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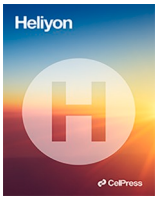

Research article

Plant extract improves quality traits of green and red lettuce cultivars

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ABSTRACT

The use of biostimulants in agriculture has been rapidly increasing in recent years, because of their positive effects on crop yield, product quality, and tolerance to abiotic stresses. In the present study the efficacy of multiple applications of a plant-derived biostimulant, obtained from the aqueous maceration of borage (*Borago officinalis*, L.) flowers on two lettuce cultivars, namely a green (*Lactuca sativa* L. cv. Expertise RZ) and red (*Lactuca sativa* L. cv. Codex RZ) Salanova® was evaluated. The treatment was applied at 10 mL L^{-1} as foliar spray three times, once a week starting from two weeks after transplant. Control plants were treated with water. Non-destructive measurements (pigments, leaf nitrogen index, chlorophyll *a* fluorescence) were taken during plant growth after at each treatment application. At the end of the experiment, destructive analyses were performed to assess qualitative traits. The research work was focused on the evaluation of physiological parameter changes during plant growth, and on primary and secondary metabolism. Foliar applications did not affect the accumulation of total sugars (4.56 mg g^{-1} in Expertise, 3.5 mg g^{-1} in Codex) in either cultivar. However, the lettuce head weight was negatively affected by the extract application in red cultivar (-10 g/plant), while no changes were observed in the green lettuce. The nitrogen-flavonol index (NFI) increased after the third application of borage extract in green cultivar (+67 %), suggesting an improvement of nitrogen nutrition status or a reduced stress condition. A different response resulted in term of maximum quantum efficiency of PSII (F_V/F_M), performance index (PI), nitrate, and anthocyanin accumulation in leaves. The F_V/F_M ratio significantly increased in green cultivar after the first application (from 0.80 to 0.84) and at harvest (from 0.79 to 0.84). The PI showed a slight but not significant increase at the same time points. On the contrary, the PI was significantly higher in red cultivar after the third application (+9.4 %). Interestingly, the borage extract induced a significant decrease of nitrate accumulation in lettuce leaves of the red cultivar (from 4149.7 to 2711.6 mg/kg, −34 %). At the same time a positive variation of anthocyanin content was observed in red lettuce (+24.7 %). The application of biostimulant products might improve the quality of some lettuce varieties as regards the accumulation of metabolites useful for the plant to overcome stress conditions and fundamental in human healthy diet, increasing the leaf concentration of Ca, Na, and Mg.

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1. Introduction

Lettuce (*Lactuca sativa* L.) is one of the most important leafy vegetables, cultivated and consumed worldwide. It is particularly appreciated for its taste and its consumption has risen owing to its high water content (95 %), low calorific value, fat and sodium, and its perception as a healthy and dietetic food [1]. Because lettuce is usually eaten raw, it is a valuable source of compounds that are important for prevention of many diseases [2]. Lettuce is rich in several antioxidants such as minerals, vitamins (vitamin C and folate), polyphenols, terpenoids, flavonoids, chlorophylls, and carotenoids [2–5]. The accumulation of components with nutraceutical properties is affected by pre-harvest [6–10] and post-harvest factors [11–15] and even more by genetic diversity among cultivars [16–20]. The high genetic diversity in *Lactuca sativa* species is the result of its polyphyletic origin and domestication process [21]. Lettuce can be classified into seven groups of cultivars called morphotypes: (a) butterhead lettuce, (b) crisphead lettuce, (c) cos lettuce, (d) cutting lettuce, (e) stalk lettuce, (f) Latin lettuce, and (g) oilseed lettuce [22]. Cultivated lettuce varieties exhibit varying leaf morphological traits such as shape, texture, and colour, which are important factors in consumer perception and appeal. According to colouring, lettuce leaves are mainly green and red, with some variegated cultivars, and this is mainly related to the contribution of chlorophylls and anthocyanins. Lettuce cultivars with red-pigmented leaves have a high concentration of phenolic compounds, particularly anthocyanins, essential oils, and carotenoids, making them particularly attractive to consumers and suggesting their health-promoting properties [4]. In addition to being a good source of bioactive compounds, lettuce, like other leafy vegetables, is considered to accumulate nitrate which may be dangerous for human health. The European Commission established maximum limits of nitrate content in selected leafy vegetable species, including lettuce, reduce the potential harmful effects of nitrate metabolites and reaction products in the human body [23]. Furthermore, different agronomic techniques have an impact on product quality, improving crop productivity, ameliorating nutritional quality, and extending post-harvest duration [19,24–27]. Among these practices, the application of various biostimulant products has become valuable to increase the qualitative traits of lettuce grown under different environmental conditions [28–32]. The application of plant biostimulants is considered an ecological approach for increasing the sustainability of agricultural practices. Thus, ensuring high-quality produce obtained with sustainable agricultural practices is essential for market competitiveness and consumer satisfaction. However, the genetic characteristics of different species and cultivars lead crops to respond differently to the application of a biostimulant. To avoid waste and ensure a truly sustainable approach, it is necessary to understand and test the effectiveness of biostimulants on various species and cultivars. Thus, this study aimed at determining the effect of a plant-derived biostimulant prototype on the growth characteristics, quality traits, and physiological aspects associated with primary and secondary metabolism of two lettuce cultivars differing for the leaf colour.

The hypothesis of this work was to enhance the quality of leafy vegetables using borage extract as biostimulant with the aim to improve of light use efficiency and the nutritional quality and increasing the antioxidant compounds.

