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Appraisal of Biodegradable Mulching Films and Vegetal-Derived Biostimulant Application as Eco-Sustainable Practices for Enhancing Lettuce Crop Performance and Nutritive Value

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Abstract: Scientists, extensions specialists, and growers are seeking sustainable agricultural practices that are able to cope with these objectives in order to ensure global food security and minimize environmental damage. The use of mulching films and plant biostimulants in agriculture seems to be a valid solution for tackling these rising concerns. A greenhouse experiment was conducted in order to elucidate the morpho-physiological and nutritive characteristics of lettuce (*Lactuca sativa* L.) in response to foliar application of a tropical plant extract (PE) biostimulant and the use of plastic mulches. Two biodegradable mulch treatments (Mater-Bi® 1 and Mater-Bi® 2) were compared to black polyethylene (LDPE) and bare soil. Biodegradable mulch film Mater-Bi® 1 produced a comparable marketable fresh yield to the commercial standard polyethylene (LDPE), whereas Mater-Bi® 2 exhibited the highest crop productivity. When averaged over biostimulant application, lettuce plants grown with biodegradable film Mater-Bi® 2 exhibited superior quality traits in terms of K, Ca, total ascorbic acid, and carotenoids content. The combination of film mulching (LDPE, Mater-Bi® 1 or Mater-Bi® 2) with the tropical plant extract biostimulant exhibited a positive and significant synergistic effect (+30%) on yield. The PE-biostimulant induced higher values of SPAD index and total chlorophyll content when compared to untreated greenhouse lettuce. The mineral content of leaf tissues was greater by 10% and 17% (for P and Ca, respectively) when compared to the untreated lettuce (no PE application). Nitrate content was significantly reduced by 23% in greenhouse lettuce plants receiving PE as compared to the untreated control. The positive effect of Mater-Bi® 2 film on the ascorbic acid content has also been highlighted when combined with the biostimulant application, where a major amplification of total ascorbic acid (+168%) was recorded in comparison to the untreated lettuce. Overall, our work can assist leafy vegetables growers in adopting good agricultural practices, such as biodegradable plastic mulches and vegetal-derived biostimulants, to improve the sustainability of greenhouse production.

Keywords: eco-friendly practices; *Lactuca sativa* L.; total ascorbic acid; tropical plant extract; Mater-Bi®; nitrate; mineral composition; SPAD index; functional quality

1. Introduction

A widespread agricultural practice across the world consists of covering the soil around plants with plastic films. The introduction of this technique in agriculture dates back to the 1970s, and its success is still linked to multiple benefits. In fact, plastic films can: (i) increase soil temperature and keep it constant throughout the first 20–30 cm layer, so that plants' roots develop faster [1,2]; (ii) reduce soil evapotranspiration and preserve moisture; (iii) prevent soil erosion and excessive leaching of nutrients from plants' rhizosphere; and, (iv) improve the performance of plants in a quantitative and qualitative manner [1,3–5]. In addition, mulching films suppress weeds growth, protect crops against pests and various diseases, and reduce the use of pesticides and herbicides. Based on their color (black, clear or white), they absorb and/or reflect sunlight, differently varying soil temperature, thus affecting crop growth and productivity [5]. Plastic films are widely used for growing vegetables under both open-field and greenhouse conditions [6]. Moreover, these films are mainly made by low-density polyethylene (LDPE) [3], having a strong resistance and high durability, even though, like all petroleum products, they are non-compostable and non-biodegradable. The presence of LDPE residues in the soil beyond the duration of a crop cycle is associated to soil contamination with phthalate and phthalic acid esters due to thermal degradation [7]. Therefore, farmers must manually or mechanically collect from the field and recycle or dispose them to comply with the legislative directives of each country. Unfortunately, the frequent illegal burning of plastic mulches by farmers is becoming a common practice, with the aim of reducing production costs by avoiding disposal expenses, which results in a consequent emission of toxic and harmful substances for humans and the environment [1,3]. In such a way, plastic mulching films increase plastic wastes that are used in agriculture, such as pipes and fittings; agricultural packaging, such as bags, liners, and containers [3]. Therefore, there is an urgent need to use compostable and biodegradable materials in modern agriculture. Nowadays, research is projected towards the creation of films made of biopolymers, such as starch, polylactic acid, and cellulose. These materials are derived from renewable resources, such as corn, potato, and rice [1,6]. Their degradation is in compliance with the European laws and Italian ones (UNI 10785, 1999) on biodegradability (EN 13432, 2000). In fact, these materials are entirely degraded by soil microorganisms and they are mineralized in carbon dioxide and methane, water, and biomass, without the production of toxic substances. Any biodegradable material is designed to disappear within the soil in 5–6 months after the end of the crop [2].

Efficient management of natural resources, such as water and soil, is needed in a scenario where the world population is growing, and agriculture must meet an increasing food demand. On the other hand, the use of plant biostimulants in agriculture has been recognized during the last two decades as an efficient tool to boost yield under optimal and sub-optimal conditions, to improve quality as well as increase nutrient uptake and use efficiency of field and horticultural crops [8–11]. Under the new European Union Regulation 2019/1009, plant biostimulants are specified based on their agronomical effects on crops (i.e., claims), and they include humic substances, protein hydrolysates, algae and plant extracts, inorganic compounds (e.g., silicon), growth-promoting bacteria, and mycorrhizal fungi. Many recent studies on vegetal-based biostimulants have shown to increase the tolerance of crops to abiotic stress (extreme temperature, drought, and salinity), and improve the quality of the produce, in terms of organoleptic and nutraceutical characteristics [11]. They have also contributed to the reduction of unwanted substances content, such as nitrates and heavy metals, in crops [12]. Among these, plant extracts that mainly contain signaling molecules (i.e., small peptides and free amino acids) can influence both primary and secondary metabolism in plants, by stimulating glycolysis enzymes' activity, Krebs' cycle, and nitrates' assimilation [13,14]. Moreover, it has been shown that vegetal-derived plant biostimulants effects involve the size modifications of roots by increasing the length and the number of root hairs, as well as the intake of both macroelements and microelements, leading to better crop performance and the nutritive value of the final produce [8,13,14].

Lettuce (*Lactuca sativa* L.) belongs to the *Asteraceae* family and it is one of the most intensively produced leafy vegetables being widespread all over the world. It is valued for its organoleptic properties and is considered an important source for health-promoting metabolites (carotenoids, chlorophylls, macro and trace elements, phenolics, and vitamins), which are crucial in human nutrition [15,16]. Lettuce has a high water and low fat content, which makes it ideal for dietary plans [15]. Italy dedicates vast areas to lettuce production, and has a broad market, which places it as a European leader in this sector [10]. More importantly, production systems and agronomic practices are pre-harvest factors that can determine the quantitative and qualitative variations in lettuce bioactive compounds [17].

