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Appraisal of Combined Applications of *Trichoderma virens* and a Biopolymer-Based Biostimulant on Lettuce Agronomical, Physiological, and Qualitative Properties under Variable N Regimes

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Abstract: The current research elucidated the agronomical, physiological, qualitative characteristics and mineral composition of lettuce (*Lactuca sativa* L. var. *longifolia*) after treatments with a beneficial fungus *Trichoderma virens* (TG41) alone or in combination with a vegetal biopolymer-based biostimulant (VBP; 'Quik-link'). The experiment consisted of lettuce plants grown in three N conditions: sub-optimal (0N kg ha^{-1}), optimal (70N kg ha^{-1}), and supra-optimal (140N kg ha^{-1}) N levels. Lettuce grown under 0N fertilization showed a significant increase in fresh yield when inoculated with TG41 alone (45%) and a greater increase with TG41 + VBP biostimulant (67%). At 48 days after transplanting, both the TG41 alone or TG41+VBP biostimulant induced higher values of CO_2 assimilation in comparison to the control. The mineral concentrations in leaf tissues were greater by 10% for K and 12% for Mg with the TG41+VBP treatments compared to the untreated lettuce. The lettuce plants receiving either TG41 alone or TG41+VBP biostimulants had a significantly lower nitrate content than any of the untreated controls. In non-fertilized conditions, plants treated with TG41+VBP biostimulants produced lettuce of higher premium quality as indicated by the higher antioxidant activity, total ascorbic acid (+61%–91%), total phenols (+14%) and lower nitrate content when compared to the untreated lettuce.

Keywords: microbial biostimulant; non-microbial biostimulant; Lactuca sativa L. var. *longifolia*; mineral profile; physiological mechanism; photosynthesis; nitrate; functional quality

1. Introduction

Rapid growth in the world population will determine an increase in global food demand that is expected to double by 2050 [1]. The intensification in agricultural production appears to be the only useful strategy to meet the rapidly growing food demand in the future, although this imposes stress to the agroecosystem [1], presents serious problems to the ecosystem and health [2–4], since it requires high-input resource cropping systems (such as greenhouse horticulture), that are not ecologically sustainable [5]. In actual fact, greenhouse farming systems use the highest amount of synthetic nitrogen (N) fertilizers per unit area of cultivated produce than any other cropping system [6–9].

Nitrogen-containing compounds are typically applied as chemical fertilizers in agriculture [10,11]. Nitrogen overuse and/or the imbalance between N and other nutrients, such as phosphorus, increases N losses while reducing nitrogen use efficiency (NUE) by the plant, which affects yield and, consequently, profit margins for farmers [12]. Moreover, the accumulation of excess nitrate in edible plant parts can be reduced to the nitrite form, which can cause diseases, such as methemoglobinemia, to which children are particularly at risk [13,14]. However, to date, the efforts to reduce N fertilizer use while at the same time attempting to increase NUE have been proven ineffective. This can be attributed to the inability of crop plants to adapt to low N availability conditions, which limit the activation of the physiological processes necessary for increasing crop production [7,15].

Recently, promising strategies that could aid a shift from N-intensive agriculture to a more eco-friendly approach that reduces the use of N fertilizers while simultaneously increasing NUE and yields, proposes the integrated use of non-chemical plant biostimulants (PBs) in cropping systems [16–20]. PBs are products able to enhance plant growth and development that include several substances with bioactive properties (seaweed and plant extracts, humic and fulvic acids, protein hydrolysates, and silicon), as well as some plant growth-promoting microorganisms (mycorrhizal fungi and plant growth-promoting rhizobacteria) [21–24]. Other plant beneficial microbes include fungi, such as *Trichoderma,* that have multiple plant beneficial capabilities, such as pathogen/pest control, increased nutrient uptake, stimulation of photosynthesis, and carbohydrate metabolism processes, that positively influence crop productivity and quality [25–29]. Several *Trichoderma* spp. are registered as microbial biological control agents in Plant Protection Products commercialized for the control of a broad-spectrum plant diseases [27,30]. Biocontrol mechanisms include direct antagonism with the production of secondary metabolites (i.e., hydrolytic enzymes, antibiotics), competition, and induced plant resistance [26–28,31–33]. Furthermore, many species, among *T. harzianum*, *T. virens, T. asperellum,* and *T. atroviride*, also act as plant biostimulants, able to enhance nutrient uptake and plant growth, or conferring plant tolerance to abiotic stress [25,34–38]. The direct and indirect benefits to the plants depend upon *Trichoderma*-plant molecular crosstalk, and exchange of diverse chemicals and small peptides that stimulate various plant responses [39,40]. These include the fungi metabolites, proven to have auxin and ethylene-like activity, that induce a reorganization of gene expression patterns in shoots and roots with significant changes in the plant metabolic machinery and a consequent improvement in plant resilience and yield [26,40–42]. These released compounds specifically modify plant root architecture, increasing root length, density and branching, and nutrient uptake (P, Fe, Mn, and Zn), in addition to acting as mediators in the plant microbiome for communication, warning signals, and pest management [25–27,43,44]. Recently, experiments conducted by Fiorentino and co-workers [25] on lettuce and rocket, grown under three different N fertilization rates and inoculated with two *Trichoderma* strains, demonstrated that, in particular, one strain of *T. virens* G41 (ex-*Gliocladium virens* GV41) was able to enhance NUE in lettuce, also favoring the uptake of native N present in the soil. Specifically, the benefits of inoculating plants with this *Trichoderma* strain were more evident when cultivation was performed under sub-optimal N conditions [25].

Another prominent category of PBs that has demonstrated beneficial effects on root stimulation, similar to those exerted by *Trichoderma*, is that of vegetal biopolymer-based products (VBP) that contain lateral root promoting peptides (LRPP) and lignosulphonates. In particular, the lignosulfonates obtained from sulfite pulping processes during cellulose extraction from wood are used in a variety

of industries, but they have also been used as fertilizers in crops [45]. They have proven auxin and gibberellin-like activities, probably due to the biological action of phenol metabolites able to interact with plant phytohormones and enzymes affecting carbon–nitrogen metabolism [45]. Lucini et al. [46] indicated that when the vegetal-based biopolymer was applied as a drench to melon, it altered the plant hormone profile by inducing an increase in ABA intermediates, brassinosteroids, and cytokinins in a dose-dependent manner. This mechanism stimulated root growth and consequently resulted in a 'nutrient acquisition response' improving resources use efficiency (RUE), thus enhancing plant biomass production and resistance to transplant stress. In addition, the authors reported that brassinosteroids may play a key role both in root system architecture changes as well as in shoot interference with hormone signaling and secondary metabolites, such as phenolic acids and carotenoids, plus the modulation of photosynthesis.