2. Materials and methods

2.1. Plant materials, experimental design, and harvesting

The experimental trial was carried out in the experimental greenhouse of the University of Milan in autumn 2022. The minimum and maximum temperatures of the air and the relative humidity in the greenhouse during culture are in Fig. 1. On October 3, 2022, plants of two lettuce cultivars, namely a green (*Lactuca sativa* L. cv. Expertise RZ) and red (*Lactuca sativa* L. cv. Codex RZ) Salanova® were transplanted in pots (14 × 14 cm, 2 L) in a commercial growing medium containing peat (Vigorplant, Fombio, Italy) considering a plant density of 8 plants m^{-2}. Lettuce plants were treated with a plant based biostimulant prototype obtained from borage flower maceration (500 g borage flower in 1 L water, kept in dark condition, at room temperature (20 °C) for 25 days, then filtered and diluted 10 mL L^{-1}), obtained as described in a previous experiment [33] and tested in other studies [34–36]. The treatment was applied as

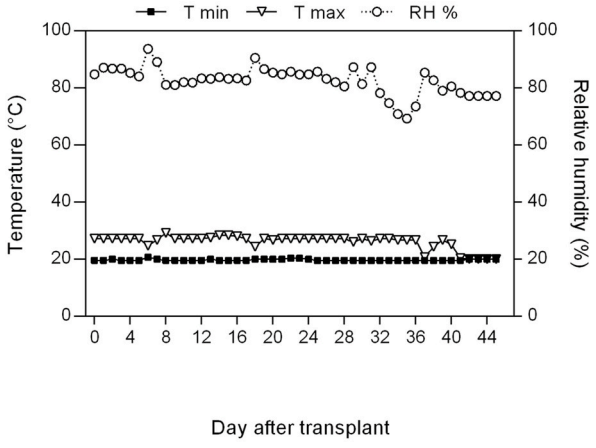

Fig. 1. Maximum and minimum air temperatures and relative humidity in the greenhouse during the experiment.

foliar spray three times once a week starting from two weeks after transplant. To discriminate the effect of borage extract applications, plants were treated with a solution containing 10 mL L^{-1} of borage extract and compared with plants sprayed with water as Control. The concentration of 10 mL L^{-1} was based on previous experiments [33]. During the growing cycle, non-destructive measurements were taken 2 days after each application to monitor the plant status over time. At the end of the experiment, all plants were harvested by cutting the whole head to determine biometric and quality parameters. The plants were immediately weighed to determine the fresh aboveground biomass, a subsample was stored at −20 °C for qualitative analysis and other leaf tissue samples were oven dried at 65 °C until constant weight was reached and calculate the dry matter percentage.

2.2. Physiological parameters in vivo: chlorophyll, flavonol, anthocyanin, NFI, and chlorophyll a fluorescence

During plant growth, specifically 2 days after each foliar application, and at harvest time, chlorophyll, flavonol, anthocyanin, and nitrogen flavonol index (NFI) were determined in vivo randomly choosing six expanded lettuce leaves, thereby 24 replicates in total. The measurements were taken by using a portable field instrument exploiting the fluorescence and transmittance properties of the leaf (multi pigment meter MPM-100, ADC BioSCientific Ltd.). The chlorophyll index was calculated as: log (T850/T720) - 1, where T is the transmittance at two wavelengths, 850 and 720 nm. The flavonol index was calculated as: log (F660/F375), where F is the fluorescence at two wavelengths, 660 and 375 nm. The anthocyanin index was calculated as: log (F660/F525), where F is the fluorescence at two wavelengths, 660 and 525 nm. The NFI was calculated as the ratio between chlorophyll and flavonol index. At the same time points the chlorophyll *a* fluorescence was measured using a hand-portable fluorimeter (Handy-PEA, Hansatech Instruments, UK). Prior to analysis, a leaf sections of six leaves each treatment, was dark-adapted for 20 min with leaf clips, then they were exposed to a saturating light (3000 μmol m^{-2} s^{-1}) provided by an array of three high-intensity light-emitting diodes for 1 s. The measured parameters included the maximum quantum efficiency of photosystem II (PSII) photochemistry (Fv/Fm ratio), the performance index (PI) and the JIP test parameters explaining the energy fluxes per cross section: the absorption flux (ABS/CSm), the trapped energy flux (TR/CSm), the electron transport (ET/CSm), the total energy dissipation (DIo/CSm) and the density of active reaction centres (RC/CSm) [37].

2.3. Leaf pigments determination: total chlorophyll, carotenoids, total phenols, and anthocyanin

Chlorophyll $a + b$ and carotenoids were extracted from red and green lettuce leaves with 99.9 % (v/v) methanol. Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer were kept in a dark room for 24 h at 4 °C into 15 mL tubes filled with 5 mL of methanol. Their content was colorimetrically determined, absorbance reading was measured at 665.2 and 652.4 nm for chlorophylls and 470 nm for total carotenoids with a spectrophotometer (Evolution 300, Thermo Electron Corporation). Pigments levels were calculated by Lichtenthaler's [38] formula and expressed on a fresh weight basis. Total phenols and anthocyanin were extracted with 3 mL of methanol acidified with HCl (1 %). Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer, were kept in dark room for 24 h at 4 °C. Then, absorbance readings were measured at 320 nm for total phenols, and at 535 nm for anthocyanin using a spectrophotometer (Evolution 300, Thermo Electron Corporation). Phenolic index was expressed as $Abs_{320\ nm}$ g^{-1} FW. Anthocyanin concentrations were expressed as cyanidin-3-glucoside equivalents using the molar extinction coefficient (ε) of 29,600 L M^{-1} cm^{-1} [39].

2.4. Total sugar, reducing sugars, and nitrate concentrations

The total sugars concentration was determined using the anthrone method with slight modifications [40]. Approximately 1 g of leaf tissue was homogenized in a mortar with 3 mL of water. The mixture was centrifuged at 4000 rpm for 15 min at RT (ALC centrifuge-model PK130R) and the supernatant was separated and used for the analyses. The anthrone reagent (10.3 mM) was prepared dissolving anthrone in 95 % H_2SO_4. The reagent was left to stand for 30–40 min before use, 0.5 mL extract was placed on top of 2.5 mL of the anthrone reagent and incubated in ice for 5 min, then mixed vigorously. The tubes were heated to 95 °C for 10 min and left to cool in ice. Readings were performed with a spectrophotometer at 620 nm. Calibration curve was carried out using a glucose standard solution.

Reducing sugars were determined on the same aqueous extract using the dinitrosalicylic (DNS) acid method [41]. This colorimetric technique consists of a redox reaction between the 3,5-dinitrosalicyclic acid and the reducing sugars in the leaf sample extract. DNS assay was performed by mixing 0.2 mL of supernatant with 0.2 mL of DNS and incubated in a water bath at 100 °C for 5 min, then 1.5 mL of water was added to samples. After cooling at room temperature, the optical density was determined spectrophotometrically at 530 nm, using a glucose standard curve.