On the basis of the above-mentioned considerations, the aim of our work was to combine two eco-sustainable agricultural practices, such as the use of biodegradable films and plant-based biostimulant (tropical plant extract), and test their effect on the morpho-physiological performance, mineral composition, and nutritive value of greenhouse lettuce plants. The films used were two biodegradable mulching films, namely Mater-Bi® with different composition, which effect was compared with that of a polyethylene film and bare soil. The findings of the study will elucidate the biostimulant × mulch interaction to select the best combination (s) able to improve crop performance and nutritive value of this important leafy vegetable. We also believe that these results will be of great interest for horticulturists, extension specialists, and scientists.

2. Materials and Methods

2.1. Greenhouse Growth Conditions, Treatments and Experimental Design

The experimental test was implemented in a protected environment made of an unheated greenhouse, which was located at the experimental farm of the Department of Agriculture, University of Naples Federico II, Portici—Naples (lat. 40°49′ N; long 14°20′ E, 37 a.s.l). The main physical and chemical characteristics of the soil at the experimental site were: sandy loam texture (74% sand, 20% silt, 6% clay), electrical conductivity of 0.5 dS m^{-1}, neutral pH-7.0, total nitrogen (N) of 0.12%, and organic matter of 1.20% (w/w). The nitrate N, ammoniacal N, Olsen phosphorus, and exchangeable potassium were 105, 12, 40, and 936 mg kg^{-1}, respectively. The butterhead lettuce F1 hybrid SINTIA RZ (42–160; Rijk Zwaan, Der Lier, The Netherlands) was used in this test. This lettuce is very resistant to tip burn and bolting and it is characterized by bright green leaves. SINTIA RZ was selected as the most representative commercial cultivar that was used in Italy during the autumn and winter growing seasons under protected environment. On 16 September 2017 three mulching films (M) were installed, two black biodegradable films, namely Mater-Bi® PC 17 N1 (15 µm thick, commercial; Novamont S.p.A, Novara, Italy) and Mater-Bi® PC 17 N2 (15 µm thick, experimental; Novamont S.p.A, Novara, Italy), and one traditional black low-density polyethylene (LDPE) plastic film (50 µm thick, Idroland s.r.l., Bari, Italy). The compositions of the two biodegradable films are composed of thermoplastic starch and copolyester. The two Mater-Bi® mulching films differ in the presence of Masterbatch (PC 17 N2), a solid additive that is used for imparting color or other properties to plastics, with innovative characteristics to improve the color of mulches with low impact on the original polymer. The soil additive is a concentrated mixture of pigments that was made through a heating process and it includes a carrier resin (e.g., wax) that is cut into granules and then added to plastics.

The greenhouse consisted of a galvanized steel frame with plastic covering material, two non-automated side openings, and a mechanized roof opening. The total greenhouse surface corresponded to an area of 162 square meters (27 m × 6 m). The soil was prepared with low energy inputs consisting of a manual grubbing-up of weeds and then a shallow hoeing (20–25 cm) to allow for a leveling of the soil in a single pass. Water was not a limiting factor, the crop evapotranspiration was calculated with the Hargreaves method, and the water deficit was fully restored by using a drip irrigation system. The irrigation system consisted of a main polyethylene pipeline with a diameter of 32 mm with a low operating pressure of 2 atm, while a series of semi-compensating dripping wings (16 mm diameter and 10 cm interpolation) were laterally attached.

The lettuce seedlings were transplanted in the greenhouse on September 25th on raised furrows. On each furrow, the lettuce seedlings were arranged in double rows, at a plant density of 12.3 plants per m^2. The antiperonosporic protection was performed with Metalaxil seven days after transplantation in order to limit the development of fungal pathogens.

Figure 1 presents the trend of minimum, maximum, and mean daily air temperature inside the greenhouse during the cropping cycle. The soil temperature measurements (minimum, maximum, and mean temperature) were also recorded with microchip sensors (0.5 °C sensitivity) that were placed at 10 cm depth. All of the measurements were collected on a data logger (Davis Vantage Pro2, CA, USA). Nitrogen fertilization was applied by fertigation with ammonium nitrate at eight and 16 days after transplantation (DAT). Half of the plots were treated with Auxym® (Italpollina USA Inc., Anderson, IN, USA) product in order to assess the action of the biostimulant (B). This biostimulant is obtained from fermented tropical plant biomass. It contains phytohormones, amino acids, vitamins, phytochelatins, and enzymes. Auxym® contains as well micro and macroelements (g/kg): N 8.3, P 4.0, K 25.0, Ca 0.9, Mg 1.2, Fe 6.6, Mn 6.4, B 4.4, Zn 0.4, and Cu 0.2 [18]. The biostimulant was applied -at a concentration of 2 mL per liter of water- on plants by a sprayer shoulder pump and application took place five times at seven days' intervals starting 10 DAT (i.e., foliar application).

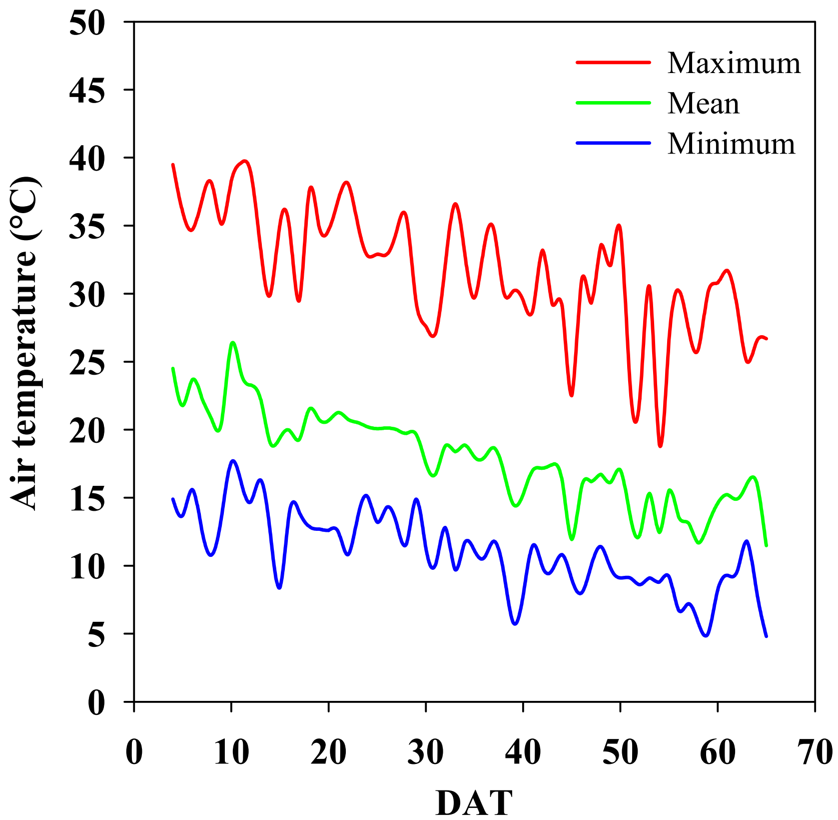

Figure 1. Daily maximum, mean, and minimum values of air temperature recorded inside the greenhouse during the growing period of lettuce.