Romaine lettuce requires varying levels of N during the 65–75-day production cycle that depends upon the plant growth and development stages, plus N availability in the rhizosphere. N availability affects the morphological and physiological plant attributes [47] that influence the marketability of the leafy produce (i.e., leaf size) and consumer perception (i.e., visual green color). From this perspective, depending on the farming conditions, growing season, and genotypes, the combined application of *Trichoderma* and vegetal-biopolymer biostimulants could be particularly useful to enhance lettuce production due to their abilities to increase NUE, favoring nutrient uptake and utilization efficiency. Furthermore, the appropriate incorporation of N in the plant is important since the nitrate content in vegetable products must be within the limits established by the market according to EU regulation no. 1258/2011, whereby the levels should not exceed 3000–5000 mg kg^{-1} fw.

In a recent opinion, Rouphael and Colla [23], indicated that the scientific community and private companies should focus on exploiting the potential synergistic biostimulatory action of microbes with non-microbial PBs combinations to design and develop second-generation plant products (biostimulant 2.0) with specific targeted biostimulant actions. A few experimental investigations have demonstrated the beneficial effects on crop performance of combining microbial inoculants (i.e., *Rhizophagus intraradices* or plant growth promoting bacteria or *R. irregulare* and *T. atroviride*) with humic acids [48,49] or protein hydrolysates [50]. Previous indications by Fiorentino et al. [25] suggested that the nutrient content of leafy horticulture crops could vary according to cultivation in diverse fertilizer conditions and in the presence/absence of a fungal inoculant. However, to date, nothing is known about the effects of *Trichoderma* alone or in combination with a vegetal biopolymer based-biostimulant on the agronomical, physiological and qualitative responses of an important leafy vegetables, such as Romaine lettuce (*Lactuca sativa* L. var. *longifolia*). This study will investigate the effect of a beneficial microbe (*T. virens* TG41) when used alone or in combination with a VBP biostimulant ('Quik-link'), under supra-optimal, optimal, and suboptimal N regimes, on Romaine lettuce production and marketability characteristics. This study will increase understanding of the processes involving these two different types of plant biostimulants and the effects on plant N acquisition response, for which the comprehension is pivotal to increasing NUE, as well as attempting to decrease N environmental inputs and reduce risks to consumer health.

2. Materials and Methods

2.1. Experimental Setup, Design, and Crop Management

An experiment was performed on lettuce (*Lactuca sativa* L. var. *longifolia* cv. 'Romana Bionda Lentissima a Montare'—Esasem, Casaleone, Verona) from November 4, 2015 to January 19, 2016, in a protected greenhouse structure (unheated) at the Department of Agricultural Sciences, University of Naples Federico II located at Portici, Italy. The soil was classified as a sandy loam texture (73% sand, 19% silt, 8% clay), with a pH of 7.0, electrical conductivity of 0.5 dS m^{-1}, an organic matter of 1.25% (w/w) and a total N of 1.1 g kg^{-1}. The NO$_3$-N, NH$_4^+$-N, available P, and exchangeable K were 95, 7, 35, and 950 mg kg^{-1}, respectively.

A split-plot design with three replicates (randomized blocks) was adopted with fertilization (3 levels) as the main factor and biostimulant applications (3 levels) as the sub-factor. The three N fertilization levels were suboptimal (0 kg ha^{-1}; 0N), optimal (70 kg N ha^{-1}; 70N) and supra-optimal (140 kg N ha^{-1}; 140N), while the three biostimulant applications were non-inoculated control, inoculated *Trichoderma virens* G41 (TG41), and *T. virens* + vegetal biopolymer-based biostimulant (TG41 + VBP). The cultivated area of each experimental plot (27 experimental plots in total) was 3.5 m^2. Lettuce were transplanted on November 4th (at the 3 true-leaf stage) in double rows with a plant density of 14 plants per square meter. A biodegradable black mulch film (15 μm thick MaterBi®, Novamont, Novara, Italy) was used and maintained throughout the entire greenhouse experiment.

N total amount was applied as ammonium nitrate (NH$_4$NO$_3$ 34%) into two identical doses, at 6 and 27 days after transplanting (DAT) by fertigation using a drip irrigation system with in-line emitters (flow rate: 3.3 L h^{-1}; distances: 35 cm). Foliar pests, such as cutworms, were controlled with two applications of Decis Evo (active ingredient 25 g L^{-1} of deltamethrin—Bayer Crop Science, Milano, Italy) at the rate of 0.4 L ha^{-1}, whereas a copper-based fungicide (Cupravit 35 WG containing 350 g kg^{-1} of copper as copper oxychloride—Bayer Crop Science, Milano) was sprayed twice at the rate of 2.5 kg ha^{-1} to control downy mildew caused by *Bremia lactucae* Regel.

2.2. Fungal and Vegetal Biostimulants

A spore suspension of *T. virens* strain G41 (final concentration 1 × 10^7 spores mL^{-1}; TG41) was used to inoculate the lettuce seedlings at time of transplant by using a root dip method (with submergence for 10 min); then a repeated inoculation was conducted at 18 DAT by watering 25 mL of the inoculum plant^{-1}. The vegetal biopolymer-based (VBP) biostimulant ('Quik-link®', Italpollina, Rivoli Veronese, Italy) was used in the current experiment. The product has a density of 1.21 kg L^{-1}, a pH (1:5) of 4.7, an electrical conductivity; EC (1:5) of 20 mS cm^{-1}, 25 g kg^{-1} of organic N as peptides and free amino acids, 160 g kg^{-1} of organic C, lignosulphonates, and micronutrients, such as iron, manganese, zinc, copper, and molybdenum, in the following concentrations 10.0, 7.0, 3.0, 1.0, and 0.2 g kg^{-1}, respectively [46]. Peptides and free amino acids were obtained through enzymatic hydrolysis of a vegetal source of proteins, as reported by Carillo et al. [7]. The peptides in the product have a high biological activity being signaling molecules (e.g., lateral root promoting peptides—LRPP). The commercial product was applied at the base of each plant (100 mL, containing 6 L ha^{-1} of 'Quik-link') at transplant, plus 17 (stage BBCH41-head beginning to form) and 45 DAT (stage BBCH45%–50% of the expected head size).

2.3. Fungal Colony Forming Units in Soil Rhizosphere and Trichoderma-VBP Compatibility

Soil samples were collected from the plant rhizosphere at the time of harvest. The number of fungal colonies forming units was determined, as indicated in Fiorentino et al. [25]. Briefly, a 1% (w/v) soil suspension was prepared in water, in serial dilutions, then 100 μL aliquots of each sample were spread on the surface of 90 mm culture plates containing Rose Bengal-Chloramphenicol agar (HiMedia Pvt. Ltd., Mumbai–India) supplemented with 0.1% (v/v) Igepal (Sigma–Aldrich, Milano, Italy), and incubated for 3–7 days at 25 °C. The emerging fungal colonies were counted daily.

In vitro tests were performed with varying doses of the VBP biostimulant and the *Trichoderma* inoculum, including the doses used for the field treatments to determine if the 'Quik-link' product inhibited the germination and growth of the fungus.