Nitrate concentration was determined in the leaf extracts by a colorimetric method [42]. In a 15 mL tube, 80 μL of 5 % (w/v) salicylic acid in concentrated H_2SO_4 were added to 20 μL of plant extract. Each sample was rapidly mixed and 3 mL of 1.5 N NaOH were added. The samples were cooled to RT and absorbance at 410 nm was measured with a spectrophotometer. Nitrate content was calculated referring to a KNO_3 standard calibration curve.

2.5. Mineral composition

Total C and total N were determined from oven-dried at 65 °C lettuce leaves by the dry combustion method using a ThermoQuest NA1500 elemental analyser (Carlo Erba, Milano, Italy). Once dry, leaf samples were ground into powder with an oscillating mill (model MM 400, Retsch, GmbH, Retsch-Allee, Haan) and a known quantity (3.5 mg) weighted into tin (Sn) capsules, subsequently

crimped. The concentrations of N and C in leaf samples were calculated based on the area of their respective peaks using an atropine calibration curve. Minerals were determined from lettuce leaves samples dried and milled as described above. Aliquots (0.2 g) of dry powdered lettuce leaves were digested by a microwave digestion system (Anton Paar MULTIWAVE-ECO) in Teflon tubes filled with 10 mL of 65 % HNO_3. Then, a one-step temperature ramp (increasing to 210 °C in 10 min, maintained for 10 min) was applied, followed by a cooling time of 20 min. The mineralized samples were transferred into polypropylene tubes and diluted to 1:40 with 1.3 M HNO_3 in MILLI-Q water. The concentration of the elements was measured by ICP-MS (Agilent 7850 ICP-MS).

2.6. Statistical analyses

Data were subjected to Two-way ANOVA and Sidak post-test ($P < 0.05$) was used to assess the differences among means. Analyses were performed using GraphPad Prism version 9 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Additional information is reported in each figure and table's caption.

3. Results

3.1. Effects on chlorophyll, flavonol, anthocyanin, NFI in vivo

Leaf chlorophyll concentration estimated in vivo was similar in both cultivars, regardless of leaf colour, after each application of the plant extract, and at harvest, no significant differences were detected (Table 1). Instead, the level of flavonol was significantly higher in red-pigmented leaves than in green lettuce at each time point, even if a decrease over time in both cultivars. A significant interaction between treatment (T) and cultivar (cv) was observed only after the first application of the borage extract inducing a significant decrease (−32 %) in flavonol concentration in the red cultivar, whereas it did not have any on effect in flavonol levels measured in green lettuce (average value of 0.42). A similar trend was observed for the anthocyanin content, in which the red cultivar had a significantly higher concentration of anthocyanin than the green one each time point, and a decrease was observed over plant growth. In contrast, the NFI increased over time in both the cultivars. The first application of the borage extract induced a decrease in the NFI of green lettuce whereas it did not affect the NFI of red lettuce. Interestingly, after the third application, NFI was significantly higher (+67

Table 1
Chlorophyll, Flavonol, Anthocyanin, and NFI measured in vivo in green (Expertise) and red (Codex) lettuce leaves. Measurements were taken 2 days after the first (I), the second (II), the third (III) application, and at harvest (IV).

Time of measurement	Cultivar (cv)	Treatment (T)	Chlorophyll (T850nm/T720nm)	Flavonol (F660nm/F325nm)	Anthocyanin (F660nm/F525nm)	NFI (T850/T720)/(F660/F325)
I	Expertise	Control	0.39 ± 0.071	0.40 ± 0.045^{Ba}	0.06 ± 0.011^{Ba}	1.29 ± 0.228^{Aa}
		Treatment	0.46 ± 0.068	0.44 ± 0.063^{Ba}	0.05 ± 0.014^{Ba}	0.85 ± 0.084^{Ab}
	Codex	Control	0.44 ± 0.056	0.97 ± 0.075^{Aa}	0.40 ± 0.025^{Aa}	0.47 ± 0.059^{Ba}
		Treatment	0.36 ± 0.040	0.65 ± 0.063^{Ab}	0.29 ± 0.020^{Ab}	0.54 ± 0.079^{Aa}
II	Expertise	Control	0.25 ± 0.046	0.46 ± 0.134	0.04 ± 0.011^{Ba}	0.69 ± 0.244
		Treatment	0.37 ± 0.113	0.43 ± 0.106	0.05 ± 0.012^{Ba}	0.79 ± 0.310
	Codex	Control	0.31 ± 0.051	0.53 ± 0.070	0.23 ± 0.020^{Aa}	0.46 ± 0.134
		Treatment	0.26 ± 0.040	0.47 ± 0.132	0.16 ± 0.032^{Aa}	1.05 ± 0.422
III	Expertise	Control	0.37 ± 0.059	0.07 ± 0.022^{Ba}	0.04 ± 0.011^{Ba}	5.25 ± 1.308^{Ab}
		Treatment	0.38 ± 0.045	0.10 ± 0.062^{Aa}	0.05 ± 0.010^{Aa}	8.79 ± 1.181^{Aa}
	Codex	Control	0.33 ± 0.035	0.23 ± 0.018^{Aa}	0.16 ± 0.015^{Aa}	1.54 ± 0.126^{Ba}
		Treatment	0.39 ± 0.062	0.16 ± 0.024^{Aa}	0.09 ± 0.008^{Ab}	3.27 ± 0.373^{Ba}
IV	Expertise	Control	0.40 ± 0.049	0.11 ± 0.015^{Ba}	0.04 ± 0.007^{Ba}	2.83 ± 0.463^{Ab}
		Treatment	0.44 ± 0.055	0.09 ± 0.009^{Ba}	0.04 ± 0.011^{Aa}	5.15 ± 0.731^{Aa}
	Codex	Control	0.40 ± 0.033	0.34 ± 0.029^{Aa}	0.14 ± 0.017^{Aa}	1.20 ± 0.127^{Ba}
		Treatment	0.33 ± 0.017	0.28 ± 0.029^{Aa}	0.11 ± 0.031^{Aa}	1.24 ± 0.095^{Ba}
Significance						
I	T x cv		ns	*	*	*
	T		ns	*	*	ns
	cv		ns	***	***	***
II	T x cv		ns	ns	ns	ns
	T		ns	ns	ns	ns
	cv		ns	ns	***	ns
III	T x cv		ns	ns	*	ns
	T		ns	ns	*	*
	cv		ns	*	***	***
IV	T x cv		ns	ns	ns	*
	T		ns	ns	ns	*
	cv		ns	***	***	***

All values are means ± SEM (n = 6). The mean is subjected to a two-way ANOVA. Different uppercase and lowercase letters indicate significant differences between varieties and treatment, respectively, at $p < 0.05$. ns, *, *** means not significant or significant at $p < 0.05$ and 0.001, respectively.