The experimental scheme provided a two factors factorial combination that resulted in eight treatments in which the factors were mulching (M; three mulching films and bare soil) and biostimulant application (B; control treatment and foliar application of biostimulant). Each treatment was replicated three times and all of the treatments were organized in a randomized complete-block design, resulting in a total of 24 experimental plots.

2.2. Growth Analysis, Yield, Harvest and Quality Analysis Sampling

The harvest was manually carried out by cutting the plants at the crown area, just when commercial weight was attained. For each replicate, a total of 15 representative plants were collected. Each plant was first weighed as a whole (leaves and stem) in order to determine the total yield, while the commercial yield was estimated after separation and weighing of leaves. In both cases, yield was expressed in g $plant^{-1}$. Finally, the leaves were counted and the leaf area (cm^2 $plant^{-1}$) was determined using a LiCor 3100C leaf area meter (LI-COR Biosciences, Lincoln, NE, USA). Five fresh plants from each treatment were randomly sampled, and then stored at −80 °C until the determination of bioactive compounds content.

2.3. Soil Plant Analysis Development (SPAD) Index and Color Measurements

Fifteen SPAD index measurements were performed by a chlorophyll meter (Minolta SPAD-502, Tokyo, Japan) and averaged to a single value on five fully expanded lettuce leaves per replicate. Leaf color (space parameters L*, a* and b*) was recorded with a Minolta chroma meter (CR-300, Minolta Camera Co. Ltd., Tokyo, Japan), on the center of the upper leaf surface with special care to avoid the central vein.

2.4. Total Chlorophyll and Carotenoid Content Determination

On 1 g of fresh leaf samples, the total chlorophylls and carotenoids content was determined following the Lichtenhaler and Buschman [19] method. Fresh sample was extracted in pure acetone, for 15 min in the dark. Subsequently, the absorbance of the extracted solutions was measured at 662, 645, and 470 nm, while using a Hach DR 2000 spectrophotometer (Hach Co., Loveland, CO, USA).

2.5. Dry Matter, Nitrate and Macromineral Content Analysis

After the determination of fresh yield, the leaves were put to a ventilated stove at a temperature of 65 °C for 72 h until constant weight for dry weight determination. The dry mater content was expressed as percentage (%). Mineral analysis was carried out in 250 mg of dry ground leaves (IKA, MF 10.1, Staufen, Germany), which were sieved and diluted in 50 mL of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany). A syringe with a 0.45 µm pore filter (Phenomenex, Torrance, CA, USA) was used to inject each sample into an ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA). For macrocations determination, an IonPac CG12A (4 × 250 mm) guard column and IonPac CS12A (4 × 250 mm) analytical column were used. While, for macroanions determination, an IonPac AG11-HC (4 × 50 mm) guard column and IonPac AS11-HC analytical column (4 × 250 mm) were used. All of the macrominerals were expressed on a dry weight (dw) basis (g kg^{-1}), while the nitrate content was expressed as mg kg^{-1} fw based on the respective leaf sample dry matter content.

2.6. Hydrophilic Antioxidant Activity Determination

In order to measure the hydrophilic antioxidant activity (HAA), 200 mg of lyophilized sample were extracted twice with distilled water, following the *N*,*N*-dimethyl-p-phenylenediamine (DMPD) method [20]. An aliquot of 20 µL of extract was combined with 2 mL of DMPD + solution. The bleaching of solution was proportional to the amount of antioxidant compounds concentration. The reduction in absorbance, as measured by UV Vis spectrophotometry at 505 nm, allows for determining the antioxidant activity. For this purpose, an ascorbate external standard calibration curve was used.

2.7. Total Phenols and Total Ascorbic Acid Content Determination

The total phenols content was assessed with the Folin–Ciocalteau procedure [21]. 250 mg of lyophilized sample were extracted with 10 mL of methanol/water (60:40 *v*/*v*). After an incubation of 90 min., the absorption was measured at 765 nm while employing a UV-Vis spectrophotometer. The results were calculated using an external gallic acid calibration curve (Sigma Aldrich Inc., St. Louis, MO, USA). Total ascorbic acid content was measured according to the method of Kampfenkel et al. [22], and it was quantified by a spectrophotometer at 525 nm against an external ascorbate standard calibration curve.

2.8. Statistical Analysis

The normal distribution of the data was verified through the Shapiro–Wilk's and Kolmogorov–Smirnov's procedures. All of the data were subjected to Two-way ANOVA using SPSS 20 software package. For mulching factor, the treatment means were confronted utilizing Duncan's Multiple Range Test that was performed at $p \leq 0.05$, while, for the biostimulant effect, the means were compared using the t-test.

3. Results

3.1. Soil Temperature Trends

The minimum, maximum and mean soil temperatures under the three tested mulches were influenced by the composition of the utilized mulching material (Figure 2). The differences between the minimum and mean soil temperatures between LDPE and the two biodegradable mulches (Mater-Bi® 1 and Mater-Bi® 2) were notable during the first 15–20 days after transplanting, whereas the differences became narrowerer towards the end of the growing cycle (Figure 2). Concerning the maximum soil temperatures, the Mater-Bi® 1 film had similar maximum soil temperature values to LDPE and slightly higher ones than Mater-Bi® 2. However, the soil minimum, maximum, and mean temperatures trends that were recorded in bare soil were regularly lower than those reached among the three tested mulch materials (Figure 2). The soil temperature trends were similar under the three mulching films in all cases, since they had the highest values at the beginning of the crop cycle and underwent a gradual decrease afterwards, especially towards the end of the growing period. The average minimum soil temperature varied between 13.2–23.9 °C in LDPE, 12.7–22.2 °C in Mater-Bi® 1, 12.7–22.2 °C in Mater-Bi® 2, and 10.6–20.8 °C in bare soil. Finally, the average maximum soil temperature fluctuated between 15.1–30.0 °C in LDPE, 15.1–29.5 °C in Mater-Bi® 1, 14.7–28.3 °C in Mater-Bi® 2, and 12.0–25.2 °C in bare soil.