2.4. Fresh and Dry Yield, SPAD index and CIE (lab) Measurements

At harvest (76 DAT), the lettuce fresh yield was assessed in sampling areas of 2 m^2 from the center of the 27 experimental plots. The shoot dry biomass was determined (after oven drying at 80 °C for 72 h). The dried leaf tissues were conserved for mineral analysis. At 45 and 75 DAT, the soil plant analysis development (SPAD) index (i.e., non-destructive measurement of chlorophyll content) was measured on undamaged and expanded lettuce leaves using a portable SPAD-502 chlorophyll meter (Konica-Minolta, Tokyo, Japan). Twelve measurements were conducted on four randomly picked

lettuce plants per experimental plot, then averaged to a single SPAD value for each replicate [51]. Subsequently, on the same date, measurements were performed using a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd., Osaka, Japan) to evaluate the *Commission Internationale de L'Eclairage* (CIE) color space parameters for L* (lightness) and chroma coordinates: a* (−a* greenness) and b* (+b* yellowness). In each experimental plot, 10 healthy leaves were measured and averaged to represent a single color value [52].

2.5. Net CO_2 Assimilation Rate and Stomatal Resistance Measurements

At 33, 40, and 48 DAT, measurements of leaf gas exchange were carried out within 2 h across solar noon on the youngest fully expanded lettuce leaves, using nine replicates for each treatment. Measurements of net CO_2 assimilation rate (A_{co2}; μmol CO_2 m^{-2} s^{-1}) and stomatal resistance (r_s; m^2 s^{-1} mol^{-1}) were recorded using a portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., UK). Photosynthetically active radiation, relative humidity, and carbon dioxide concentration (PAR, 850 ± 100, 1000 ± 100, and 600 ± 100 μmol m^{-2} s^{-1}, RH 60 ± 5, 55 ± 5, and 60 ± 5%, and 400 ± 5, 410 ± 5, and 400 ± 5 ppm, at 33, 40, and 48 DAT, respectively) were set at ambient value, and the airflow rate was 400 mL s^{-1}.

2.6. Mineral Composition Analysis

Plant material was dried and pulverized using a cutting–grinder head (IKA, MF10.1, Staufen, Germany), then the powder was extracted in Milli-Q water (Merck Millipore, Darmstadt, Germany) for 10 min at 80 °C in a thermostatic bath (ShakeTemp SW22, Julabo, Seelbach, Germany) and centrifuged at 6000 rpm for 10 min as indicated in Rouphael et al. [50]. A Dionex ICS-3000 system (Sunnyvale, CA, USA) equipped with suppressed conductivity detection was used to determine the ion content of the samples. The ion separation of the samples was carried out with two different ion-exchange columns: An IonPac CS12A column (250 × 4 mm) was used for the cation separation eluted with 20 mM methanesulfonic acid (flow rate 1 mL min^{-1}), and an IonPac AS11-HC column (250 × 4 mm) was used for the anion separation eluted with a potassium hydroxide gradient (flow rate 1.5 mL min^{-1}). Nitrogen (total N) concentration in leaf tissue was determined according to the Kjeldahl method [53].

2.7. Antioxidant Capacity, Total Phenols, and Total Ascorbic Acid Analysis

Lipophilic and hydrophilic antioxidant capacity and total phenols were determined on freeze-dried tissue samples, whereas the total ascorbic acid was assessed on fresh material and measured using a spectrophotometer (Hach DR 2000, Hach Co., Loveland, CO, USA) according to the protocols of Re et al. [54], Fogliano et al. [55], Singleton et al. [56], and Kampfenkel et al. [57], respectively. Solution absorbances were assessed at 505, 734, 525, and 765 nm for the lipophilic and hydrophilic antioxidant fractions, total polyphenols, and total ascorbic acid, respectively.

2.8. Data Elaboration, Statistical Analysis, Principal Component Analysis, and Heat Map

The statistical analyses were all carried out using the software IBM SPSS Statistics 21. All data were subjected to two-way analysis of variance, and mean values were separated according to Duncan test with $p < 0.05$. Principal component analysis (PCA) was performed on the whole morphological and physiological data set, and the eigen values, total variance of the first three principal components (PCs) as well as the loading scores and plots were determined [58–60]. A heat map summarizing the agronomical, physiological, and qualitative responses of lettuce to plant biostimulant applications and N fertilization levels was also generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage [6].

3. Results

3.1. Fungal Concentration in the Soil

The total number of fungal colonies (including *Trichoderma*) recovered from soil rhizosphere in the nine treatments ranged between 2.0×10^5 and 6.5×10^5 colony forming units (CFU) g^{-1} of soil and was significantly ($p < 0.05$) influenced by the interaction of the two tested factors: N fertilization level (N) and VBP biostimulant application. In particular, results indicated that the highest fungal CFU was observed in soils from lettuce plants inoculated with TG41 under suboptimal 0N conditions (6.5×10^5 CFU g^{-1} of soil), in comparison to any of non-inoculated plants under suboptimal, optimal, or supra-optimal N conditions (average 2.5×10^5 CFU g^{-1} of soil), whereas the treatments with TG41 (at 70N and 140N) or TG41 +VBP biostimulant (at 0N and 70N) exhibited intermediate values (average 3.9×10^5 CFU g^{-1} of soil) (data not shown). Moreover, the in vitro tests performed with the beneficial microbe (TG41) and non-microbial VBP biostimulant at the dose applied in the greenhouse experiment did not demonstrate any inhibition of the germination and growth of the fungi concentration (69.2 CFU in the absence and 68.5 CFU in the presence of the 'Quik-link-product), suggesting compatibility between the two biostimulants.

3.2. Growth Responses, SPAD Index and Leaf Colorimetry

A significant ($p < 0.01$) interaction between N fertilization level and biostimulant application was observed on fresh yield and dry biomass. For instance, the use of the TG41-based biostimulant alone or in combination with the VBP biostimulant positively affected both fresh and dry yield of lettuce plants under both sub-optimal (0 kg ha^{-1}) and optimal (70 kg ha^{-1}) N conditions, but the beneficial effect was not apparent in the over N fertilization condition (140 kg ha^{-1}) (Figure 1). Lettuce grown in the absence of N fertilization demonstrated a highly significant increase in fresh yield of 67% when inoculated with the combined TG41+VBP biostimulants. Instead, a more moderate increase of 45% was observed over the untreated 0N condition with the inoculation of *T. virens* G41 alone. Moreover, under optimal N fertilization (70 kg ha^{-1}), only lettuce plants inoculated with TG41 alone exhibited significantly higher fresh yields. Treatments with TG41 alone or TG41+VBP increased marketable dry yield by 16% when compared to the untreated control, but no significant differences were noted between the two different biostimulant inoculations (Figure 1). No effects on lettuce yield were observed with either of the biostimulants at the supra-optimal 140N fertilization.

The SPAD index in *Lactuca sativa* L. var. *longifolia*, as an indication of chlorophyll content, was significantly affected by N fertilization levels (at 75 DAT) and by biostimulant applications (at 45 and 75 DAT), with effects in the N × T interaction (Table 1). At 75 DAT, the highest SPAD index values were recorded with TG41 + VBP biostimulant combination (Table 1). The visual appearance, particularly the greenness of leaf color, is a primary parameter used by the consumer in product preference and selection choice [61]. In general, neither the N fertilization level nor biostimulant application had a significant effect on the leaf greenness (−a* values) in lettuce (Table 1). Overall, the N application levels resulted in a greater lightness in the color of the lettuce leaves, with the lowest L* values recorded in the 140 kg N ha^{-1} treatment, which also corresponded to a decrease in the chroma coordinate (b*; Table 1).

