 a	11.2 ± 0.2 a	20.2 ± 0.2 a	52.3 ± 0.9 b	150.1 ± 8.1	1.7 ± 0.4	9.0 ± 1.5	29.0 ± 0.5	8.3 ± 0.1 a
	MLE	1.4 ± 0.0 b	2.8 ± 0.0 b	9.9 ± 0.1 b	17.5 ± 0.4 b	48.2 ± 1.0 c	148.2 ± 10.0	1.1 ± 0.3	8.7 ± 0.5	23.0 ± 1.0	8.0 ± 0.1 ab
Genotypes (G)	BN	1.1 ± 0.0 b	2.5 ± 0.0 b	8.8 ± 0.2 b	15.4 ± 0.4 b	54.6 ± 1.7 a	138.5 ± 6.2	1.9 ± 0.3	7.9 ± 0.6	26.0 ± 1.4	7.9 ± 0.1
	CL	1.6 ± 0.1 a	3.1 ± 0.1 a	10.5 ± 0.3 a	18.9 ± 0.6 a	50.2 ± 1.1 b	149.2 ± 5.8	1.4 ± 0.2	8.9 ± 0.7	26.0 ± 1.4	8.1 ± 0.1
Treatment (T)	C	1.4 ± 0.2 a	3.0 ± 0.2 a	9.8 ± 0.6 a	17.5 ± 1.2 a	51.7 ± 0.6	143.7 ± 6.0	1.9 ± 0.3	8.5 ± 0.8	26.5 ± 1.3	8.0 ± 0.1
	MLE	1.3 ± 0.1 b	2.7 ± 0.1 b	9.5 ± 0.2 b	16.8 ± 0.4 b	53.1 ± 0.4	144.0 ± 6.9	1.4 ± 0.2	8.2 ± 0.6	25.5 ± 1.5	8.0 ± 0.1
Significance	*G*	***	***	***	***	**	ns	ns	ns	ns	ns
	T	***	***	*	*	ns	ns	ns	ns	ns	ns
	$G \times T$	***	***	***	***	**	ns	ns	ns	ns	*

All data are expressed as mean ± standard error ($n = 3$). Means (±SE) in columns not sharing the same letters are significantly different according to LSD test ($p < 0.05$). ns = not significant; asterisk(s) = significant at 0.05 (*), 0.01 (**) or 0.001 (***) level of significance.

The first three principal components (PC1, PC2 and PC3), accounting for 65.3%, 20.7% and 14.1% of the 100% of the total variance, were associated with eigenvalues higher than one. In the PCA, PC1 vs. PC2 a negative correlation was observed in total sugars, Mn, dry biomass, carotenoids, chl *a*, *b* and total, Fv/Fm, TPC and ascorbic acid, while a positive correlation was detected in DPPH, height, leaf number, Na, Ca, K, Mg, P, and total nitrate (Figure 6). Principal component analysis (PCA) exhibited clear separation between the groups. The lower right quadrant (the positive side of PC1) included Cl control representing plants with high total nitrate and mineral elements (Figure 6). A second group clustered in the lower left quadrant included plants of BN control and MLE treated characterized by good concentration of chlorophyll, carotenoids and total sugars. Finally, the treated CL plants had the highest nutritional quality (TPC, ascorbic acid and DPPH) (Figure 6).