3.2. Yield and Biometric Parameters

The combination of LDPE or biodegradable mulching materials (Mater-Bi® 1 or Mater-Bi® 2) with the PE-based biostimulant positively affected the total and marketable yields of greenhouse lettuce when compared to the untreated plants, although the beneficial effect of biostimulant application was not apparent for bare soil treatment (Table 1). According to the average effect of the mulching films, a tendency to higher total yield values was recorded for Mater-Bi® 2 (319.5 g plant^{-1}), with a 22% increase as compared to bare soil (261.3 g plant^{-1}), even though no significant differences were recorded between the three mulching treatments. However, this trend became apparent for marketable yield with significantly higher values for Mater-Bi® 2 when compared to Mater-Bi® 1 or LDPE and especially to bare soil (Table 1). The positive effect of PE-treated lettuce plants that were cultivated under the three mulching materials (LDPE, Mater-Bi® 1, or Mater-Bi® 2) was mainly attributed to an increment in the total leaf area and not to an increase in the plant leaf number based on the M × B interaction (Table 1). Moreover, the effect of PE foliar application, when averaged over all mulching treatments, was shown to affect leaf number, which was higher by 10% in PE-treated than in untreated greenhouse lettuce plants. Finally, our findings demonstrated that lettuce plants that were grown under LDPE or Mater-Bi® 2 elicited a significant increment in the number of leaves confronted to the bare soil treatment, whereas the plants cultivated under Mater-Bi® 1 exhibited intermediate values (Table 1).

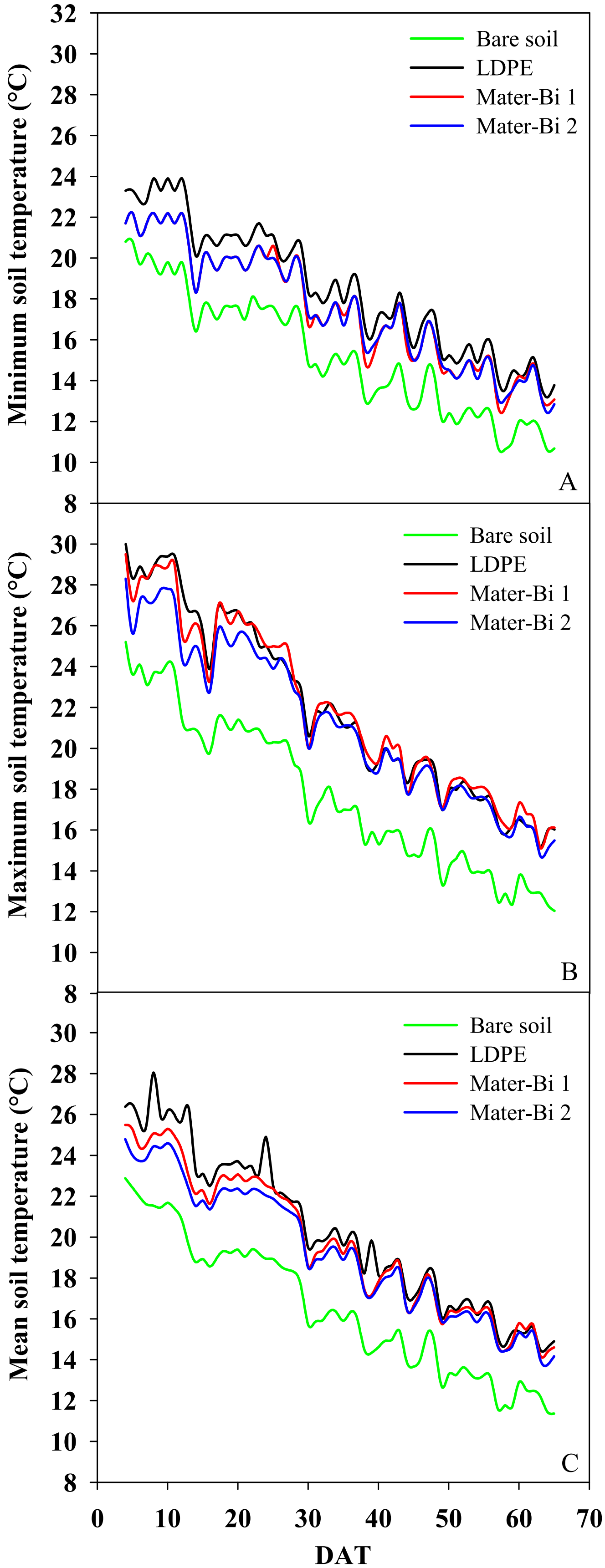

Figure 2. Daily minimum (**A**), maximum (**B**), and mean (**C**) values of soil temperature recorded at a depth of 10 cm in bare soil, LDPE, Mater-Bi® 1 and Mater-Bi® 2 mulches.

Table 1. Mean comparison and analysis of variance for total and marketable yield, leaf number, and total leaf area of untreated and biostimulant-treated greenhouse lettuce grown under low-density polyethylene (LDPE) mulch and biodegradable (Mater-Bi® 1 and Mater-Bi® 2) mulching materials in relation to bare soil.

Source of Variance	Total Yield (g plant^{-1})	Marketable Yield (g plant^{-1})	Leaf Number (no. plant^{-1})	Leaf Area (cm^2 plant^{-1})
Mulch (M)				
Bare soil	261.3 ± 7 b	243.9 ± 7 c	36.5 ± 0.4 c	3414 ± 58 c
LDPE	298.0 ± 18 a	274.3 ± 17 b	44.5 ± 1.2 a	3885 ± 193 a
Mater-Bi® 1	302.8 ± 21 a	274.7 ± 19 b	40.6 ± 1.4 b	3566 ± 168 b
Mater-Bi® 2	319.5 ± 17 a	296.5 ± 16 a	41.9 ± 1.0 a	3667 ± 168 ab
	***	***	***	*
Biostimulant (B)				
Control	266.4 ± 6	243.3 ± 5	38.9 ± 0.8	3375 ± 73
Tropical plant extract (PE)	324.5 ± 12	301.4 ± 10	42.8 ± 1.1	3891 ± 100
t-test	0.000	0.000	0.009	0.000
M × B				
Bare soil without biostimulant	260.6 ± 14 b	238.0 ± 13 b	35.5 ± 0.1	3433 ± 109 b
LDPE without biostimulant	263.9 ± 15 b	240.3 ± 12 b	42.7 ± 1.6	3545 ± 241 b
Mater-Bi® 1 without biostimulant	256.8 ± 5 b	232.9 ± 4 b	38.6 ± 0.5	3198 ± 47 b
Mater-Bi® 2 without biostimulant	284.8 ± 11 b	262.0 ± 8 b	38.9 ± 0.6	3323 ± 103 b
Bare soil + PE	262.0 ± 8 b	249.9 ± 6 b	37.4 ± 0.2	3395 ± 67 b
LDPE + PE	332.2 ± 13 a	308.4 ± 13 a	46.4 ± 1.0	4226 ± 114 a
Mater-Bi® 1 + PE	348.7 ± 9 a	316.5 ± 7 a	44.9 ± 0.3	3934 ± 57 a
Mater-Bi® 2 + PE	354.9 ± 4 a	331.0 ± 4 a	42.6 ± 0.8	4011 ± 112 a
	**	*	NS	*

NS, *, **, *** Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters in the same column indicate significant differences according to DMR test ($p = 0.05$). Means of biostimulant effect are compared according to Student's *t*-test ($p = 0.05$). All data are expressed as mean ± SE.