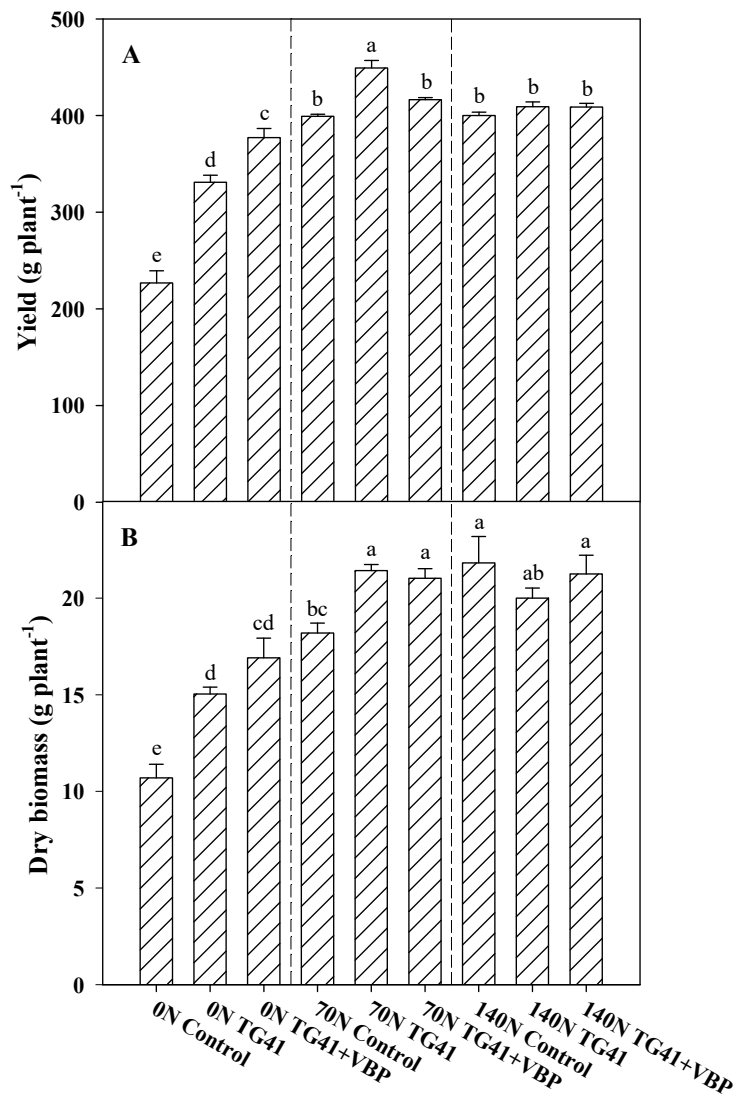**Figure 1.** Fresh yield (**A**) and dry biomass (**B**) of Romaine lettuce grown in greenhouse in relation to N fertilization level (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated = Control, TG41 = *T. virens* G41, and TG41+VBP = vegetal biopolymer-based biostimulant). Mean values with the same letter were not different, according to Duncan's test ($p < 0.05$).

Table 1. Effects on Romaine lettuce soil plant analysis development (SPAD) index and *Commission Internationale de L'Eclairage* (CIE) color space parameters for: L* (lightness) and chroma coordinates: a* (−a* greenness) and b* (+b* yellowness) in relation to N fertilization level (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated=Control, TG41=*T. virens* G41, and TG41+VBP=vegetal biopolymer-based biostimulant) during the cultivation cycle.

Treatments	SPAD Index		L	a*	b*
	45 DAT	75 DAT			
Nitrogen rate (N)	NS	***	**	NS	*
Biostimulant (B)	*	**	NS	NS	NS
N × B	NS	NS	NS	NS	*
Nitrogen rate (kg ha^{-1})					
0	38.27	37.92 b	42.81 a	−16.61	24.53 a
70	38.94	39.35 a	42.60 a	−16.37	24.20 ab
140	38.47	39.24 a	41.54 b	−16.23	23.62 b
Biostimulant					
Control	37.54 b	38.39 b	41.94	−16.33	23.86
TG41	39.50 a	38.56 b	42.65	−16.49	24.37
TG41+VBP	38.63 a	39.50 a	42.36	−16.38	24.13
N × B					
0N Control	37.23	38.00	42.08	−16.25	23.50 c
0N TG41	39.30	37.45	43.08	−16.93	25.18 a
0N TG41+VBP	38.27	38.30	43.28	−16.65	24.93 ab
70N Control	37.50	38.69	42.95	−16.53	24.55 abc
70N TG41	40.60	39.15	42.98	−16.53	24.53 abc
70N TG41+VBP	38.73	40.15	41.88	−16.05	23.53 bc
140N Control	37.90	38.48	40.80	−16.20	23.53 bc
140N TG41	38.60	39.23	41.90	−16.03	23.40 c
140N TG41+VBP	38.90	40.14	41.93	−16.45	23.93 abc

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant. Mean values with the same letter in each column were not different according to Duncan's test ($p < 0.05$). DAT: days after transplanting.

3.3. Leaf Gas Exchange: Net CO₂ Assimilation Rate and Stomatal Resistance

The physiological parameters, in particular, the net CO_2 assimilation rate (A_{CO2}) and stomatal resistance (r_s) in the Romaine lettuce plants throughout the cultivation cycle in the greenhouse, were evaluated as a function of N fertilization level and biostimulant application as displayed in Table 2. The A_{CO2} was significantly affected by the biostimulant treatments for all measured data, and to a lesser degree, by the N fertilization level (only at 48 DAT). Irrespective of the N fertilization level (N × B interaction= ns) at 33 and 40 DAT, both the TG41 alone or in combination with the VBP-based biostimulant induced higher values of A_{CO2} in comparison to the control treatment that was not significantly different between the two biostimulant treatments. At 48 DAT, the A_{CO2} increased in the following order with the applications: TG41+VBP > TG41 > control (Table 2). On the other hand, augmenting the N fertilization level resulted in a linear increase in A_{CO2} from 0 to 140 kg ha^{-1} but only at 48 DAT (Table 2).

Contrary to A_{CO2}, the r_s was not affected neither by N fertilization level nor by biostimulant application at 33 and 48 DAT, while at 40 DAT, the r_s was only influenced by the two biostimulant applications (Table 2). Particularly, on this date, the r_s was significantly lower on average by 26% when lettuce plants were inoculated with *Trichoderma* alone or in combination with the commercial product 'Quik-link' (Table 2).