%) in green lettuce treated with borage extract (8.79) than in the control (5.25). No significant difference was observed in the red-pigmented cultivar; however, the NFI in treated plants doubled compared with the non-treated samples. The same trend was observed at harvest where the NFI of green-treated plants was significantly higher than that of control plants.

3.2. Effects on chlorophyll a fluorescence parameters

The graphs of chlorophyll *a* fluorescence parameters show the overall response of photosynthesis to the application of borage extract in green (Fig. 2) and red (Fig. 3) lettuce cultivars after each application and at harvest. Data obtained were normalized to 0 – dotted line representing the non-treated plants. In the green-pigmented cultivar, the borage extract strongly affected a large number of parameters after the first application, as shown by the distance of the solid black line from the reference line (Fig. 2). In particular, PI inst. and PI total, as well as the ABS/CSm, TRo/CSm, ETo/CSm, REo/CSm, and Area were higher in treated plants than in control plants, whereas DIo/CSm and Fo were similar to the control. After the second and third applications the highest distance between solid

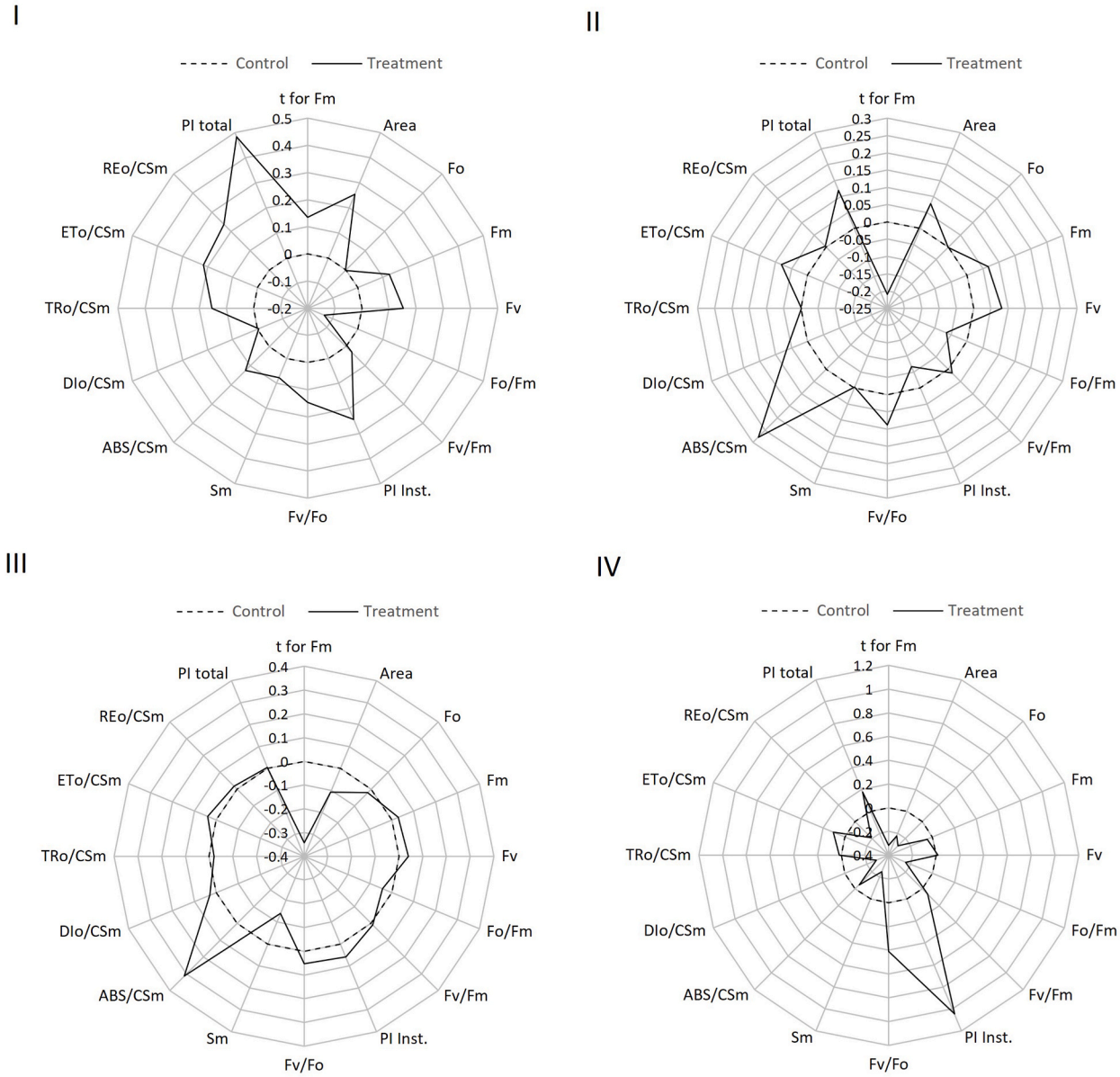

Fig. 2. Chlorophyll *a* fluorescence parameters measured in vivo in green (Expertise) lettuce leaves. Measurements were taken 2 days after the first (I), the second (II), the third (III) application, and at harvest (IV). Values are means ± SE (n = 6). (Ft – Fc)/Fc, was used to normalize the data. In the formula, "Ft" and "Fc" represent the values of treated plants and control plants treated with water, respectively. Values of "Fc" plants were normalized to 0 (control plants treated with water, dotted line circle = 0).