3.3. SPAD index, Chlorophyll Content and Colorimetric Indices

The non-destructive (SPAD index) and destructive measurement of chlorophylls content were significantly affected by mulching materials and biostimulant applications, with no significant effects from the M × B interaction (Table 2). The PE-based biostimulant provoked greater values of SPAD index and chlorophyll content (+6% and 30%, respectively) in comparison to the untreated control, irrespective of the mulching materials (Table 2). Moreover, when averaged over biostimulant applications, the total chlorophyll content was enhanced by 33% in mulched lettuce plants (avg. 51.3 mg 100 g^{-1} fw) when compared to bare soil (avg. 38.6 mg 100 g^{-1} fw), with no significant differences being observed among the three mulching materials (Table 2).

Concerning the Hunter color parameters, the ANOVA highlighted no significant M x B interaction for all of the examined color parameters (Table 2). In general, neither mulching nor biostimulant application had a significant effect on leaf yellowness (+b*; avg. 33.4) of greenhouse lettuce. Moreover, the use of LDPE as a mulching material resulted in greater lightness (i.e., lowest L* values) of greenhouse lettuce leaves (Table 2). Finally, the foliar application of PE-based biostimulant provoked greater values of brightness and greenness, in comparison to the untreated control, irrespective of the mulching materials (Table 2).

Table 2. Mean comparison and analysis of variance for Soil Plant Analysis Development (SPAD) index, total chlorophyll content, and Hunter color parameters L* (brightness), a* (−a* = green) and b* (+b* = yellow) of untreated and biostimulant-treated greenhouse lettuce grown under LDPE mulch and biodegradable (Mater-Bi® 1 and Mater-Bi® 2) mulching materials in relation to bare soil.

Source of Variance	SPAD Index	Total Chlorophyll (mg 100 g^{-1} fw)	L*	a*	b*
Mulch (M)					
Bare soil	27.3 ± 0.4 c	38.6 ± 0.0 b	54.4 ± 1.7 b	−19.4 ± 0.4	33.6 ± 0.4
LDPE	29.4 ± 0.4 b	50.3 ± 0.1 a	51.8 ± 2.1 c	−19.8 ± 0.4	33.3 ± 0.6
Mater-Bi® 1	30.3 ± 0.6 a	51.8 ± 0.1 a	60.2 ± 1.1 a	−18.6 ± 0.6	33.9 ± 0.9
Mater-Bi® 2	29.2 ± 0.4 b	51.7 ± 0.1 a	56.5 ± 2.0 b	−18.9 ± 2.0	32.8 ± 0.4
	***	*	***	NS	NS
Biostimulant (B)					
Control	28.3 ± 0.4	41.9 ± 0.0	52.1 ± 1.2	−19.9 ± 0.2	33.9 ± 0.3
Tropical plant extract (PE)	30.0 ± 0.5	54.4 ± 0.0	59.3 ± 0.9	−18.3 ± 0.3	32.9 ± 0.5
t-test	0.043	0.039	0.000	0.000	0.084
M × B					
Bare soil without biostimulant	26.7 ± 0.2	34.5 ± 0.0	50.6 ± 0.3	−20.4 ± 0.2	34.3 ± 0.4
LDPE without biostimulant	28.7 ± 0.9	39.3 ± 0.1	47.7 ± 0.5	−20.6 ± 0.3	33.6 ± 0.6
Mater-Bi® 1 without biostimulant	29.5 ± 0.7	40.8 ± 0.1	58.0 ± 1.0	−19.3 ± 0.3	34.8 ± 0.4
Mater-Bi® 2 without biostimulant	28.4 ± 0.1	52.9 ± 0.0	52.1 ± 1.2	−19.7 ± 0.5	33.2 ± 0.7
Bare soil + PE	27.9 ± 0.1	42.7 ± 0.1	58.1 ± 0.7	−18.5 ± 0.2	33.0 ± 0.5
LDPE + PE	30.0 ± 0.4	61.4 ± 0.1	56.0 ± 2.1	−19.0 ± 0.4	33.2 ± 1.1
Mater-Bi® 1 + PE	31.1 ± 0.6	62.9 ± 0.1	62.3 ± 0.5	−17.9 ± 1.0	32.9 ± 1.9
Mater-Bi® 2 + PE	30.0 ± 0.2	50.5 ± 0.1	60.9 ± 0.4	−18.0 ± 0.2	32.6 ± 0.3
	NS	NS	NS	NS	NS

NS, *, *** Non-significant or significant at $p \leq 0.05$ or 0.001, respectively. Different letters in the same column indicate significant differences according to DMR test ($p = 0.05$). Means of biostimulant effect are compared according to Student's *t*-test ($p = 0.05$). All data are expressed as mean ± SE.

3.4. Dry Matter Percentage and Leaf Mineral Profile

The leaf dry matter percentage and nitrate content were significantly influenced by M × B interaction (Table 3). The recorded leaf dry matter percentage across the eight experimental treatments ranged from 3.5 to 4.2%, with the lowest values being recorded in bare soil without biostimulant application (Table 3). The recorded nitrate content across the eight experimental treatments (836–2685 mg kg^{-1} fw) was within the limits set by the EU Commission Regulation No 1258/2011 for the commercialization of fresh lettuce (3000–5000 mg kg^{-1} fw). Our results also demonstrated that the presence of mulching materials, in particular, the use of Mater-Bi® 2, evoked a significant increment in nitrate content confronted to bare soil in both untreated and biostimulant-treated lettuce plants. Interestingly, the nitrate content was significantly reduced by 23% in greenhouse lettuce plants receiving foliar application with tropical plant extract (1566 mg kg^{-1} fw) confronted to the control (2037 mg kg^{-1} fw) (Table 3).

Table 3. Mean comparison and analysis of variance for leaf dry matter percentage and mineral composition of untreated and biostimulant-treated greenhouse lettuce grown under LDPE mulch and biodegradable (Mater-Bi® 1 and Mater-Bi® 2) mulching materials in relation to bare soil.