3.4. Mineral Composition in Leaf Tissue

The results regarding the mineral profile in Romaine lettuce leaves are presented in Table 3. For all the macronutrients and sodium analyzed, no significant differences were observed in the N fertilization level and biostimulant application interaction. In particular, neither N fertilization rate nor biostimulant treatment had a significant effect on Ca and Na concentrations in lettuce leaves (average 7.0 and 1.4 g kg^{-1} dry weight, respectively; Table 3). The concentrations of N and P in leaf tissues were significantly affected by N fertilization rate. Concentrations of N and P increased as the N fertilization level increased, with the highest values recorded at 140 kg ha^{-1} for N and at 70 and 140 kg ha^{-1} for P (Table 3).

The effects of TG41 and TG41+VBP biostimulant, when averaged over all N fertilization rates, affected the K and Mg concentrations in leaf tissues which were higher by 10% and 12%, respectively, than in untreated lettuce plants, but with no significant difference noted between the two biostimulant treatments (Table 3).

Table 2. Net CO_2 assimilation rate and stomatal resistance of greenhouse Romaine lettuce plants measured during the production cycle in relation to N fertilization level (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated=Control, TG41=*T. virens* G41, and TG41+VBP=vegetal biopolymer-based biostimulant).

Treatments	Net CO_2 Assimilation Rate (μmol CO_2 m^{-2} s^{-1})			Stomatal Resistance (m^2 s^1 mol^{-1})		
	33 DAT	40 DAT	48 DAT	33 DAT	40 DAT	48 DAT
Nitrogen rate (N)	NS	NS	***	NS	NS	NS
Biostimulant (B)	***	***	***	NS	*	NS
N × B	NS	NS	NS	NS	NS	NS
Nitrogen rate (kg ha^{-1})						
0	14.51	21.26	13.20 c	3.32	4.06	4.35
70	15.16	20.93	14.12 b	3.94	4.40	3.80
140	14.61	20.45	15.78 a	2.96	4.52	3.36
Biostimulant						
Control	11.74 b	18.49 b	12.48 c	4.59	5.25 a	4.27
TG41	15.25 a	21.60 a	14.76 b	2.77	4.03 b	3.63
TG41+BVP	17.34 a	22.55 a	15.86 a	3.02	3.70 b	3.61
N × B						
0N Control	10.92	18.59	10.93	4.01	5.05	4.83
0N TG41	15.08	22.12	13.23	2.64	3.37	4.20
0N TG41+VBP	17.52	23.08	15.44	3.29	3.77	4.01
70N Control	12.44	18.73	12.06	5.80	4.91	3.98
70N TG41	15.17	21.67	14.54	3.11	4.49	3.61
70N TG41+VBP	17.86	22.38	15.76	2.93	3.79	3.82
140N Control	11.94	18.15	14.44	3.67	5.79	4.01
140N TG41	15.60	21.01	16.52	2.46	4.23	3.08
140N TG41+VBP	16.29	22.20	16.37	2.74	3.53	3.01

* $p < 0.05$; *** $p < 0.001$; NS, not significant. Mean values with the same letter in each column were not different according to Duncan's test ($p < 0.05$). DAT: days after transplanting.

Table 3. Total nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sodium (Na) concentrations of greenhouse Romaine lettuce plants at time of harvest (75 DAT) in relation to N fertilization level (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated=Control, TG41=*T. virens* G41, and TG41+VBP=vegetal biopolymer-based biostimulant).

Treatments	N	P	K	Ca	Mg	Na
	(mg g^{-1} dw)	(mg g^{-1} dw)	(mg g^{-1} dw)	(mg g^{-1} dw)	(mg g^{-1} dw)	(mg g^{-1} dw)
Nitrogen rate (N)	**	***	NS	NS	NS	NS
Biostimulant (B)	NS	NS	**	NS	*	NS
N × B	NS	NS	NS	NS	NS	NS
Nitrogen rate (kg ha^{-1})						
0	36.90 b	2.02 b	55.02	6.83	3.63	1.52
70	37.63 b	2.58 a	53.71	7.53	3.69	1.42
140	39.04 a	2.93 a	53.40	6.62	3.42	1.35
Biostimulant						
Control	37.43	2.48	50.65 b	6.47	3.31 b	1.54
TG41	37.57	2.59	55.90 a	7.06	3.70 a	1.34
TG41+VBP	38.58	2.46	55.57 a	7.46	3.73 a	1.41
N × B						
0N Control	37.03	1.98	51.36	6.39	3.46	1.60
0N TG41	35.70	2.03	56.17	6.27	3.55	1.52
0N TG41+VBP	37.98	2.05	57.53	7.84	3.90	1.43
70N Control	37.60	2.45	52.59	7.18	3.46	1.44
70N TG41	37.38	2.55	54.09	7.88	3.92	1.38
70N TG41+VBP	37.93	2.75	54.45	7.53	3.69	1.45
140N Control	37.65	3.00	48.01	5.85	3.01	1.58
140N TG41	39.63	3.20	57.45	7.02	3.62	1.13
140N TG41+VBP	39.85	2.58	54.75	7.00	3.62	1.35

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant. Mean values with the same letter were not different according to the Duncan's test ($p < 0.05$). DAT: days after transplanting.

3.5. Nitrate, Antioxidant Capacity, and Bioactive Content

The registered nitrate content among the 9 experimental conditions (890–1496 mg kg^{-1} fresh weight) was within the limits imposed by the European Regulation No. 1258/2011 for the commercialization of fresh lettuce (3000–5000 mg kg^{-1} on a fresh weight basis). In our study, nitrate content was affected by both N fertilization level and biostimulant application, without significant effects in the N×B interaction (Table 4). As expected, our results demonstrated that increasing N fertilization from 0 to 140 kg ha^{-1} elicited a significant increase in nitrate content compared to non-fertilized plants, whereas lettuce plants cultivated under optimal N fertilization (70 kg ha^{-1}) exhibited intermediate values (Table 4). Interestingly, the nitrate content was significantly lowered in lettuce plants receiving treatments of either TG41 alone and the combined TG41+VBP biostimulants (not significant between them) compared to the untreated control (Table 4).

The hydrophilic and lipophilic antioxidant fractions of greenhouse lettuce ranged from 1.44 to 1.61 mmol ascorbic acid eq. 100 g^{-1} dw and from 2.69 to 4.62 mmol trolox 100 g^{-1} dw, respectively. Neither N fertilization level nor the biostimulant application had a significant effect on the hydrophilic antioxidant activity. Moreover, significant effects were noted on lipophilic antioxidant activity (LAA) with both N and biostimulant treatments, but not the N×B interaction. Irrespective of N fertilization treatments, the application of TG41+VBP demonstrated a significant increase in LAA (+13%) compared to the treatment of TG41 alone and the non-inoculated control (Table 4). Moreover, antioxidant molecules, in particular, total phenols and total ascorbic acid, were significantly influenced by either tested factors of N fertilization and biostimulant application. When averaged over the nitrogen treatments, the lettuce plants cultivated under supra-optimal conditions (i.e., 140 kg ha^{-1}) were characterized by low-quality bioactive compounds in terms of both total phenols and total ascorbic acid (Table 4). Interestingly, the biostimulants-treated plants with TG41 alone and particularly in the combination of TG41+VBP, produced a major amplification of total phenols (+14%) and total ascorbic acid (+61%–91%) in comparison to untreated lettuce plants (Table 4).