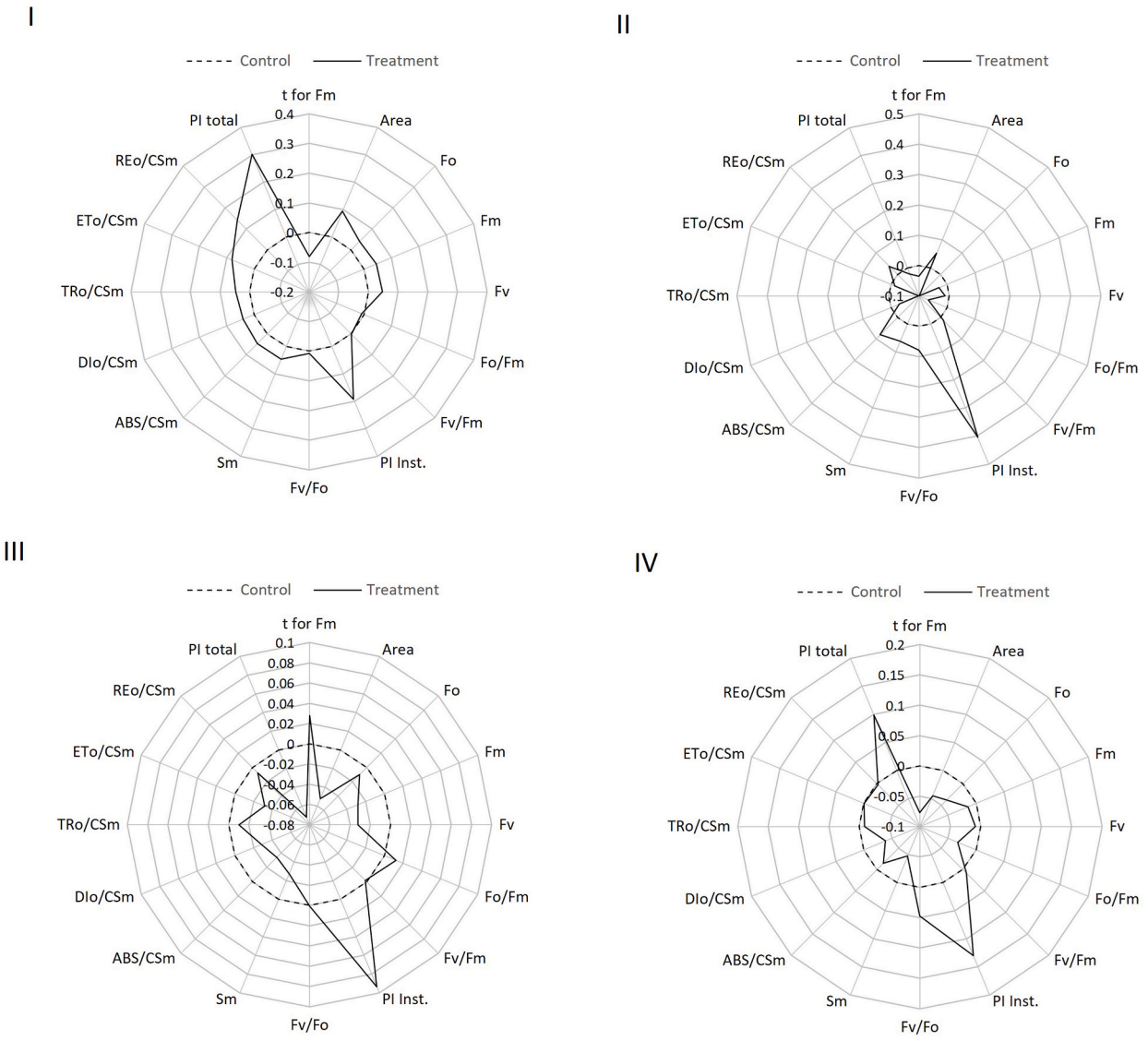

Fig. 3. Chlorophyll *a* fluorescence parameters measured in vivo in red (Codex) lettuce leaves. Measurements were taken 2 days after the first (I), the second (II), the third (III) application, and at harvest (IV). Values are means ± SE (n = 6). (Ft – Fc)/Fc, was used to normalize the data. In the formula, "Ft" and "Fc" represent the values of treated plants and control plants treated with water, respectively. Values of "Fc" plants were normalized to 0 (control plants treated with water, dotted line circle = 0).

line representing the treated plants and the spotted line was observed for ABS/CSm, instead at harvest treated plants showed a value of PI inst. higher than that in the control plants. Moreover, the Fv/Fm index was generally higher than the reference threshold 0.83 in treated plants at each time point whereas, the Fv/Fm index was generally below the threshold in control plants.

Chlorophyll *a* fluorescence parameters showed a different pattern in red-pigmented cultivar in response to the application of borage extract (Fig. 3). The first application induced an increase in PI inst. and PI total, similar to those observed in green lettuce. However, all parameters related to energy fluxes were not affected. The peak in PI inst. was constant in treated plants after each application and at harvest. None of the other parameters was strongly affected by the borage extract.

3.3. *Effects on pigment: total chlorophyll, carotenoids, total phenols, and anthocyanin*

The concentration of chlorophyll *a*+b was significantly affected by cultivar and treatment (Fig. 4A). The level of total chlorophyll was higher in red leaf lettuce (1.40 μg mg^{-1}) than in green lettuce (0.98 μg mg^{-1}). At the same time, the application of the borage extract induced a significant increase of approximately 16 % in chlorophyll concentration in the Expertise cultivar, whereas it did not show any effect on the Codex cultivar. Similarly, the concentration of carotenoids was significantly higher in Codex than in Expertise.

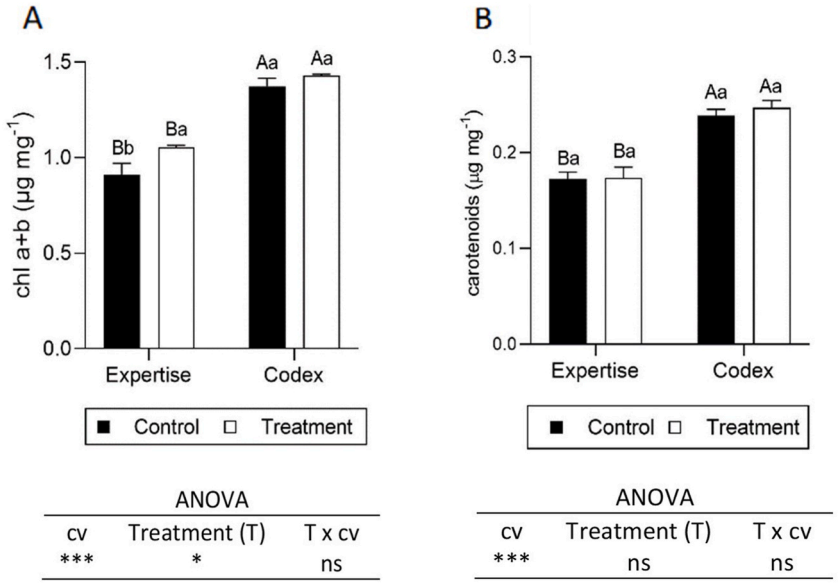

Fig. 4. Chlorophyll *a* + b (A) and Carotenoids (B) concentration in green (Expertise) and red (Codex) lettuce leaves cultivars. Measurements were taken at harvest. Values are means ± SE (n = 4). The mean is subjected to a two-way ANOVA. Different uppercase and lowercase letters indicate significant differences between varieties and treatment, respectively, at $p < 0.05$. ns, *, *** means not significant or significant at $p < 0.05$ and 0.001, respectively.