Source of Variance	Dry Matter (%)	Nitrate (mg kg^{-1} fw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	S (g kg^{-1} dw)	Na (g kg^{-1} dw)
Mulch (M)								
Bare soil	3.7 ± 0.1 c	955 ± 54 d	7.9 ± 0.3 b	90.2 ± 2.1 a	6.4 ± 0.3 b	3.5 ± 0.2	0.8 ± 0.1	2.2 ± 0.1 b
LDPE	4.2 ± 0.0 a	1898 ± 74 c	9.4 ± 0.4a	77.3 ± 2.7 b	4.8 ± 0.4 c	2.7 ± 0.2	1.0 ± 0.1	2.3 ± 0.2 b
Mater-Bi® 1	4.1 ± 0.1 ab	1984 ± 152 b	9.0 ± 0.2 a	79.6 ± 1.2 b	5.9 ± 0.4 bc	3.3 ± 0.1	1.1 ± 0.1	2.3 ± 0.1 b
Mater-Bi® 2	3.9 ± 0.1 bc	2368 ± 142 a	7.5 ± 0.3 b	88.7 ± 1.2 a	8.3 ± 0.6 a	4.0 ± 0.1	1.1 ± 0.1	2.7 ± 0.0 a
	***	***	***	***	***	NS	NS	*
Biostimulant (B)								
Control	3.9 ± 0.1	2037 ± 180	8.1 ± 0.3	82.1 ± 2.3	5.8 ± 0.5	3.3 ± 0.2	0.9 ± 0.1	2.5 ± 0.1
Tropical plant extract (PE)	4.0 ± 0.1	1566 ± 135	8.9 ± 0.3	85.9 ± 1.8	6.8 ± 0.4	3.5 ± 0.2	1.1 ± 0.1	2.2 ± 0.1
t-test	0.758	0.048	0.050	0.206	0.049	0.320	0.157	0.024
M × B								
Bare soil without biostimulant	3.5 ± 0.1 c	1075 ± 2 f	7.3 ± 0.3	88.6 ± 2.7	6.0 ± 0.6	3.4 ± 0.3	0.8 ± 0.1	2.5 ± 0.1
LDPE without biostimulant	4.2 ± 0.1 a	2063 ± 10 c	8.7 ± 0.5	72.8 ± 2.7	4.1 ± 0.0	2.5 ± 0.1	1.0 ± 0.1	2.5 ± 0.1
Mater-Bi® 1 without biostimulant	4.2 ± 0.1 a	2325 ± 8 b	8.9 ± 0.2	78.9 ± 2.4	5.7 ± 0.4	3.3 ± 0.2	1.0 ± 0.1	2.4 ± 0.2
Mater-Bi® 2 without biostimulant	3.9 ± 0.2 b	2685 ± 3 a	7.3 ± 0.2	88.0 ± 2.4	7.9 ± 1.2	3.9 ± 0.2	0.9 ± 0.1	2.7 ± 0.1
Bare soil + PE	3.9 ± 0.1 b	836 ± 9 g	8.5 ± 0.2	91.9 ± 3.6	6.9 ± 0.4	3.6 ± 0.2	0.9 ± 0.1	2.0 ± 0.1
LDPE + PE	4.2 ± 0.1 a	1733 ± 10 d	10.0 ± 0.3	81.8 ± 3.1	5.5 ± 0.8	2.9 ± 0.3	1.1 ± 0.1	1.9 ± 0.3
Mater-Bi® 1 + PE	3.9 ± 0.1 b	1643 ± 13 e	9.0 ± 0.4	80.4 ± 0.5	6.2 ± 0.7	3.3 ± 0.2	1.1 ± 0.1	2.2 ± 0.2
Mater-Bi® 2 + PE	3.8 ± 0.0 b	2051 ± 3 c	7.9 ± 0.5	89.4 ± 1.0	8.7 ± 0.4	4.2 ± 0.1	1.1 ± 0.1	2.7 ± 0.1
	*	***	NS	NS	NS	NS	NS	NS

NS, *, *** Non-significant or significant at $p \leq 0.05$ and 0.001, respectively. Different letters in the same column indicate significant differences according to DMR test ($p = 0.05$). Means of biostimulant effect are compared according to Student's *t*-test ($p = 0.05$). All data are expressed as mean ± SE.

Neither mulching materials nor PE-application had a significant influence on Mg and S concentrations in greenhouse lettuce leaves (avg. 3.4 and 1.0 g kg^{-1} dw, respectively). The concentrations of target macronutrients and sodium in leaf tissues were significantly affected by mulching materials, with the highest values of K, Ca, and Na being recorded in lettuce plants that were grown under Mater-Bi® 2 mulching material (Table 3). Interestingly, PE biostimulant treatment, as averaged over mulching materials (M × B interaction = ns), affected P, Ca, and Na leaf tissues concentrations, which were greater by 10% and 17% (for P and Ca, respectively) and lower by 12% (for Na) when compared to the untreated lettuce (Table 3).

3.5. Antioxidant Activity and Bioactive Compounds

The hydrophilic antioxidant fraction of lettuce ranged from 5.6 to 7.5 mmol ascorbate eq. 100 g^{-1} dw (Table 4). Regardless of mulching materials, the antioxidant capacity in lettuce that was treated with the commercial biostimulant Auxym® was significantly higher (+9%) as compared to the untreated control (Table 4). Neither mulching materials nor PE-application had a significant influence on total phenols content in lettuce leaves (avg. 3.4 mg gallic acid eq. 100 g^{-1} dw). Moreover, phytochemicals with antioxidant properties, such as total ascorbic acid and carotenoids, were affected by both the tested factors (mulching materials, biostimulant application, and their combination). When averaged over the biostimulant application, the use of the Mater-Bi® 2 film evoked a significant increase in the biosynthesis and the accumulation of carotenoids (Table 4). The positive effect of Mater-Bi® 2 film on total ascorbic acid content has also been highlighted in the interaction with the biostimulant, where a major increase of total ascorbic acid (+168%) was recorded in comparison to the untreated and PE-treated lettuce grown in bare soil (Table 4).

Table 4. Mean comparison and analysis of variance for hydrophilic antioxidant activity, total phenols, total ascorbic acid and carotenoid contents of untreated and biostimulant-treated greenhouse lettuce grown under LDPE mulch and biodegradable (Mater-Bi® 1 and Mater-Bi® 2) mulching materials in relation to bare soil.