Table 4. Nitrate content, hydrophilic (HAA), and lipophilic (LAA) antioxidants activities, total phenols and total ascorbic acid (TAA) content of greenhouse Romaine lettuce at time of harvest in relation to N fertilization level (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated = Control, TG41 = *T. virens* G41, and TG41+VBP = vegetal biopolymer-based biostimulant).

Treatments	Nitrate	HAA	LAA	Phenols	TAA
	(mg kg^{-1} fw)	(mmol eq. ascorbic acid 100g^{-1} dw)	(mmol eq. trolox 100g^{-1} dw)	(mg eq. gallic acid g^{-1} dw)	(mg 100g^{-1} fw)
Nitrogen rate (N)	*	NS	***	***	***
Biostimulant (B)	**	NS	***	*	***
N × B	NS	NS	NS	NS	***
Nitrogen rate (kg ha^{-1})					
0	1019.09 b	1.54	4.06 b	55.94 a	22.66 a
70	1119.46 ab	1.56	2.84 c	54.44 a	13.81 b
140	1319.38 a	1.47	4.88 a	47.38 b	11.20 c
Biostimulant					
Control	1356.88 a	1.56	3.66 b	49.39 b	10.53 c
TG41	1052.41 b	1.47	3.85 b	52.28 ab	16.97 b
TG41+VBP	1048.64 b	1.53	4.26 a	56.10 a	20.17 a
N × B					
0N Control	1152.55	1.56	3.74	52.84	13.88 de
0N TG41	890.63	1.47	3.99	56.07	21.73 b
0N TG41+VBP	1014.10	1.58	4.44	58.91	32.36 a
70N Control	1422.03	1.63	2.69	53.09	9.54 ef
70N TG41	1063.18	1.61	2.95	54.06	19.27 bc
70N TG41+VBP	873.18	1.44	2.88	56.18	12.61 def
140N Control	1496.08	1.51	4.56	42.23	8.15 f
140N TG41	1203.43	1.35	4.62	46.71	9.91 ef
140N TG41+VBP	1258.65	1.55	5.45	53.19	15.54 cd

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant. Mean values with the same letter in each column were not different according to the Duncan's test ($p < 0.05$). DAT: days after transplanting.

3.6. Heat Map Analysis of all Measured Plant Parameters

An aggregated data heat-map analysis of the measured agronomic and physiological parameters was conducted to produce a visual comparison of the effects determined by the tested treatment factors on the Romaine lettuce plants. In Figure 2, the analysis revealed two dendrograms: on the top (Dendrogram 1), a classification that corresponded principally to the biostimulant applications, and on the left (Dendrogram 2), the parameters that influenced this distribution. Dendrogram 1 revealed two main groups: on the left, the cluster corresponded to controls for each of the three N levels that were all untreated with the biostimulant conditions; then on the right of the heat map, two clusters that contained the other six treatments, consisting of a mix of the N levels receiving the biostimulant applications (Figure 2).

In particular, in the left cluster of Dendrogram 1, the 140N Control was well separated from the other two controls (0N and 70N) due to the higher r_s at 40 and 48 DAT; nitrate, Na, P, and dry biomass (in the first/highest cluster of Dendrogram 2), as well as the lower values for the parameters in the second cluster, mainly for the parameters of L*, total phenols, Mg, and K content. On the right side of Dendrogram 1, two clusters were identified, the first on the left included treatments 70N TG41+VBP biostimulant, separated from the 140N level with the biostimulants TG41 or TG41+VBP, that showed in particular lower Na, r_s at 40 and 48 DAT, hydrophilic (HAA), b* and total phenols parameters, but higher LAA, a* value, P and N content, SPAD index and A_{CO2} at 33 and 48 DAT. The grouping on the right included 70N TG41, 0N TG41, and 0N TG41+VBP treatments. Within this cluster, the 0N treatments with the biostimulants were clearly separated from the 70 N TG41 by higher HAA, leaf number (LN), and lower LAA in this latter treatment. Instead, the two 0N levels receiving the biostimulants were distinguished by the parameter groupings found in Dendrogram 2, whereby 0N TG41 could be attributed to the lower values for the parameters found in the third cluster (mainly due to a*, SPAD Index, N), as well as the lower r_s 40 DAT and nitrate, but higher b*; whereas 0N TG41+VBP biostimulant could principally be identified by the all the higher parameters found in the second cluster—specifically total ascorbic acid (TAA). Interestingly, the first cluster in Dendrogram 2 clearly demonstrated the differential effects of the biostimulant treatments (untreated ones had high parameters for all N levels), while the second cluster clearly revealed the consequence of supra-optimal N levels (all parameter values were low), and the outcome of the combined biostimulants in the low N level condition (all parameter values were high), comparatively to the *Trichoderma* alone (i.e., TG41) at 0N.
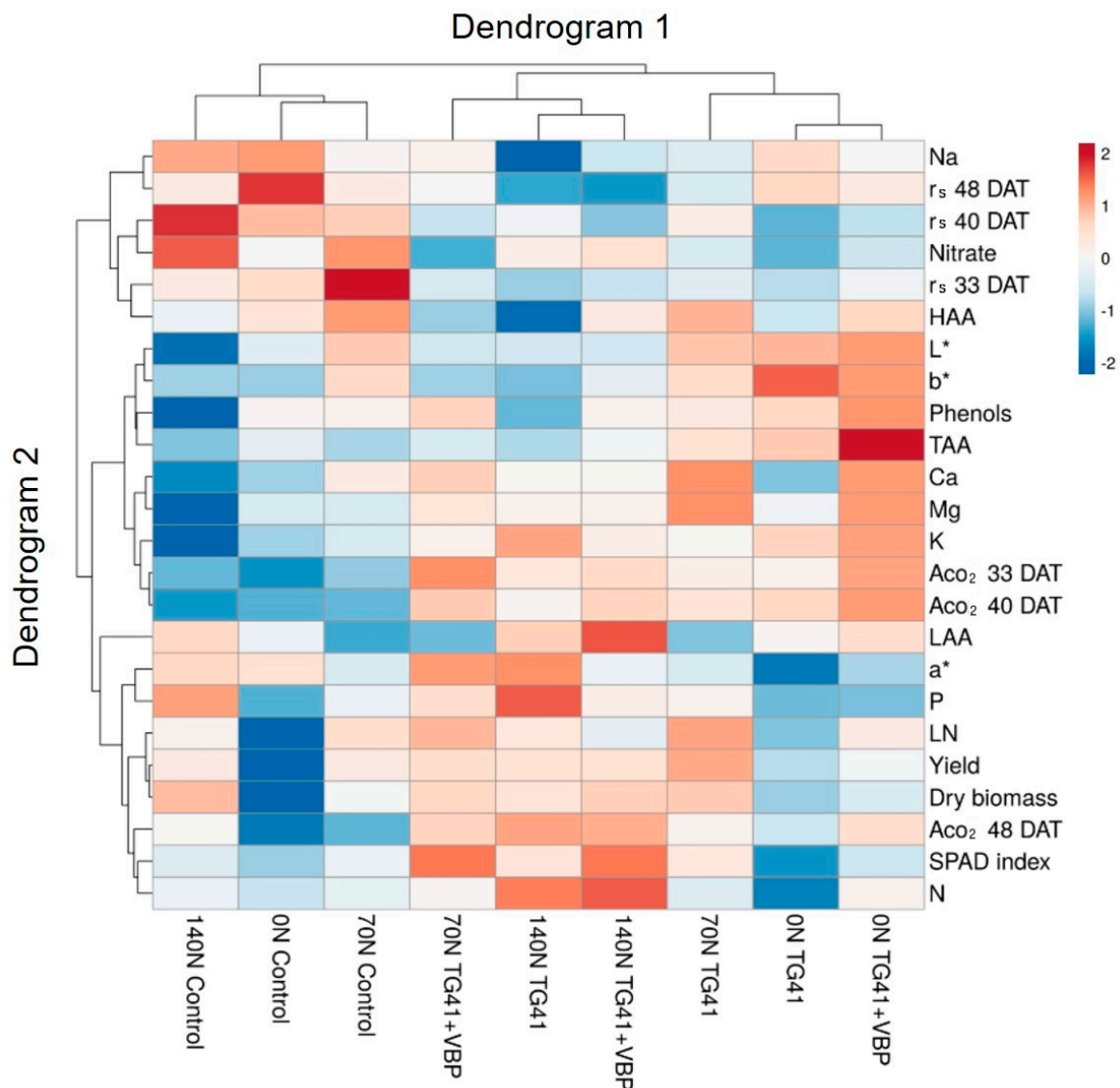
Figure 2. Cluster heat map analysis summarizing greenhouse lettuce plant responses to a factorial experiment with three N fertilization levels (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated=Control, TG41=*T. virens* G41, and TG41+VBP=vegetal biopolymer-based biostimulant). Control plants were not treated with TG41 and/or VBP. The figure was generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage. A$_{CO2}$: net CO_2 assimilation rate; r_s: stomatal resistance; HAA: hydrophilic antioxidant activity; LAA: lipophilic antioxidant activity; TAA: total ascorbic acid; SPAD: soil plant analysis development; DAT: days after transplanting.