However, the borage extract did not affect carotenoid levels in any of the cultivars (Fig. 4B). For the analyses of phenol index, there was a significant interaction between treatment (T) and cultivar (cv) (Fig. 5A). Moreover, the phenol index measured in Expertise leaves (393.6 Abs_{320nm} g^{-1}) was almost 70 % lower than that measured in Codex leaves (1283.5 Abs_{320nm} g^{-1}). The application of borage extract induced a significant increase (+23 %) in this index in red lettuce and treated plants reached 1418.1 Abs_{320nm} g^{-1}. Likewise, the concentration of anthocyanin was higher in the red-pigmented cultivar than in the green one, and borage extract had a positive effect on their accumulation (Fig. 5B), inducing an increase of about 30 % from 23 to 30 mg Cyanidin eq. 100 g^{-1} in treated plants of the Codex cultivar.

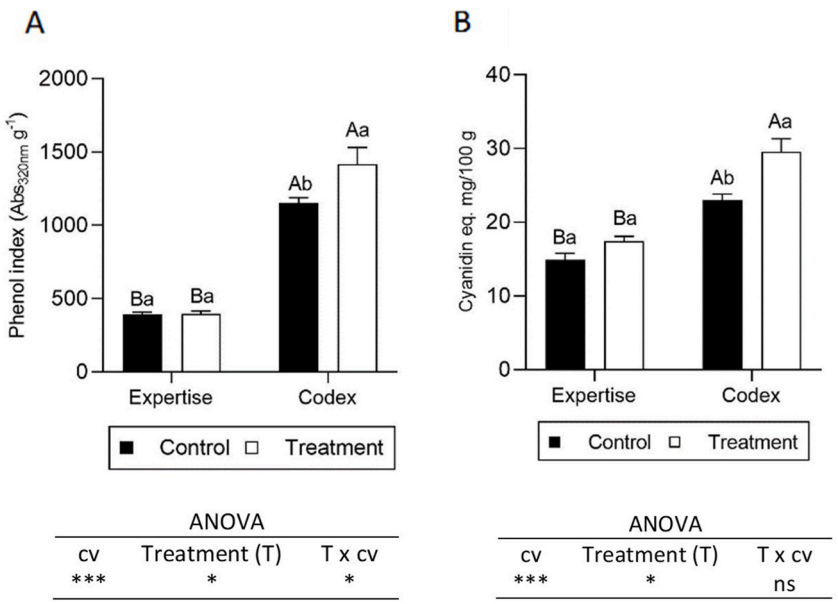

Fig. 5. Phenol index (A) and Anthocyanin (B) concentration in green (Expertise) and red (Codex) lettuce leaves cultivars. Measurements were taken at harvest. Values are means ± SE (n = 4). The mean is subjected to a two-way ANOVA. Different uppercase and lowercase letters indicate significant differences between varieties and treatment, respectively, at $p < 0.05$. ns, *, *** means not significant or significant at $p < 0.05$ and 0.001, respectively.

3.4. Effects on fresh biomass, yield, total sugar, reducing sugars, and nitrates

Lettuce head weight was negatively affected by extract application in the red cultivar, whereas it did not change in the green cultivar. The decrease in fresh biomass observed in the Codex cultivar was approximately 10 g. The same trend has been observed in lettuce yield (Table 2). Borage extract applied as a foliar spray on lettuce leaves did not significantly alter the concentration of total and reducing sugars in either cultivar. The green lettuce cultivar had a generally higher concentration of total sugars than the red cultivar, with a mean value of 4.56 and 3.5 mg g^{-1} FW of glucose, respectively. Such difference was not observed in the concentration of reducing sugars and the average value was around 1.46 mg g^{-1} FW of glucose. Interestingly, the accumulation of nitrates in red lettuce leaves was significantly affected by the application of borage extract, with a decrease of about −34.7 %. In particular, the nitrate concentration in the control plants was around 4300 mg g^{-1} FW, regardless of the cultivar, whereas the treated plants of the codex cultivar showed a lower nitrate concentration (2711.6 mg g^{-1} FW). The same effect was not observed in green cultivar leaves, where the concentration of nitrates in the treated plants was comparable that to in the control.

3.5. Effects on C and N and minerals

Cultivars and treatments had a significant effect on both total N and total C measured in lettuce leaves (Table 3). In particular, total C was significantly higher in red cultivar (38.11 %) than in the green lettuce (36.59 %). In contrast, total N showed the opposite trend, and it was significantly higher in green lettuce (5.21 %) than in red lettuce (4.79 %). The application of the borage extract induced a significant increase in total C in the green leaf cultivar and a significant increase in total N in the red leaves.

Table 4 reports the concentration of nine mineral elements. A significant interaction between cv and T resulted only for the concentration of Ni. The two cultivars had similar levels of K, Ca, Mn, Ni, and Zn, whereas significant differences between cv were found for Na, Mg, P, and Se. At the same time, the application of the borage extract induced a significant effect in the accumulation of Na, Mg, Ca, Mn, and Ni. In particular, treated plants of Expertise and Codex cultivar showed levels of Ca and Mn almost two times higher than control ones. Treated plants of red leaves cultivar showed the highest concentration of Ni and almost equal to zero of Se.