Source of Variance	Hydrophilic Antioxidant Activity (mmol ascorbate eq. 100 g^{-1} dw)	Total Phenols (mg gallic acid eq. 100 g^{-1} dw)	Ascorbic Acid (mg 100 g^{-1} fw)	Carotenoids (mg g^{-1} fw)
Mulch (M)				
Bare soil	7.3 ± 0.1	3.9 ± 0.2	7.2 ± 0.6 b	18.4 ± 2.2 ab
LDPE	6.4 ± 0.3	3.1 ± 0.3	8.1 ± 1.1 b	13.1 ± 1.4 b
Mater-Bi® 1	6.8 ± 0.4	3.4 ± 0.3	7.7 ± 2.3 b	17.4 ± 1.4 ab
Mater-Bi® 2	7.2 ± 0.3	3.3 ± 0.1	13.7 ± 3.1 a	21.0 ± 2.1 a
	NS	NS	*	*
Biostimulant (B)				
Control	6.6 ± 0.2	3.3 ± 0.2	6.0 ± 0.7	16.0 ± 1.3
Tropical plant extract (PE)	7.2 ± 0.2	3.6 ± 0.2	12.3 ± 1.6	18.9 ± 1.6
t-test	0.036	0.241	0.002	0.158
M × B				
Bare soil without biostimulant	7.1 ± 0.2	3.7 ± 0.5	6.1 ± 0.3 c	14.3 ± 1.9
LDPE without biostimulant	5.6 ± 0.1	3.0 ± 0.3	7.1 ± 0.8 bc	12.7 ± 3.1
Mater-Bi® 1 without biostimulant	6.7 ± 0.3	3.3 ± 0.6	3.0 ± 0.4 c	16.2 ± 1.1
Mater-Bi® 2 without biostimulant	6.9 ± 0.2	3.2 ± 0.2	8.0 ± 1.4 bc	20.8 ± 1.5
Bare soil + PE	7.5 ± 0.1	4.1 ± 0.0	8.3 ± 0.6 bc	22.5 ± 2.0
LDPE + PE	7.0 ± 0.1	3.1 ± 0.6	9.1 ± 1.9 bc	13.4 ± 0.6
Mater-Bi® 1 + PE	6.9 ± 0.8	3.6 ± 0.1	12.4 ± 2.0 b	18.6 ± 2.6
Mater-Bi® 2 + PE	7.5 ± 0.4	3.5 ± 0.1	19.3 ± 3.7 a	21.2 ± 4.3
	NS	NS	*	NS

NS, * Non-significant or significant at $p \leq 0.05$, respectively. Different letters in the same column indicate significant differences according to DMR test ($p = 0.05$). Means of biostimulant effect are compared according to Student's *t*-test ($p = 0.05$). All data are expressed as mean ± SE.

4. Discussion

The use of biodegradable mulching films and plant-based biostimulants has revolutionized modern agriculture in the last two decades. Nevertheless, no scientific studies have assessed the combinatorial effect of these two agricultural practices on crop performance and nutritional value of an important greenhouse leafy vegetable, such as lettuce. Our findings indicated that biodegradable mulching film Mater-Bi® 1 produced comparable marketable fresh yield to the commercial standard polyethylene (LDPE), while Mater-Bi® 2 exhibited the highest crop productivity. It is well established that plastic mulching films increase soil temperature in comparison to bare ground. This was the case in the current experiment, since the soil minimum, maximum, and mean temperature trends that were recorded in bare soil were always lower by 2.3–3.3 °C, 3.5–4.2 °C, and 2.8–3.8 °C, respectively, than those that were observed among the three tested mulching materials. The differences in fresh yield could be also associated to differences in soil temperatures, when temperature is a limiting factor (autumn–winter growing season; [23]). The results that were recorded in this greenhouse experiment endorse the previous study, where the span of soil temperature under the different mulching materials had a pronounced effect on marketable lettuce yield [24–27]. Our findings concerning the beneficial effect of mulching versus bare soil were also reported in previous studies on open-field and greenhouse vegetables. For instance, melon plants had more fruits and higher fruit mean weight when grown with biodegradable films and LDPE, as compared to bare soil [2]. An increase in marketable yield in the presence of polyethylene and biodegradable (Mater-Bi®) films when compared to bare soil was also observed in pumpkin [24], tomato [1,4,25], strawberry [3,26], garlic chives [5], as well as lettuce [27]. The use of plastic films may have preserved soil moisture and prevented water evaporation and the excessive leaching of nutrients in the rhizosphere [1].

Interestingly, the combination of film mulching (LDPE, Mater-Bi® 1, or Mater-Bi® 2) with the tropical plant extract biostimulant exhibited a positive and important synergistic effect (+30%) on both total and marketable yield. Particularly, the higher marketable production that was observed in greenhouse lettuce plants that were grown under mulching films and treated with PE-biostimulant, was due to an increase in the leaf area and not to the number of leaves per plant. The increase in crop productivity and biometric parameters of lettuce plants grown under protected cultivation has been previously reported in several research studies testing the action of this tropical plant extract biostimulant on leafy and fruit vegetables, such as tomato, jute, wall rocket, and lettuce [18,28,29]. The biostimulant action of the commercial product Auxym® on PE-treated lettuce plants could be associated to the presence of signaling molecules, such as carbohydrates, vitamins, but especially free amino acids and soluble peptides [14,18,30]. The hormone-like activity of plant-derived peptides that are contained in Auxym® has been proposed in many scientific papers, where the foliar application of vegetal-based biostimulants elicited auxin- and gibberellin-like activities and, thus, boosted yield [31,32]. Since many other signaling peptides have been identified in plant cells controlling growth, development, and stress responses of plants [33], it is expected that more signaling-peptide based PE will be developed in the near future. Furthermore, some indirect effects of amino acids can be postulated. The amino acid L-tryptophan is a precursor of indole compounds (thus including auxins), while L-methionine is known as the precursor of ethylene [30]. Finally, these bioactive compounds that are present in the plant-based biostimulants can act on the primary metabolism, increasing the photosynthetic activity of the plants, and it can act as well on root growth, which might increase water and nutrient absorption efficiency, thus resulting in a yield increase [18]. This was the case in the current study, where plants that were grown under plastic mulching films and treated with tropical plant extract were characterized by better physiological and biochemical status. The greater SPAD index and chlorophyll content of lettuce leaves corroborated this, thus confirming the better photosynthetic efficiency that leads to better plant performance. Similar results on the stimulation of the physiological and biochemical status of biostimulant-treated plants were also previously observed in greenhouse tomato [18], spinach [34], lettuce [35], and jute [28].

The leaf appearance in peculiar color is among the visual characteristics of leafy vegetables that steadily govern consumer preference and selection choice [36]. Lettuce green color is directly dependent upon chlorophyll synthesis in leaf tissue. Plant extract-biostimulant application affected lettuce greenness color (−a*) to the extent it affected chlorophyll content, as observed earlier in a broad span of leafy greens, such as spinach, lamb's lettuce, and baby lettuce [29,37].