3.7. Principal Component Analysis of all Measured Plant Parameters

Principal component analysis was carried out on the whole experimental data set, and the loading plot and scores are reported in Figure 3. The analysis indicated that the variables in the first three principal components (PCs) were highly correlated, with eigen values greater than 1, thus explaining for 80.4% of the total variance, with PC1, PC2, and PC3 accounting for 36.1%, 32.0%, and 12.4%, respectively. The variable distribution along PC1 was clearly attributed to the biostimulant treatments, while N fertilization levels contributed to that on PC2 (Figure 3). TG41 and TG41+VBP biostimulant treated plants were distributed in the positive quadrants of PC1 except for 0N TG41, while all control treatments (untreated lettuce plants) were distributed in the negative side of PC1. In particular, 0N TG41+VBP biostimulant and 70N TG41 were in the upper right quadrant, while

70N TG41+VBP biostimulant, 140N TG41+VBP biostimulant, and 140N TG41 were in the lower right quadrant. Moreover, in PC2, 0N TG41 was positioned in the positive side of the upper left quadrant, with 0N and 70N untreated control treatments, while the 140N control was in the lower left negative quadrant (Figure 3). PC1 was positively correlated to A_{CO2} at 33, 40, and 48 DAT, K, Mg, and Ca content, yield (fresh weight), dry biomass, and SPAD index. PC1 was also negatively correlated with r_s at 33, 40, 48 DAT, and also with nitrate content. PC2 was positively correlated with L* and b* colorimetric parameters, total phenols, and TAA, while it was negatively correlated to P content and a* colorimetric parameter. In addition, the treatments with 70N TG41+VBP biostimulant and 140N TG41 produced lettuce with a higher yield, leaf number, SPAD index, and A_{CO2} at 48 DAT. Interestingly, the non-fertilized 0N lettuce plants treated with TG41+VBP produced lettuce with higher premium quality (higher total phenols and TAA and lower nitrate content) (Figure 3). Finally, the upper and lower left quadrant depicted the three non-treated control treatments with the lowest quality characteristics (high Na and nitrate content; Figure 3).
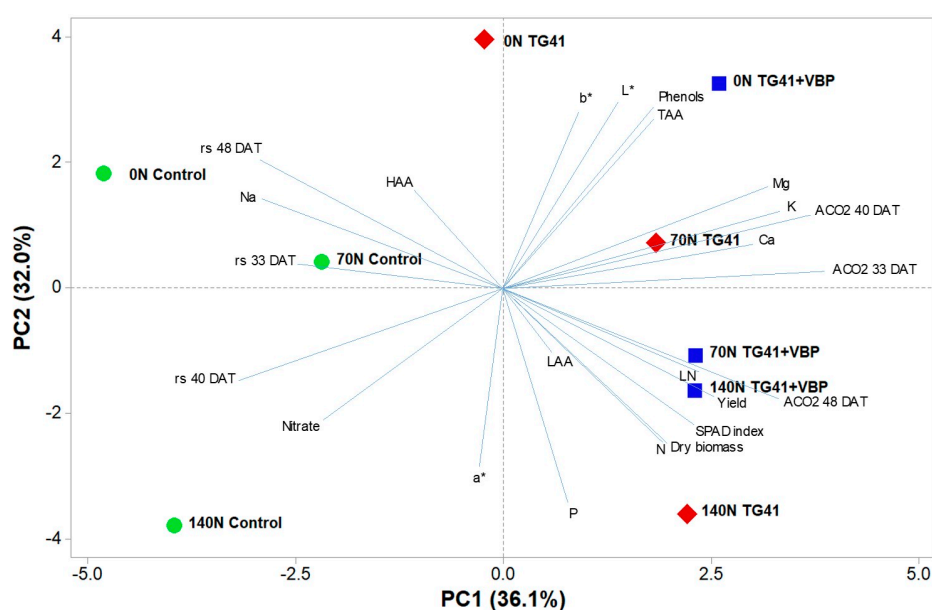

Figure 3. Principal component loading plot and scores of principal component analysis of all morpho-physiological and qualitative parameters analyzed in Romaine lettuce plants submitted to a factorial experiment with three N fertilization levels (0N = 0 kg ha^{-1}, 70N = 70 kg ha^{-1}, 140N = 140 kg ha^{-1}) and biostimulant application (Untreated = Control, TG41 = *T. virens* G41, and TG41+VBP = vegetal biopolymer-based biostimulant). A_{CO2}: net CO_2 assimilation rate; r_s: stomatal resistance; HAA: hydrophilic antioxidant activity; LAA: lipophilic antioxidant activity; TAA: total ascorbic acid; SPAD: soil plant analysis development; DAT: days after transplanting.