4. Discussion

The nutritional quality of lettuce is regulated by both genetic characteristics and crop management practices [43]. This study aimed to investigate the responses of two leaf-coloured lettuce cultivars to the application of a plant extract previously tested under different experimental conditions [33–36]. Lettuce is a leafy vegetable, and because it is mostly eaten raw, its nutritional value is associated with the presence of antioxidant compounds and minerals. Antioxidants, such as chlorophylls, carotenoids, and anthocyanins, also play a role as pigments, being relevant in the determination of colour and thus, in visual quality, having an important impact on consumer appreciation. In a scenario where interest in food quality is growing and consumers are willing to pay more for products with a high nutritional value, finding a treatment that can increase these traits might represent an added value [44]. The red-leaf lettuce tested in this study showed a higher concentration of anthocyanin and flavonols than the green-leaf variety, as expected, owing to the genetic properties and because they are responsible for the red colour. Similar results have been observed by other authors comparing green and red lettuce cultivars [45,46]. The non-destructive measurement of these pigments, performed with a multi pigment meter and based on leaf fluorescence, did not reveal any strong change in response to each treatment application during the growing cycle, probably indicating that from an aesthetic point of view, leaf colour did not change significantly. However, destructive analyses performed at harvest showed an increased concentration of anthocyanin and phenols in the red-leaf cultivar treated with the borage

Table 2
Fresh biomass, Yield, Total sugars, Reducing sugars, and Nitrates measured in green (Expertise) and red (Codex) lettuce leaves. Measurements were taken at harvest.

Cultivar (cv)	Treatment (T)	Fresh biomass (g $plant^{-1}$)	Yield (g m^{-2})	Total sugars (Glucose mg g^{-1} FW)	Reducing sugars (Glucose mg g^{-1} FW)	Nitrate (mg kg^{-1} FW)
Expertise	Control	58.8 ± 0.49^{Aa}	470 ± 3.9^{Aa}	4.69 ± 0.447	1.63 ± 0.187	4522.4 ± 304.55^{Aa}
	Treatment	49.4 ± 3.13^{Aa}	395 ± 25.1^{Aa}	4.43 ± 0.341	1.27 ± 0.082	4447.0 ± 404.44^{Aa}
Codex	Control	53.8 ± 3.73^{Aa}	430 ± 29.9^{Aa}	3.71 ± 0.605	1.50 ± 0.190	4149.7 ± 171.49^{Aa}
	Treatment	43.8 ± 0.90^{Ab}	350 ± 7.2^{Ab}	3.29 ± 0.423	1.42 ± 0.053	2711.6 ± 375.77^{Bb}
Significance						
T x cv		ns	ns	ns	ns	ns
T		*	*	ns	ns	*
cv		ns	ns	*	ns	*

All values are means ± SEM (n = 4). The mean is subjected to a two-way ANOVA. Different uppercase and lowercase letters indicate significant differences between varieties and treatment, respectively, at $p < 0.05$. ns, *, *** means not significant or significant at $p < 0.05$ and 0.001, respectively.

Table 3
Total C and N in green (Expertise) and red (Codex) lettuce leaves. Measurements were taken at harvest.

Cultivar (cv)	Treatment (T)	C (%)	N (%)
Expertise	Control	36.4 ± 0.05^{Ab}	5.03 ± 0.022^{Aa}
	Treatment	36.8 ± 0.17^{Aa}	5.38 ± 0.083^{Aa}
Codex	Control	37.9 ± 0.05^{Ba}	4.59 ± 0.026^{Bb}
	Treatment	38.3 ± 0.07^{Ba}	4.99 ± 0.163^{Ba}
Significance			
T x cv		ns	ns
T		*	*
cv		***	*

All values are means ± SEM (n = 3). The mean is subjected to a two-way ANOVA. Different uppercase and lowercase letters indicate significant differences between varieties and treatment, respectively, at $p < 0.05$. ns, *, *** means not significant or significant at $p < 0.05$ and 0.001, respectively.

Table 4
Content of Na, Mg, P, K, Ca, Mn, Fe, Cu, Ni, Se, and Zn in green (Expertise) and red (Codex) lettuce leaves. Measurements were taken at harvest.

Cultivar (cv)	Treatment (T)	Na (mg/g DW)	Mg (mg/g DW)	P (ng/g DW)	K (mg/g DW)	Ca (mg/g DW)	Mn (μg/g DW)	Ni (μg/g DW)	Se (μg/g DW)	Zn (μg/g DW)
Expertise	Control	1.41 ± 0.092^{Ba}	3.35 ± 0.274^{Aa}	1.57 ± 0.225	64.2 ± 4.79	5.46 ± 1.299^{Aa}	12.65 ± 0.113	0.36 ± 0.205^{Aa}	1.25 ± 0.240^{Aa}	39.47 ± 6.226
	Treatment	1.94 ± 0.034^{Bb}	4.52 ± 0.179^{Ab}	1.86 ± 0.084	62.9 ± 0.78	10.71 ± 0.36^{Ab}	29.60 ± 9.302	0.45 ± 0.139^{Aa}	1.98 ± 0.755^{Aa}	63.06 ± 3.307
Codex	Control	1.12 ± 0.075^{Aa}	3.71 ± 0.139^{Aa}	2.23 ± 0.228	61.8 ± 2.25	6.33 ± 0.707^{Aa}	12.07 ± 0.250	0.25 ± 0.146^{Aa}	0.24 ± 0.138^{Aa}	61.65 ± 17.790
	Treatment	1.40 ± 0.061^{Ab}	5.20 ± 0.217^{Ab}	2.14 ± 0.098	61.6 ± 1.99	10.34 ± 0.753^{Ab}	16.80 ± 0.739	3.03 ± 1.122^{Bb}	0.00 ± 0.000^{Ba}	53.79 ± 5.853
Significance										
T x cv		ns	ns	ns	ns	ns	ns	*	ns	ns
T		*	***	ns	ns	***	*	*	ns	ns
cv		*	*	*	ns	ns	ns	ns	***	ns

All values are means ± SEM (n = 3). The mean is subjected to a two-way ANOVA. Different uppercase and lowercase letters indicate significant differences between varieties and treatment, respectively, at $p < 0.05$. ns, *, *** means not significant or significant at $p < 0.05$ and 0.001, respectively.