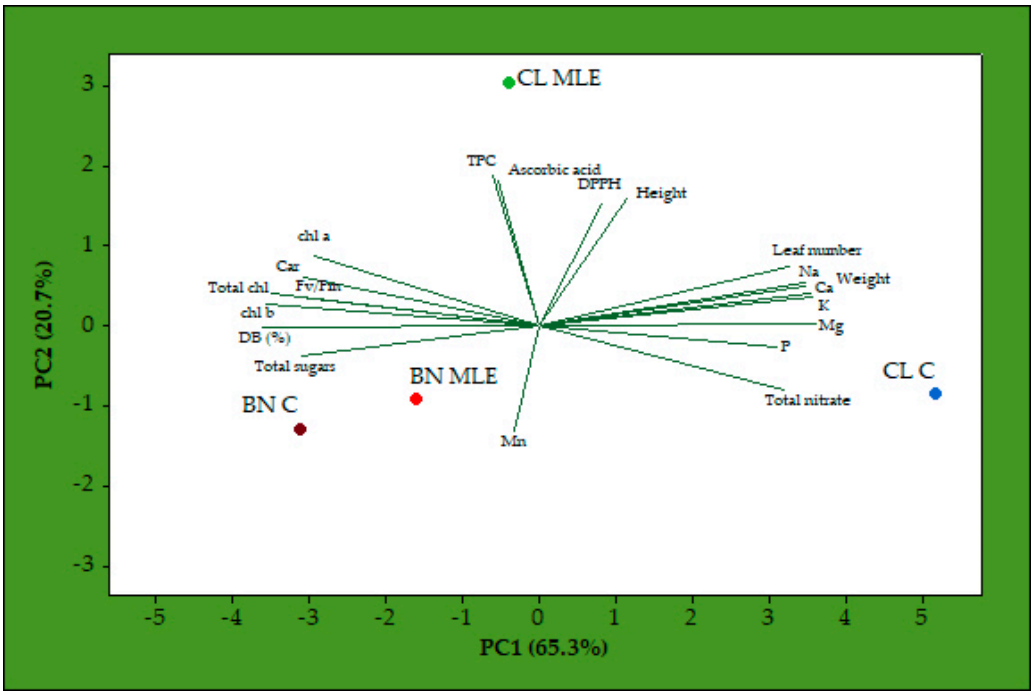


Figure 6. Principal component loading plot and scores of principal component analysis (PCA) of morphometric and nutraceutical traits of BN and CL response to MLE treatment.

4. Discussion

The "baby leaves" represent a new typology of leafy vegetables used in their juvenile state alone or as a mix of different species. These include Amaranthaceae, Asteraceae, and Brassicaceae, and plants of other botanical families [8].

It has been shown that leafy vegetables can take advantage of biostimulant applications, which are able to reduce the nitrate concentration [40], increase the yield, and the concentration of many antioxidant compounds with potential benefits for human health [41].

Among the various biostimulants, moringa leaf extract has received particular attention in recent years thanks to its positive effects on plant productivity [27,42]. The moringa aqueous extract is very simple to prepare, inexpensive, and safe for the environment and adopted by farmers as a biostimulant to increase growth and productivity in various crops. This extract is considered an excellent alternative to organic fertilizers [43] thanks to the high content of proteins, essential amino acids and minerals [42].

Culver et al. [44] found that MLE significantly increased growth and yield of tomato, cultivated both in the greenhouse and open field. It enhanced dry matter yield, root dry matter biomass and plant height. The growth acceleration could be due to a high content of crude protein (43.5%) and growth-promoting hormones, e.g., cytokinins and auxins,

in moringa leaf and twig extracts [45,46]. MLE foliar application improved BN height compared to untreated plants with an increase by 10%.

This was confirmed by Merward [47] in a study conducted on pea, where the treatment with MLE, at different concentrations, enhanced growth characteristics (plant fresh and dry biomass, leaf area and number, plant height, etc.). Similarly, Culver et al. [44] showed that MLE significantly enhanced the growth and yield of tomato cultivated both in greenhouse and open field. The treatment increased the yield and root dry matter, biomass, and plant height.

Chlorophyll *a* fluorescence, in particular, Fv/Fm ratio strictly related to the photochemical efficiency of PSII, is indicated as a useful tool to signal any stress in plants [48]. Our data reveal no stress state in the studied plants treated with MLE treatments of the experiment. The results of our experiment showed that MLE did not damage the photosynthetic apparatus efficiency; on the contrary, in the case of CL the Fv/Fm showed a significant increase in the treated plants while no significant difference was observed instead in BN.

This was also confirmed by the analysis of photosynthetic pigments. Our results showed that the highest concentrations of photosynthetic pigments in CL were registered by MLE spray treatment, with an increase in chlorophyll *a*, *b* and carotenoids. Increasing carotenoids and chlorophyll concentration may be related to the high level of chemicals involved in chlorophyll structure in moringa (leaves, seeds, and pods) such as the carotenoids (lutein, α-carotene, ß-carotene, and xanthin) and chlorophyll [49]. Similar results were found in maize [50], common bean [20], fennel [51], broccoli [33]. Furthermore, MLE contains a high level of Mg, which is a key element for the biosynthesis of chlorophyll [52]. Similar results were found in a study on *Ocimum basilicum* leaves treated with MLE, which leads an increase in the amounts of chlorophyll *a* and chlorophyll *b* thanks several macro elements such as magnesium contained in the leaves [53].