4. Discussion

Our findings indicated that the suboptimal fertilizer condition (0 kg N ha^{-1}) sharply reduced yield, dry biomass, and A_{CO2}, particularly at 48 DAT, whereas r_s and sodium content increased. In fact, at 0 and 70 kg ha^{-1}, the lower leaf N availability may affect photosynthetic performance and rate due to N remobilization from photosynthetic enzymes and pigments [62]. The decreased SPAD index, which is significantly correlated to chlorophyll concentration as indicated by absorbance measurements [63], corresponded to the decrease in photosynthetic capacity, and an increase in the sensitivity to photo-inhibition [64]. However, the application of TG41 alone, but especially in combination with the VBP, to lettuce grown in sub-optimal N induced significant changes in morphology and physiology, as noted with increased yield and dry biomass. Therefore, under low-input conditions (0 kg N ha^{-1}), the combination of the microbial inoculant with the biopolymer-based biostimulant exhibited an important synergistic effect, thus confirming the beneficial effects on crop productivity as

previously reported by several authors [48–50]. Both PB treatments enhanced photosynthetic activity, SPAD index, and leaf nutritional status, as reflected by higher K and Mg and lower Na concentrations, that indicate a more efficient accumulation and translocation of assimilates to photosynthetic sinks that improve crop performance, but are not associated to the external N fertilization level applied [50]. Under optimal N conditions (i.e., recommended rate of 70 kg ha^{-1}), the treatment with TG41 alone had the best effect on fresh yield, combined with high Mg and antioxidant contents, as well as low nitrate and Na. In this N regime, the addition of the VBP-based biostimulant to the fungal inoculant did not improve the morpho-physiological parameters, nor the mineral profile in the leaves. As mentioned above, growth under suboptimal N conditions increased leaf cell susceptibility to light-induced oxidative damages, a condition that plants are not capable of overcoming. However, the application of the combined microbial and VBP PBs induced a strong production of TAA, phenols, and probably glutathione, a metabolite that works cooperatively with ascorbic acid to generate antioxidant effects that safely detoxify accumulated reactive oxygen species (ROS), thus protecting the plant and increasing the photosynthetic rate [6,7].

The application of 140 kg N ha^{-1} to lettuce was an excess condition that determined a plateau in yield and dry biomass but not in the N content, although there was an increase in nitrate and Na, as well as r_s at 40 DAT. This demonstrated that plants supplied with high levels of N were not able to assimilate and reduce all the nitrate supplied, risking negative consequences by the accumulation of these compounds in the vacuoles. This was also reported by Di Mola et al. [65] in rocket plants and by Wang et al. [66] in leafy vegetables, whereby optimal and particularly supra-optimal N treatments were not always characterized by the best quality traits in the produce, but on the contrary, resulted in damage to the commercial, nutritional, and functional quality traits. These effects were similar to those noted in our lettuce plants under supra-optimal N conditions, i.e., low macronutrients and total ascorbic acid, high nitrate and sodium content. The application of both PBs under supra-optimal N level (e.g., 140 kg ha^{-1}) significantly enhanced the N content and SPAD index while reducing nitrate content without affecting the CO_2 assimilation rate and the accumulation of beneficial nutrients. The strong increase in the SPAD index at 140 kg ha^{-1} in plants inoculated with TG41 or TG41+VBP biostimulants was also observed at 70 kg ha^{-1}, suggesting that the biostimulants were able to increase the number and efficiency of photosynthesis systems and light-harvesting complexes (LHC), that allowed plants to "fine-tune" photosynthesis in the fluctuating spectral quality and light intensity conditions, thus avoiding ROS formation and photo-oxidation. This also allowed a higher use efficiency of nitrate, as confirmed by the lower concentration of this ion in leaf tissues when compared to the untreated control because of a more efficient reduction and assimilation processes [6,7].

Our results correspond to previous findings on the plant growth-promoting effect of fungi inoculants containing *Trichoderma* [25,26,29,30,33,38,67]. The presumed mechanisms behind the beneficial morpho-physiological effects on lettuce plants by TG41 could be due to the release of signaling molecules with auxin and ethylene-like activity [28], in particular, bioactive volatile compounds [43], which increased nutrient bioavailability to the plant, that improved their uptake, translocation, and accumulation within the plant [35]. In addition, it has also been demonstrated that *Trichoderma* in the rhizosphere stimulates root growth and reshapes its architecture, morphological changes which are pivotal for improving nutrient uptake, in particular, nitrate, Ca, Mg, and K [29,30,35,41]. The synergistic action of TG41 with the VBP biostimulant is of particular interest because it resulted in the production of premium quality lettuce traits, as is clearly exhibited by the PCA. The vegetal-biopolymer biostimulant action was probably due to the presence of phenol metabolites with auxin and gibberellin-like activities, that interacted with phytohormones and enzymes stimulating the activity of carbon–nitrogen metabolism and plant development [45,46]. Another putative mechanism behind the stimulation of plant growth and yield in response to VBP drench application could involve the increased presence of bioactive molecules, such as signaling peptides (LRPP) and lignosulphonates, which are typical compounds present in VBP [46]. A previous study reported that lignosulphonate treatments can improve N uptake and assimilation in plants through the stimulation of glutamate synthase and

glutamine synthetase, as well as by triggering photosynthetic activity through the stimulation of both rubisco enzyme activity, thus improving plant performance [45]. The improved NUE in lettuce treated with PBs enhanced not only the chlorophyll content (as represented by the increased SPAD index) but also the synthesis of antioxidant metabolites that were capable of re-activating photosynthetic activity that under sub-optimal N conditions without PBs, would be severely compromised. Finally, the synergistic beneficial effect on root system architecture, as previously shown by Colla et al. [38,68], determined a '*nutrient acquisition response*' improving resource use efficiency (RUE) that enhanced plant biomass production and the quality of the produce.

5. Conclusions

Our study on the leafy vegetable crop Romaine lettuce confirmed that inoculations with *Trichoderma* TG41 under optimal N conditions (70 kg ha^{-1}) were able to improve the leaf nutritional status as indicated with the higher potassium and magnesium content and lower sodium content, plus providing the best yield performance of all tested conditions in terms of plant fresh and dry weight. Interestingly, the combined biostimulant applications of *Trichoderma* with the vegetal biopolymer-based product, in suboptimal fertilizer conditions of low N availability (0N kg ha^{-1}), was more effective than the treatment of the microbial inoculant alone not only in improving yield but also in producing a premium quality marketable lettuce with higher lipophilic antioxidant activity and total ascorbic acid content. Together these biostimulants positively influenced plant morpho-physiological processes that improved the assimilation of nitrate and macronutrients and stimulated root system architecture reshaping, thus permitting increased bioabsorption or '*nutrient acquisition response*'. Moreover, the assimilatory pathways were stimulated, for which nitrate was used to synthesize chlorophyll (increased SPAD index) and the antioxidant metabolites, which, in turn, re-activated the CO_2 assimilation activity normally decreased under sub-optimal N conditions. Therefore, the combination of microbial and non-microbial plant biostimulants represents a promising, efficient, and sustainable strategy for improving yield and quality of horticultural crops, such as lettuce, as well as improving cultivation in N compromised fields or low fertilizer input scenarios.

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