extract. These results along with the phenolic compounds increase indicated that borage extract activate the phenylpropanoids pathway [47]. In our study, anthocyanins were quantified as equivalent to Cyanidin-3-glucoside, the most important anthocyanidin in red lettuce, with strong radical scavenging activity [48]. These results suggest that lettuce cultivars with a genetically higher concentration of anthocyanin respond more strongly to the application of the borage extract in terms of accumulation of these compounds. Interestingly, the same extract, obtained by the maceration of borage flowers and applied at a concentration of 10 mL L^{-1} increased total phenols and flavonoids in a different lettuce variety with green leaves after two applications [33]. These results provide evidence of variance among cultivars regarding the effectiveness of these products. Similarly, foliar application of plant-derived biostimulants to red and green lettuce cultivars revealed a significant interaction between cv and the treatment in the content of specific phenols and flavonoids [49]. In particular, a higher concentration of these molecules was found in the red cultivar than in the green cultivar, and a significant increase in specific compounds in response to plant-based products was observed, especially in the red cultivar. At the same time, the red-leaf cultivar 'Codex' studied in our experiment showed a significantly higher concentration of carotenoids and total chlorophylls than the green cultivar 'Expertise'. A literature review reported that the levels of pigments such as carotenoids and chlorophyll are sensitive to plant growth conditions, but there could also be variation among cultivars [50]. In our experiment, the two cultivars grew under the same environmental conditions, thus, the differences observed could be attributed to the genetic differences between the cultivars. Interestingly, screening of the composition of metabolites in 23 diverse lettuce cultivars showed a higher accumulation of carotenoids in red lettuce cultivars than in green/red and green ones [48]. Carotenoids are necessary components of the photosynthetic apparatus and act as antioxidants and light-harvesting pigments. Moreover, they are responsible for the red, purple, yellow, and orange colours of fruits and vegetable [51]. However, the application of the borage extract did not affect the concentration of carotenoids in either the green or red cultivars. In contrast, a significant increase (+16 %) in total chlorophyll concentration in response to foliar application of the plant extract was observed only in green lettuce 'Expertise'. Chlorophylls, carotenoids, vitamins, and other bioactive compounds are associated with the health benefits of lettuce. Chlorophyll has been claimed to play an important role in anticarcinogenic and antimutagenic activities as a cancer-preventing agent [52]. Plant biostimulants of different origins have been shown to improve the accumulation of chlorophyll in leafy vegetables [31]. In addition to the effect on chlorophyll content, an important analysis for determining the health status of plants is the measurement of chlorophyll *a* fluorescence. An increase in the density of active reaction centres (RC/CSm) and the performance index was observed in green leaf cultivar after almost every

application. In contrast, the red-leaf cultivar only showed an increase in the performance index after the application of the borage extract. The two cultivars showed different response patterns in terms of the analysed parameters. In particular, borage extract seemed to affect green leaf lettuce, both in terms of chlorophyll content and increasing the efficiency of photosynthesis.

One of the most important aspects when discussing the quality of leafy vegetables is the concentration of nitrate in leaves. They are regulated at the European level by European Regulation No. 915/2023 setting the maximum thresholds of nitrates due to their potential negative effects on human health. According to this regulation, fresh lettuce harvested between October and March and grown under protect cultivation must not exceed a threshold of 5000 mg NO_3 kg^{-1} FW. The concentrations of nitrates in the green and red leaf cultivars used in our experiment were below the legal limit. Interestingly, the application of borage extract induced a significant decrease of nitrates concentration in 'Codex' cultivar. A similar effect was previously reported in response to the application of the same extract in rocket salads [36]. The authors observed that lower nitrates were linked to a higher activity of the enzyme nitrate reductase in the same samples. Despite the decrease in nitrate concentration, red lettuce leaves showed an increase in leaf nitrogen following treatment with borage extract. These findings suggest better assimilation of nitrates without affecting the total amount of nitrogen. Both nitrogen and carbon were higher in the green-leaf cultivar than in the red-leaf cultivar. These results are partially in line with those obtained in another study where the authors found higher levels of carbon and lower levels of nitrogen in the red cultivar than in the green one [53]. The application of borage extract induced an increase in both red and green leaf cultivars of essential minerals (Na, Mg, and Ca) for human health and metabolism. Sodium is important for several metabolic functions. However, when consumed in large quantities, it may lead to physiological disorders associated with high blood pressure. Thus, the WHO recommends less than 2000 mg/day of sodium for adults [54]. The Na concentration in lettuce varies among cultivars ranging from 0.8 mg g^{-1} DW to 44 mg g^{-1} DW [2], and its contribution to Na is still very low. In this experiment, the highest concentration of Na was 1.94 mg g^{-1} DW. Magnesium is essential for the health of the nervous system, muscles, and bones [55]. Lettuce is generally poor in Mg, even though its concentration differs according to variety. The Mg concentrations in our cultivars are in line with those reported in other studies, and the increase observed in response to the application of borage extract may indicate an increase in the quality of the final product. Interestingly, the application of borage extract induced a significant increase in Ca concentration both red (+63.4 %) and green (+96.2 %) cultivars. Indoor cultivation using artificial lighting may cause necrosis of the leaf marginal apex, known as tipburn. This phenomenon is generally considered a physiological disorder associated with Ca deficiency [56]. The higher concentration measured in leaves after the application of the borage extract suggests not only a higher nutritional value of the product but also a lower probability of developing tipburn. Interestingly, the nickel concentration significantly increased only in red leaf lettuce after the application of borage extract. This microelement is essential for plant growth and its concentration in vegetables is generally very low, with values between 0.05 and 10 μg g^{-1} DW [57]. The highest amount of Ni measured in the red lettuce leaves was 3.03 μg g^{-1} DW. Although the concentration of Ni in food products is not regulated by law, it is of great importance because of the health risks associated with its consumption and skin allergic reactions [58]. Biostimulant applications can also be used to improve the quality of produce, such as leaf pigments that affect the visual appearance and antioxidant compounds, which can have potential benefits on human health.

5. Conclusion

Results obtained demonstrated that the borage extract can be applied to increase the secondary metabolism in lettuce. The increase of phenolic compounds and anthocyanins may suggest an enhanced capacity of plants to cope with various abiotic stresses. At the same time, at nutritional level the application of the borage extract can be useful for enhancing the antioxidant potential and lowering nitrate concentration in leafy vegetables, with potential benefits for the human health. These promising results can be useful for suggesting the commercialization of borage extract as biostimulant. However, further research should be carried out for the evaluation of the product stability during storage and transportation.

CRediT authorship contribution statement

Giulia Franzoni: Writing – original draft, Investigation, Formal analysis, Data curation. **Antonio Ferrante:** Writing – review & editing, Validation, Supervision, Conceptualization.

Ethical Statement

Not applicable.

Data availability statement

The data that support the findings of this study are available.

Founding

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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