A negative aspect in the quality of leafy vegetables is, certainly, the high content of nitrates, as they are involved in the onset of different diseases [38]. Generally, vegetables that belong to the *Brassicaceae*, *Chenopodiaceae*, and *Asteraceae* families [18] may accumulate nitrates in their leaves. Significant genotypic variations in nitrate accumulation are shown for lettuce [39–41]. The nitrate concentration in plants is closely related to nitrate reductase activities [42]. This reality has prompted the European Commission to regulate the nitrate limits for lettuce. In our experiment, nitrate concentrations for plants cultivated with LDPE films, Mater Bi® 1, and Mater Bi® 2 films (1898–2368 mg kg^{-1} fw), were within the set limits for fresh lettuce according to Commission regulation (EU) No 1258/2011 (3000–5000 mg kg^{-1} fw) [43]. The PE-biostimulant application decreased nitrates concentration in lettuce leaves by 23% (avg. 1566 mg kg^{-1}), as compared to the control (avg. 2037 mg kg^{-1}). This positive effect could be linked to the presence of a high content of free amino acids in the biostimulant product, which, once absorbed by the plant, might exert the inhibition of the nitric ion transporters that are present in the root. On the other hand, the ability of the plant-based biostimulant Auxym® to reduce nitrates accumulation could be associated with the regulation of nitrogen metabolism in plants, which involves the activity of nitrate and nitrite reductase, glutamate synthase, as well as glutamine synthetase [14,18,44]. Various studies confirmed our results, such as that of Bulgari [45] performed on iceberg lettuce, which showed that a biostimulant of vegetal origin enriched with micro-elements (one), kept nitrate levels well under the limit required by the EC. Similar results were also obtained in spinach, on which the effect of amino acid-based biostimulant (Aminoplant) was evaluated [46]. Other studies on corn, soy, and wheat also showed that exogenous amino acids application can significantly reduce nitrate absorption [18].

Scientists recommend that people should consume fruits and vegetables daily, because they satisfy 11%, 35%, 7%, and 24% of the daily intake of P, K, Ca, and Mg, respectively [10]. These macronutrients help against certain diseases, such as blood pressure imbalances, hypertension (K), and osteoporosis (P, Ca, and Mg) [15]. For lettuce, several authors reported a potassium content between 48–72 mg g^{-1}, phosphorus 4–6 mg g^{-1}, magnesium 1.4–2.8 mg g^{-1}, and calcium 4–10 mg^{-1} on a dry weight basis [15,16,47]. In our work, the use of biodegradable films influenced the biofortification of macronutrinets in lettuce leaves. In particular, the use of polyethylene film Mater-Bi® 1 increased P content, confronted to the control, whereas lettuce plants that were grown under Mater-Bi® 2 exhibited higher values of K and Ca when compared to the bare soil treatment. Our results match with previous studies on the 'nutrient acquisition response' of plant-based biostimulant application on tomato [18], jute [28], and spinach [29]. In addition to the accumulation of macronutrients in leaf tissues of biostimulant-treated plants, the use of PE reduced sodium concentration in lettuce leaves by 12%, confronted to the control, which is in harmony with Carillo et al. [28] findings. This is a very important aspect, because Na causes hypertension and cardiovascular diseases [48].

Furthermore, lettuce is considered to be a good source of nutraceutical molecules, such as vitamin C and carotenoids [15]. These molecules represent the radical scavenging power that protects plants from the oxidative damage caused by free radicals. In our work, when averaged over biostimulant application, lettuce plants that were grown under Mater-Bi® 2 had the highest total ascorbic acid and carotenoids content. Similarly, Morra et al. [3] recorded a higher antioxidant activity, total polyphenols, and anthocyanins in two strawberry cultivars grown under biodegradable Mater-Bi film as compared to those cultivated with LDPE or in bare ground. These results are also confirmed for melon plants that are grown with biodegradable mulching films [27,49]. The Mater Bi® 2 behavior could be related to the fact that below this film there is a greater evaporation of the soil, which results in a lower accumulation of water in plants. Therefore, this mild condition of stress might trigger the plant to synthesize defensive molecules [27,49]. More compelling, these secondary metabolites are also crucial to human well-being [50,51].

In our work, the foliar application of PE on greenhouse lettuce also influenced antioxidant activity and health-promoting secondary metabolites, since the antioxidant potential increased by 10% when compared to the untreated control plants. The latter is a notable qualitative functional parameter in leafy vegetables, since it is correlated to the synergetic effect of low-molecular weight biologically active compounds, such as phenolic compounds and carotenoids [50]. Ertani et al. [52] showed an increase in antioxidant activity, lycopene, phenols, and ascorbic acid of *Capsicum chinense* L., in response to the application of plant extract based biostimulants. The synergistic action of Mater-Bi® 2 with tropical plant extract is of significant interest for scientists and nutritionists, because it resulted in the production of superior greenhouse lettuce leaves in terms of vitamin C content (+168% as compared to the control). In fact, as also shown by Carillo et al. [28], signaling compounds that are present in the tropical plant extract Auxym®, like glutamic and aspartic acids are involved in the stimulation of primary and secondary metabolism, thus, leading to a greater synthesis of antioxidant molecules, such as vitamin C [28].

5. Conclusions

In recent years, horticultural research has focused on improving farming practices in the framework of a more sustainable agricultural, including the use of biodegradable mulching films and vegetal-based plant biostimulants to improve the crop performance and nutritive quality of the produced commodities. Our greenhouse experiment on lettuce confirmed that the use of biodegradable plastic mulching materials, especially Mater-Bi® 2, could be considered as an alternative to LDPE and bare soil cultivation. This biodegradable mulching material increased marketable yield irrespective of the biostimulant application, due to many agronomic benefits, in particular, the better microclimate (minimum and maximum soil temperatures) in the rhizosphere. Our results also demonstrated, that lettuce plants grown under biodegradable film especially Mater-Bi® 2 exhibited superior quality traits in terms of K, Ca, total ascorbic acid, and carotenoids. Interestingly, the foliar application of PE-biostimulant in the presence of mulching materials was able to improve the total and marketable yield and biometric traits. The synergistic effect of mulching with plant-based biostimulant was linked to better physiological and biochemical status (higher SPAD index and chlorophyll content) and a higher nutrient acquisition response (higher P and Ca and lower Na content). The PE-biostimulant treated lettuce had a lower nitrate content and higher antioxidant scavenging capacity than the non-treated control, while the combination of Mater-Bi® 2 and PE-biostimulant resulted in the production of premium greenhouse lettuce leaves in terms of vitamin C content. The outcomes of the current study can encourage leafy vegetables producers to replace LDPE films with biodegradable ones in combination with plant-based biostimulants in order to attain high productivity and reach consumer expectations for high quality produce. In addition, the substitution of plastic mulching with biodegradable ones can significantly tackle the environmental issues that are related to the disposal of mulching materials at the end of the cropping cycles. The absence of dumping costs for farmers could likely offset the higher costs due to biodegradable mulching, favoring the application of biodegradable mulching materials on a wide scale.

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