Biostimulants can activate the primary and secondary metabolism of the plant. The antioxidant potential of vegetables, flowers, and fruits can be enhanced by biostimulants, as reported by several studies [14]. The physiological properties of this group of phytochemicals are very wide, and includes anti-inflammatory, anti-allergic, anti-oxidant, anti-microbial, cardioprotective, and vasodilatory effects. The antioxidant activity of these substances is linked to their capacity to eliminate free radicals and donate hydrogen atoms or electrons. Their structure, including their conjugation with other phenols, degree of glycosylation or acylation, molecular size, and solubility, and their absorption and metabolism determine their health benefits [54].

In our study, it was observed that the treatment with moringa had a positive effect by an increase of 40% of the antioxidant activity in CL compared to the control. The relevance of antioxidant activity, as a parameter of functional quality of food, is linked to the beneficial effects of antioxidant molecules (hydrophilic and lipophilic) on human health; these molecules, able to delay or/inhibit oxidative damage, can avoid a wide range of stress or age-related diseases [55–57].

The polyphenol content also showed different trends in the two genotypes. In fact, in CL, moringa extract increased the total polyphenol content, and this can be attributed to the high phenol content in MLE [58], which could influence the endogenous content of total phenols in our baby leaf. The scientific articles on the impact of botanical extracts on the polyphenol content and the antioxidant activity of cultivated plants are few. Ertani et al. [17] have observed, in plants of *Zea mays*, a reduction of the level of phenols to values comparable to those measured in the control (by 19.6%) by the application of biostimulants derived from alfalfa plants.

Sugars are essential metabolites for plant metabolism, and they also play a fundamental role as molecular signal. In fact, leafy vegetables, represent an energy resource during preservation and have a fundamental value in the human diet, etc.

In our work, it was observed that the moringa treatment had a negative effect in BN, while the sugar level was significantly increased in the treated CL plants, showing an increase of 37%.

Vitamin C is usually one of the main vitamins presented in leafy vegetables, but it is also one of the most unstable. It is a fundamental compound for humans for its antioxidant properties [59]. Our results showed that the treatment with MLE enhanced the content of this compound. Our results are in accordance with those found by Vallejo et al. [60] in a broccoli experiment, where levels 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). The content of vitamin C between the different brassicas varies significantly between and within each species and interspecific genotype [61]. Vitamin C is a relevant important water-soluble food antioxidant, which significantly reduces the free radical negative effects; the latter can cause oxidative damage to macromolecules, such as lipids, DNA and proteins, which in turn are involved in chronic diseases, such as cardiovascular disease, cancer, neurodegenerative diseases, cataractogenesis, and stroke [2].

Another essential aspect, especially in leafy vegetables, concerns nitrate accumulation. Nitrates above a certain threshold are considered potentially dangerous for human health, both in adults and in newborns. Biostimulants can reduce the nitrate levels in several species of leafy vegetables [62–64]. The biostimulants can enhance the assimilation pathway acting through the primary metabolism and the increase of sugar biosynthesis. There is a clear relationship between C source and N assimilation. The application of borage extracts as biostimulants showed the effect of applications on nitrate accumulation [41].

It was interesting to observe how the CL treated with moringa showed a significant reduction (−70%) compared to the control plants. Negative effect was instead observed in BN, where the samples treated with moringa showed an increase in the nitrate content of 60% but these always remain below the maximum permitted threshold of European Commission Regulation.

The results of our work showed that the response to treatment with biostimulant is species-specific. This agrees with those obtained by Kocira et al. [65] where, in a study on different bean cultivars treated with *Ecklonia maxima* extract, the result was dependent on cultivar and frequency of application and concentration of the biostimulant.

5. Conclusions

Results confirm that it is not possible to generalize the effect of plant-based natural biostimulants on plants as they consist of different substances with heterogeneous composition. In fact, a biostimulant can have a variable effect from species to species and in some cases from cultivar to cultivar [14]. The results of this study showed how the foliar application of moringa extract was effective in improving various nutraceutical parameters, in particular in kale; the extract seems to have optimized the responses at the level of the whole plant, thus improving the quality and nutraceutical properties of this product. Further in-depth investigation on the composition of the plant-based biostimulant and the molecules of interest present in the aqueous extract also to define the optimal dose will be necessary. These studies will be replicated among different genotypes because there appears to be a species-specific response.

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